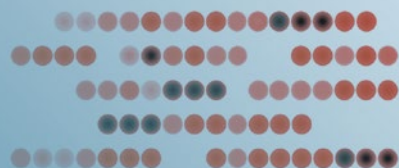
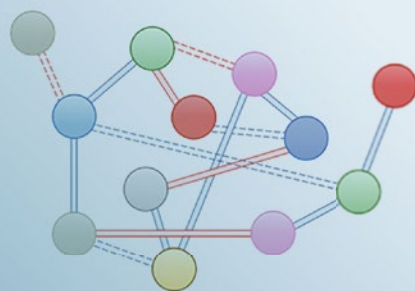


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Rodrigo Barderas
Joshua LaBaer
Sanjeeva Srivastava *Editors*



Protein Microarrays for Disease Analysis

Methods and Protocols

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Protein Microarrays for Disease Analysis

Methods and Protocols

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 **Humana Press**

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Preface

During the last years, the application of protein microarray-based proteomics to the analysis of human diseases has paved the way to profile proteomes in normal and pathological states, increasing our understanding of human biology.

The themes discussed within this book, *Protein Microarrays for Disease Analysis: Methods and Protocols*, are mainly focused on protein analysis covering a wide spectrum of the utility of protein microarrays for disease analysis. This book brings a collection of detailed protocols written by top experienced professionals around the world to cover a variety of protein microarray-based approaches. The chapters encompass different stages of protein microarrays from their construction to their use, including the different types of protein microarrays (recombinant proteins, antibody, phage, and NAPPA protein microarrays) in planar format or in solution by means of bead arrays with their multiple applications discussed, and the development of the next generation of protein microarrays and their applications to human diseases. These approaches have proved useful to decipher disease proteomes and characterize protein networks and interactomes to help shed light on mechanisms of many diseases (allergy, ocular diseases, characterization of the humoral immune response, etc.). Finally, the last chapter focuses on protein microarrays by bioinformatics for data analysis and statistics.

Collectively, this volume will offer readers key advice and procedural details to empower their needs and successfully achieve their own scientific and experimental goals applied to their specific research.

Protein Microarrays for Disease Analysis covers all the technical aspects of protein microarrays, and it will serve as a thorough, comprehensive, and valuable resource for graduate and postdoctoral fellows interested in protein microarrays as well as senior researchers interested in getting further insights into the growing field of protein microarrays.

Madrid, Spain
Tempe, AZ, USA
Mumbai, Maharashtra, India

Rodrigo Barderas
Joshua LaBaer
Sanjeeva Srivastava

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RODRIGO BARDERAS received his PhD in Chemistry (Biochemistry and Molecular Biology) from the Complutense University of Madrid (Spain) in 2004. Since 2004, he has been working in the field of proteomics and high-throughput screening techniques. In 2017, he got a Tenured Scientist position at the Instituto de Salud Carlos III. He is currently the Head of the Functional Proteomics Unit of the Chronic Disease Programme. He has contributed more than 100 original research publications, reviews, and chapters. His areas of interest include the identification of diagnostic and prognostic markers and new targets of intervention in chronic diseases of high prevalence by using proteomics, high-throughput techniques, and phage display, with a special focus on colorectal cancer.



JOSHUA LABAER is one of the foremost investigators in the rapidly expanding field of personalized diagnostics. His efforts focus on the discovery and validation of biomarkers—unique molecular fingerprints of disease—which can provide early warning for those at risk of major illnesses, including cancer and diabetes. Formerly founder and director of the Harvard Institute of Proteomics, LaBaer was recruited to ASU's Biodesign Institute as the first Piper Chair in Personalized Medicine in 2009. The Virginia G. Piper Center for Personalized Diagnostics (VGPCPD) has a highly multidisciplinary staff of molecular biologists, cell biologists, biochemists, software engineers, database specialists, bioinformaticists, biostatisticians, and automation engineers. VGPCPD applies open reading frame clones to the high-throughput (HT) study of protein function. In addition, his group invented a novel protein microarray technology, nucleic acid programmable protein array, which has been used widely for biomedical research, including the recent discovery of a panel of 28 autoantibody biomarkers that may aid the early diagnosis of breast cancer. LaBaer earned his medical degree and a doctorate in biochemistry and biophysics from the University of California, San Francisco. He completed his medical residency at the Brigham and Women's Hospital and a clinical fellowship in oncology at the Dana-Farber Cancer Institute, both in Boston. He has contributed more than 150 original research publications, reviews, and chapters. LaBaer is an associate editor of the *Journal of Proteome Research*, a recent member of the National Cancer Institute's Board of Scientific Advisors, Chair of the Early

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Part I

Introduction



Chapter 1

Protein Microarray-Based Proteomics for Disease Analysis

Rodrigo Barderas, Sanjeeva Srivastava, and Joshua LaBaer

Abstract

As we approach the twentieth anniversary of completing the international Human Genome Project, the next (and arguably most significant) frontier in biology consists of functionally understanding the proteins, which are encoded by the genome and play a crucial role in all of biology and medicine. To accomplish this challenge, different proteomics strategies must be devised to examine the activities of gene products (proteins) at scale. Among them, protein microarrays have been used to accomplish a wide variety of investigations such as examining the binding of proteins and proteoforms to DNA, small molecules, and other proteins; characterizing humoral immune responses in health and disease; evaluating allergenic proteins; and profiling protein patterns as candidate disease-specific biomarkers. In *Protein Microarray for Disease Analysis: Methods and Protocols*, expert researchers involved in the field of protein microarrays provide concise descriptions of the methodologies that they currently use to fabricate microarrays and how they apply them to analyze protein interactions and responses of proteins to dissect human disease.

Key words Disease analysis, Proteomics, Protein microarrays, Protein science, Functional analysis, Microarray data analysis

1 Introduction

With their ability to combine 20 different amino acids, in contrast to only 4 nucleotides in polynucleotide chains, proteins have enormous potential for complexity. The complexity of the human proteome is further expanded to an estimated 10^6 proteoforms, well beyond the 19,100 human protein-coding genes [1–3]. These values inform the challenge to study proteins' functions, their molecular behavior in comparison to genes, genomes, and transcriptomes. In this sense, about 20% of human proteins remain without known function and/or structure and even their interacting proteins are yet to be elucidated (referred to as the “dark proteome”) [4, 5], which may play significant roles in health and disease.

The exploding interest in proteomics-based research has enabled the simultaneous examination of thousands of proteins using high-throughput techniques and innovative analytical tools for data analysis [6–8]. Among proteomic approaches, protein microarrays provide a suitable tool for the functional characterization of proteins, identification of protein-protein interactions, determination of DNA- and small-molecule-binding protein and proteoform specificity, identification of protein targets of infectious disease antibodies or disease-specific autoantibodies (i.e., neurodegeneration, cancer, or autoimmune diseases), etc.

Here, the editors' intent for the volume *Protein Microarrays for Disease Analysis: Methods and Protocols* is to provide not only an introduction to protein array technology but also an extensive display of applications for screening and validation, epitope mapping, detection of protein-protein interactions, analysis of PTMs, and characterization of humoral immune responses, among others. Therefore, we have introduced the readers to descriptions of state-of-the-art protein microarrays with multiplexed detection platforms to study proteins in any field of life sciences, with an emphasis on clinical applications.

The first part of the volume includes four chapters, mainly focused on the design and production of protein microarrays, either in planar format (Chapters 2 and 3), in nanowells (Chapter 4), or in solution using bead arrays (Chapter 5). These have been used for the identification of antibody biomarkers, for the screening of humoral immune responses, or for the validation of proteomic profiles in body fluids.

The next set of chapters focus on the different applications that protein microarrays excel at. In this section readers can become familiarized with methods for the specific detection of protein-protein interactions using recombinant protein microarrays (Chapter 6), production of peptide microarrays with posttranslational modifications for detection of autoantibodies (Chapter 7), epitope mapping of allergenic lipid transfer proteins by means of HaloTag NAPP (Chapter 8), or epitope mapping of food allergens by peptide/protein microarrays printed using noncontact piezoelectric microarray printers (Chapter 9).

The most common applications of protein microarrays are described in the next five chapters, especially related to deciphering immune responses in health and disease. In these chapters, a description of how protein microarrays can be used to elucidate immune responses in infectious diseases (Chapters 10 and 11) or noninfectious human chronic diseases (Chapters 12–14) is given. In particular, the screening of protein microarrays for the identification of immune responses to malaria and bacterial infections is described. Chapter 12 describes protocols for discovering protein biomarker candidates in cardiovascular and atherosclerotic diseases using suspension bead arrays with technology developed by the

Human Protein Atlas. In addition, protocols for screening humoral immune responses in osteoarthritis using NAPPA and screening autoantibodies against brain tumors with high-density recombinant protein microarrays are thoroughly described in Chapters 13 and 14, respectively.

The remaining four chapters are related to the use of protein and antibody microarrays for the characterization of disease-specific protein profiles, and bioinformatics for the data analysis of protein microarrays. First, a thorough description of the methodology for deciphering intracellular altered signaling pathways in tumor development is discussed (Chapter 15). Then, the combination of recombinant proteins and antibody arrays to characterize functional features of dark proteins in human cell cultures is provided (Chapter 16). Chapter 17 focuses on the application of protein and antibody microarrays in planar format or in solution bead-array format for the characterization of ocular diseases. Finally, an overview of protein microarray bioinformatics for data analysis is provided (Chapter 18). In this last chapter, statistical methods for analyzing protein microarray data using R are provided. Using a publicly available protein microarray dataset, this chapter describes practical applications for how to sort the findings based on their significance.

In summary, this volume provides unique methodologies for each of the steps of protein array, from its construction to its evaluation, which will give the readers the idea of the versatility of protein microarrays to be used in their specific field to accelerate their research. Finally, we would like to thank all of the contributors of the *Protein Microarrays for Disease Analysis: Methods and Protocols* for sharing their insights and protocols of this emerging field, where different creative approaches have been used in multiple applications to solve biological and clinical problems, and associated diseases.

References

1. Piovesan A, Antonaros F, Vitale L, Strippoli P, Pelleri MC, Caracausi M (2019) Human protein-coding genes and gene feature statistics in 2019. *BMC Res Notes* 12(1):315. <https://doi.org/10.1186/s13104-019-4343-8>
2. Ezkurdia I, Juan D, Rodriguez JM, Frankish A, Diekhans M, Harrow J, Vazquez J, Valencia A, Tress ML (2014) Multiple evidence strands suggest that there may be as few as 19,000 human protein-coding genes. *Hum Mol Genet* 23(22):5866–5878. <https://doi.org/10.1093/hmg/ddu309>
3. Smith LM, Kelleher NL, Consortium for Top Down Proteomics (2013) Proteoform: a single term describing protein complexity. *Nat Methods* 10(3):186–187. <https://doi.org/10.1038/nmeth.2369>
4. Perdigao N, Rosa A (2019) Dark Proteome Database: studies on dark proteins. *High Throughput* 8(2). <https://doi.org/10.3390/ht8020008>
5. Perdigao N, Heinrich J, Stolte C, Sabir KS, Buckley MJ, Tabor B, Signal B, Gloss BS, Hammang CJ, Rost B, Schafferhans A, O'Donoghue SI (2015) Unexpected features of the dark proteome. *Proc Natl Acad Sci U S A* 112(52):15898–15903. <https://doi.org/10.1073/pnas.1508380112>
6. LaBaer J, Ramachandran N (2005) Protein microarrays as tools for functional proteomics.

- Curr Opin Chem Biol 9(1):14–19. <https://doi.org/10.1016/j.cbpa.2004.12.006>
7. Barderas R, Babel I, Casal JI (2010) Colorectal cancer proteomics, molecular characterization and biomarker discovery. *Proteomics Clin Appl* 4(2):159–178. <https://doi.org/10.1002/prca.200900131>
8. Chandra H, Reddy PJ, Srivastava S (2011) Protein microarrays and novel detection platforms. *Expert Rev Proteomics* 8(1):61–79. <https://doi.org/10.1586/epr.10.99>

Part II

Design and Production of Protein Microarrays



Chapter 2

Systematic and Rational Design of Protein Arrays in Noncontact Printers: Pipeline and Critical Aspects

Pablo Juanes-Velasco, Alicia Landeira-Viñuela,
Angela-Patricia Hernandez, and Manuel Fuentes

Abstract

The systematic design and construction of customized protein microarrays are critical for the further successful screening of biological samples in biomedical research projects. In general protein microarrays are classified according to the content, detection method, and printing methodology, among others. Here, we are focused on the type of printing: contact and noncontact. Both approaches have advantages and disadvantages; however, in any of the approaches, a prior well design and systematic preparation of materials and/or instruments required for the customized antibody arrays is critical. In this chapter, the process for an antibody microarray by a noncontact printer is described in detail from the preparation of array content to the analysis, including quality control steps.

Key words Noncontact printers, Protein microarrays, Array design, Array workflow, Array pipeline

1 Introduction

Recently, protein microarray technology has been successfully employed for multiplex biomarker determination (diagnostic and prognostic) in tumoral pathologies [1–4], discovery of potential vaccine candidates [5–7], or seeking for autoantibodies in autoimmune diseases [8–11], among others. The usefulness of arrays in these areas is given by the ability to generate high-content information with minimal amount of sample. There are several types of arrays depending on what you want to study. It is essential to know the different types of arrays to develop new and diverse applications.

In general, protein microarrays are classified according to their features such as content, format, or detection method (Fig. 1). According to their content or their nature of the capturing agent, the arrays can be divided into three main groups: (1) analytical

Pablo Juanes-Velasco and Alicia Landeira-Viñuela contributed equally with all other contributors.

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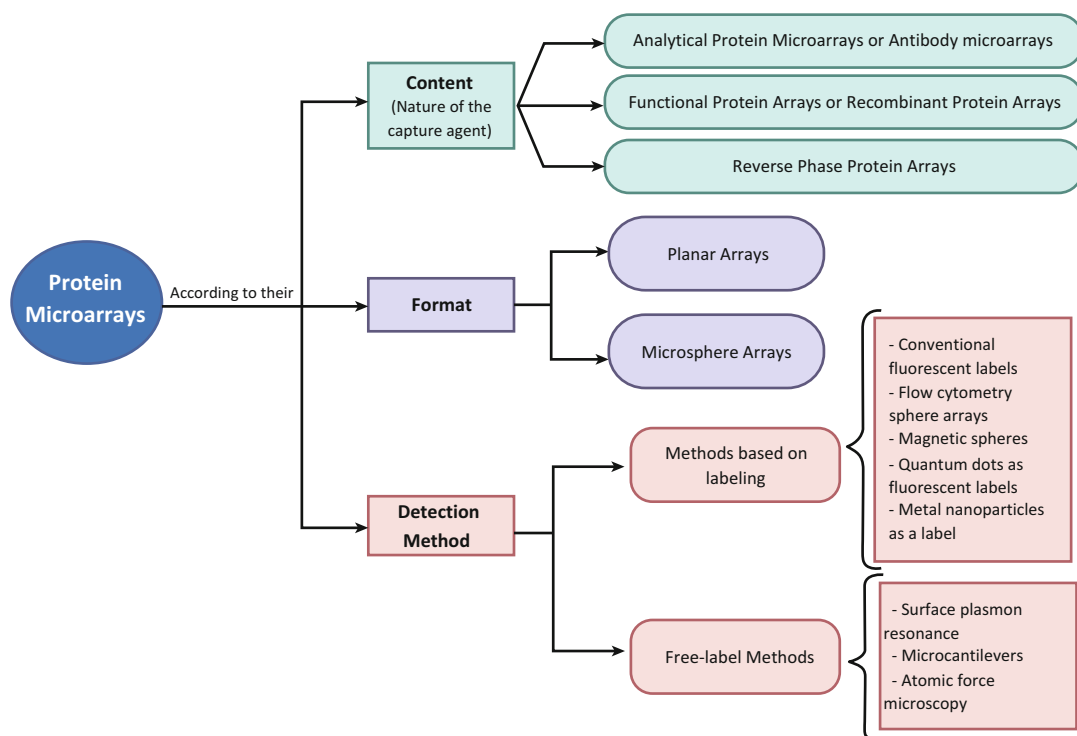


Fig. 1 Different types of protein microarrays according to their content, format, and detection method. (Created with BioRender.com)

protein microarrays or antibody microarrays, in which proteins are specifically and selectively detected in complex biological samples by immunoaffinity [12–14]; (2) functional protein arrays or recombinant protein arrays, to identify the interactions of proteins with different molecules such as DNA, lipids, other proteins, or small molecules [14]; and (3) reverse-phase protein arrays, in which a biological sample is printed and its proteins are detected by the binding with antibodies [12, 14, 15].

According to the format, protein arrays are divided into two main groups: (1) planar arrays, in which high-density microspots containing different and multiple ligands are deposited on a solid support (2D (i.e., silica-activated surface) or 3D (i.e., polymer-coated glass surface)) and separated by a minimal distance. In this format, it is necessary to define the size, morphology, and reproducibility of the spots, as well as the capacity of the ligand and the background signal, being that they are parameters that influence on the performance of the assay [14, 16]. (2) Microsphere arrays are based on the simultaneous use of different populations of microspheres color coded with different fluorochromes. Each single-color-coded microsphere will be covered by different antibodies to incubate and study a biological sample [14, 15].

Regarding the detection method, they can be classified into two large groups: methods based on labeling with reporter molecules (i.e., fluorescence dyes) and label-free methods. Among the methods based on labeling are (1) conventional fluorescent labels such as fluorochromes or radioisotopes (such as rhodamine, coumarin, cyanine); (2) flow cytometry microsphere arrays to detect soluble proteins from cell lysates or in human proximal fluids; (3) antibodies coupled to magnetic microspheres to detect proteins; (4) quantum dots that function as fluorescent tags by binding to antibodies or peptides for the recognition of cellular components; and (5) metal nanoparticles as label agents, such as gold nanoparticles that have good optical properties, quantum efficiency, and compatibility with a wide range of wavelengths. Among the label-free methods there are (1) surface plasmon resonance, based on the generation of plasmons on a well-controlled chemically activated and characterized surface, which allows measuring changes in the refractive index of the sensor surface. It will be possible to determine if there are molecular associations or dissociations, as well as the affinity and specificity of the biomolecules with each other, according to the variation in the intensity of the reflected light and/or the angle of incidence. (2) Microcantilevers are thin sheets of silicon coated with a gold surface associated with nanomechanical biomolecular recognition systems. The interaction between the antibodies or proteins immobilized in said sheets, with their possible ligands, can be observed with different optical and electronic systems. (3) Atomic force microscopy is a microscopic analysis to observe the topological variations of a surface and thus is able to evaluate the immobilization of biomolecules and detect protein interactions on the surface of the arrays [14].

Among others, there are currently different deposition methods useful for generating protein arrays, which can be divided into two principal groups: contact and noncontact printing. Both printing technologies have been highly reliable for biomarker discovery and validation in many biomedical research projects [17] (Fig. 2a).

In contact printing, there is a direct contact between the deposition tool (i.e., stainless needle or pin) and the chemically activated planar microarray surface. This method is based on printing with tips/pins in which probe solutions are loaded by capillarity and the deposition is by contacting the tips on the support [18]. Contact printing can be performed with micro-stamps based on the principle of soft lithography [19, 20] or as nano-tip based on the dip-pen nanolithography [21, 22]. The diameter of the spots will be influenced by the surface properties, viscosity of the probe solution, and geometry of the tips. Although it is a very simple method, its drawbacks are cross-contamination and damage to the array surface [18].

On the other side, noncontact printing methods have in common the preservation of the sample. In these groups of methods are included electrospray technique in which a strong electric field is

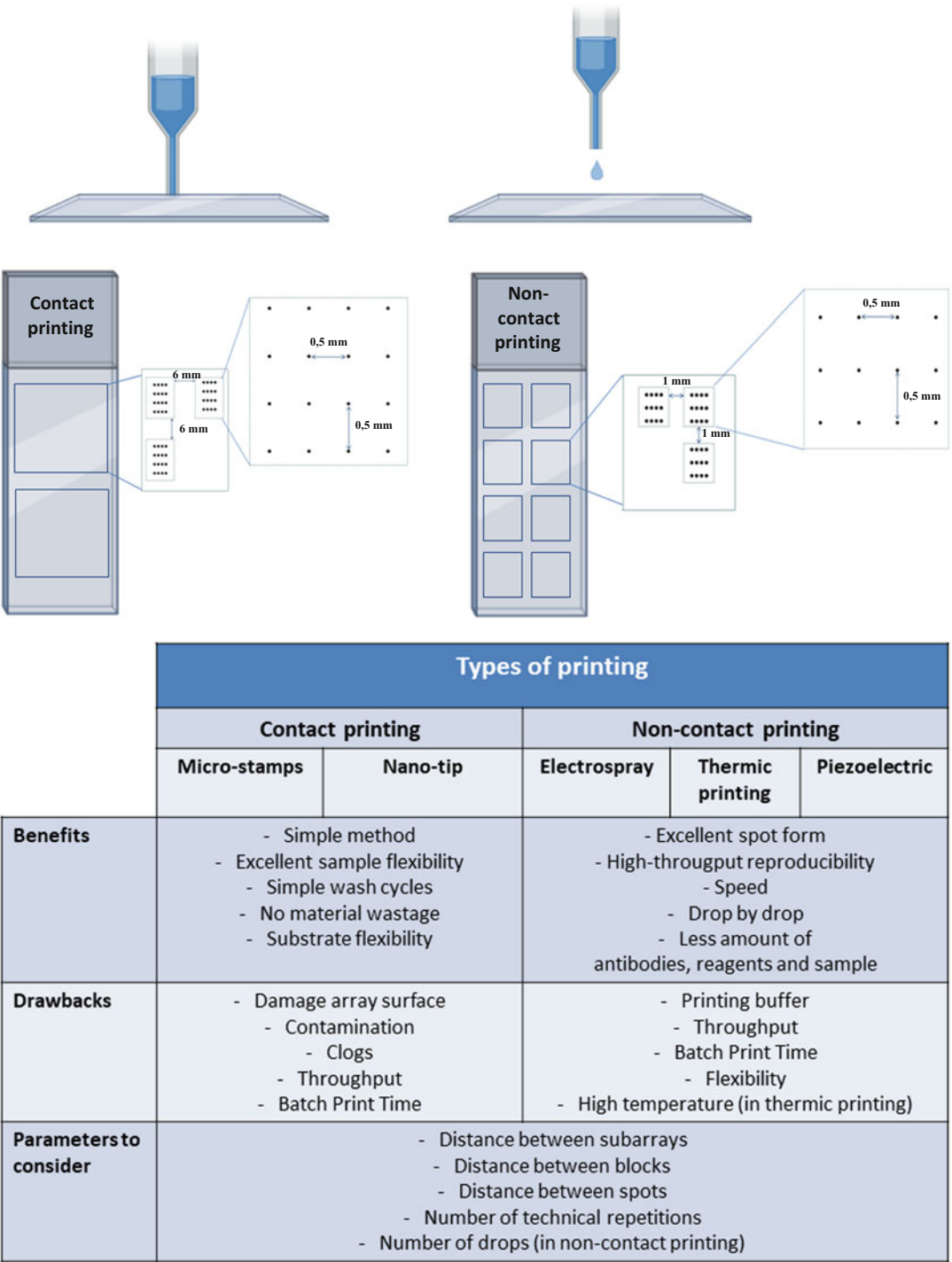


Fig. 2 (a) Different types of printing. (b) Different types of printing, their benefits, drawbacks, and some parameters to take into account in their construction and design

applied to the solution, microdroplets are formed, and the solution dries instantly [23]. Another method is thermo printing in which the ejection of the drop is based on inkjet printing, in which the sample is heated to a temperature of around 200 °C, avoiding its evaporation. And the last noncontact technique is piezoelectric printing, similar to the previous method but avoiding heating of the sample, in which the drop is expelled by the vibration of a piezoelectric membrane at the printing tip [18], so it is generally preferred and is the one that we will describe as a systematic pipeline to generate a tailor-made array with high reproducibility.

Both deposition methods have advantages and disadvantages regarding compatibility with complex biological compounds, real-time detection systems, intra- and inter-array reproducibility, and surface format, among others (Fig. 2b). However, it appears that noncontact printing is the best strategy to process a large number of samples with high reproducibility, reducing the number of antibodies, reagents, and samples required [15].

In this chapter, it is described in detail how a customized antibody microarray is designed and constructed with the noncontact nanoinjection piezoelectric printer “ArrayJet Printer Marathon v1.4” (ArrayJet, Roslin, UK), and all the steps of the workflow, included from the beginning to the final experimental design with labeling-based detection methods.

2 Materials

2.1 Source Plate Preparation

1. JetStar™ Microarray-Specific 384-well Microplates (ArrayJet).
2. JetStar™ Optimum Microarray Protein Printing Buffer (ArrayJet).
3. DMSO (Sigma-Aldrich).
4. BS3 (bis(sulfosuccinimidyl)suberate) (Thermo Fisher Scientific).
5. Thermoblock (Thermo Fisher Scientific).
6. Thermal mixer (Thermo Fisher Scientific).
7. Phosphate-buffered saline (PBS) pH 7.4: 3.6 g Na_2HPO_4 , 0.2 g KCl, 0.24 g KH_2PO_4 , 8 g NaCl in 1 L.
8. Glycerol 47% (v/v): Prepared from glycerol 99% (Sigma-Aldrich).

2.2 Chemical Functionalization of Microarray Silica Surface [24]

1. Acetone.
2. 3-(2-Aminoethylamino)-propyldimethoxymethylsilane (Thermo Fisher Scientific).
3. DEPC-treated water.

4. Ground slides 76×26 mm (LineaLab, Barcelona, Spain).
5. Orbital shaker.

2.3 Noncontact Printing

1. Slides with chemically activated glass surfaces.
2. Nanoinjection printer Marathon ArrayJet (ArrayJet).
3. JetSpyder™ 12 samples (ArrayJet).
4. Sonicator.

3 Methods

The generation of a customized microarray is a process that involves multiple steps in a specific sequential planning (Fig. 3). Each of them is essential to obtain perfectly designed and successful screening with this set of customized microarrays. This section details the methodology and recommendations for designing microarrays.

3.1 Design of Customized Array Layout

The first step in the production of the array is the design of the arrangement of the spots. In this chapter, we used Microsoft Excel as the spreadsheet program to generate the array layout sample file. This command allows to generate the appropriate comma-delimited file format (.csv) that will be exported to the ArrayJet software.

1. Open a new sheet of Microsoft Excel and design the microarray according to the final sample printing (*see Note 1*).
An example of a design can be seen in Fig. 4.
2. Save as .CSV document.
3. Open the new document with a text editor and replace the semicolon (“;”) with comma (“,”).
4. Save the document with the replacement.

3.2 Array Map Generation and Array Content Preparation

Antibody solutions and controls for printing must be properly arranged on the source plate to be used in the process. Likewise, the samples require a previous preparation in order to set a constant viscosity in all the solutions to be aspirated and injected correctly and reproducibly on the slide.

3.2.1 Originate the Well Plate Map

The well plate map is the document that provides the information to prepare the source plate (*see Note 2*).

1. Open the software “*Command Centre™ for Marathon.*”
2. Go to File→Generate Microplate Map File.
3. In the new tab titled “*Microplate Mapping from Array Layout File,*” select the file, the type of plate we will use to contain the

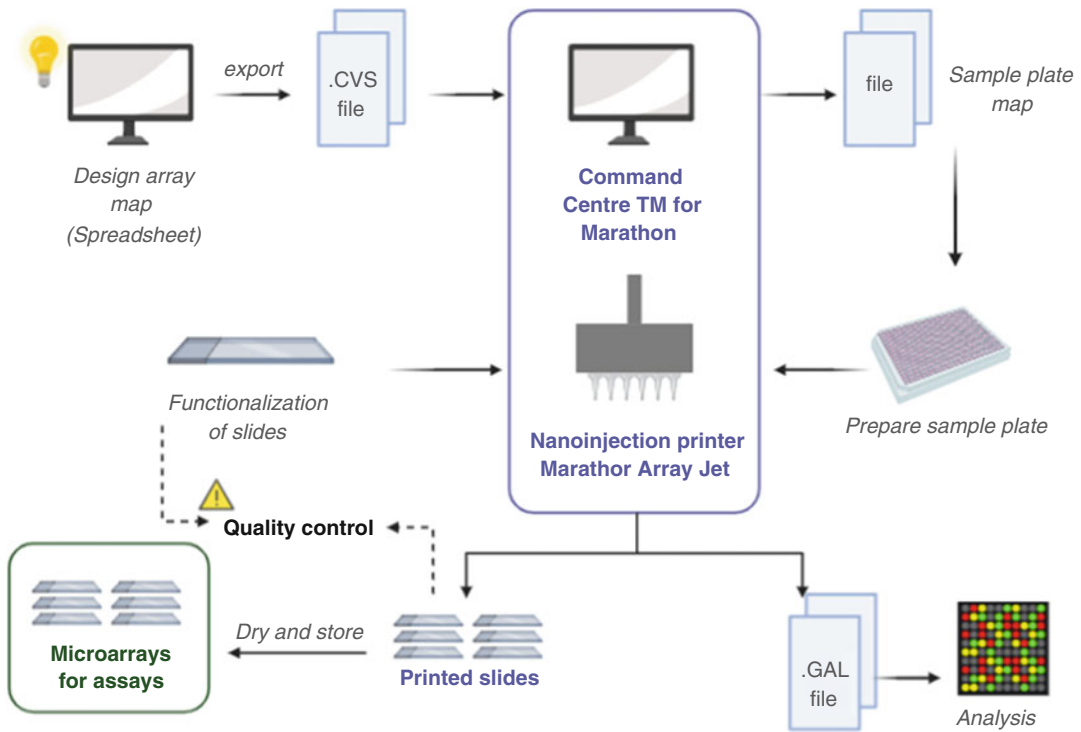


Fig. 3 Workflow of design and construction of a microarray

12	11	10	9	8	7	6	5	4	3	2	1
Landmark	Sample11	Sample22	Sample34	Sample42	Sample53	Buffer	Sample72	Landmark	Sample89	Sample101	Positive control
Sample1	Sample12	Sample23	Positive control	Sample43	Sample54	Sample65	Buffer	Sample81	Sample90	Sample102	Sample112
Sample2	Sample13	Sample24	Sample35	Sample44	Sample55	Sample66	Sample73	Sample82	Sample91	Sample103	Sample113
Sample3	Sample14	Sample25	Sample36	Sample45	Sample56	Buffer	Sample74	Landmark	Sample92	Sample104	Negative control
Sample4	Sample15	Sample26	Negative control	Buffer	Sample57	Sample67	Buffer	Sample83	Sample93	Buffer	Sample114
Sample5	Buffer	Sample27	Sample37	Sample46	Sample58	Sample68	Buffer	Sample84	Sample94	Sample105	Sample115
Sample6	Sample16	Sample28	Sample38	Sample47	Sample59	Buffer	Sample75	Landmark	Sample95	Sample106	Sample116
Sample7	Sample17	Sample29	Positive control	Sample48	Sample60	Sample76	Sample85	Sample96	Sample107	Sample117	
Sample8	Sample18	Sample30	Sample39	Sample49	Sample61	Sample77	Sample86	Sample97	Sample108	Sample118	
Landmark	Sample19	Sample31	Sample40	Sample50	Sample62	Buffer	Sample78	Landmark	Sample98	Sample109	Sample119
Sample9	Sample20	Sample32	Negative control	Sample51	Sample63	Buffer	Sample79	Sample87	Sample99	Sample110	Sample120
Sample10	Sample21	Sample33	Sample41	Sample52	Sample64	Sample71	Sample88	Sample96	Sample100	Sample111	Landmark

Fig. 4 Microarray design of 12 row $\times$ 14 column

samples to be printed, the direction (vertical or horizontal), and the type of JetSpyder of your equipment as could be seen in Fig. 5.

4. Click on the button “Browse” and select the location and folder where the .Csv file (Subheading 3.1, step 3) was saved.
5. Once selected, click on the open button.
6. In the section “Print run information” select:
 - (a) Type of microplate where the samples will be located (96/384 wells).
 - (b) In the section “Microplate filling” select the direction in which we want to fill the microplate with our samples.

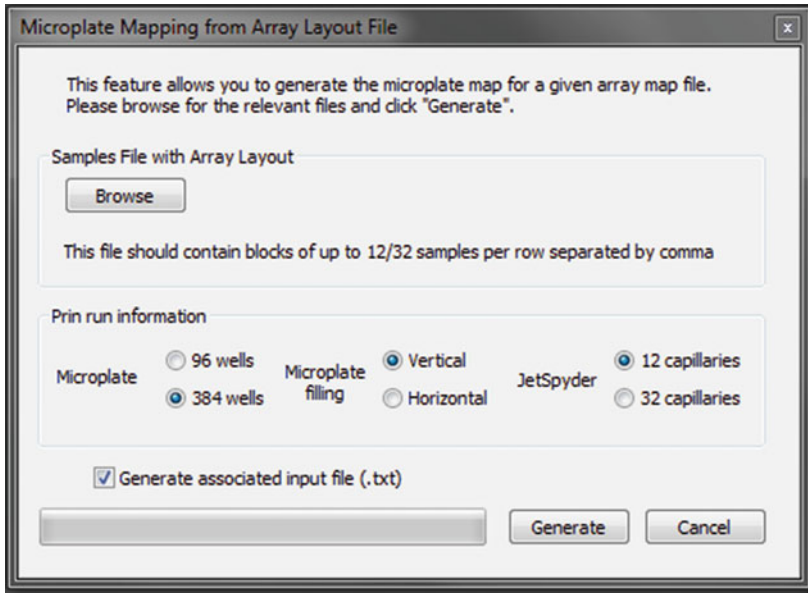


Fig. 5 Tab for the selection of characteristics of the ArrayMap

	1	2	3	4	5	6	7	8	9	10	11	12
A	Sample89	Sample91	Sample53	Sample55	Sample11	Sample13	Sample7	Sample39	Sample61	Sample63	Sample18	Sample20
B	Sample90	Sample92	Sample54	Sample56	Sample12	Sample14	Sample38	Sample100	Sample62	Sample64	Sample19	Sample21
C	Sample34	Sample35	Positive control	Sample13	Sample72	Sample73	Sample39	Negative control	Sample118	Sample120	Sample77	Sample79
D	Positive control	Sample36	Sample112	Negative control	Buffer	Sample74	Sample40	Sample41	Sample119	Landmark	Sample78	Sample80
E	Sample42	Sample44	Landmark	Sample2	Sample82	Sample49	Sample51	Sample5	Sample9	Sample86	Sample87	Sample89
F	Sample43	Sample45	Sample1	Sample3	Sample81	Landmark	Sample50	Sample52	Landmark	Sample10	Landmark	Sample88
G	Sample101	Sample103	Buffer	Sample56	Sample22	Sample24	Sample108	Sample110	Sample70	Buffer	Sample30	Sample32
H	Sample102	Sample104	Buffer	Sample65	Sample23	Sample25	Sample109	Sample111	Buffer	Sample71	Sample31	Sample33
I	Sample93	Sample95	Sample57	Sample59	Sample15	Sample16	0	0	0	0	0	0
J	Sample94	Sample96	Sample58	Sample60	Buffer	Sample17	0	0	0	0	0	0
K	Negative control	Sample38	Sample114	Sample116	Buffer	Sample75	0	0	0	0	0	0
L	Sample37	Positive control	Sample115	Sample117	Buffer	Sample76	0	0	0	0	0	0
M	Buffer	Sample47	Sample4	Sample6	Sample83	Landmark	0	0	0	0	0	0
N	Sample46	Sample48	Sample5	Sample7	Sample84	Sample85	0	0	0	0	0	0
O	Buffer	Sample105	Sample67	Buffer	Sample26	Sample28	0	0	0	0	0	0
P	Sample105	Sample107	Sample68	Sample69	Sample27	Sample29	0	0	0	0	0	0

Fig. 6 Wellplate map to prepare the samples

(c) In the section “*JetSpyder*” select type of instrument available for the printing.

7. Click on the “*Generate associated input file (.txt)*” (see **Note 3**).
8. Finally, click on the button “*Generate*” (see **Note 4**). The program will generate a file that can be opened as a separate spreadsheet, obtaining a map as the example depicted in Fig. 6. This file is also necessary for generating the corresponding document to analyze the array as it will be explained at the end of this method’s section.

3.2.2 Source Plate Preparation

Once the well plate map is generated, prepare the samples with the corresponding antibody dilutions and dispositions:

1. Prepare dilutions of protein samples in PBS (see **Note 5**).
2. Wells must be prepared with the right viscosity required by the printer:

- *Option 1:* protein sample (15 μ L) and optimum protein printing buffer (15 μ L).
 - *Option 2:* protein sample (15 μ L) and glycerol at 47% v/v crosslinker containing BS3 at a final dilution of 0.1 mmol (15 μ L).
3. Once the plate is charged with the samples, spin it down briefly to remove air bubbles. When the plate is not going to be used immediately after being prepared, cover it with foil and store it in the dark at 4 °C.

3.3 Chemical Functionalization of Microarray Glass Surface

Surface of slide must be chemically functionalized before the printing to promote molecule binding by adsorption or covalent link [24].

1. Select slides without notches or scratches. Clean the slides with a paper or tissue that does not leave any dust.
2. Leave the slides parallel on a metal rack.
3. Prepare the silica functionalization solution: dissolve 6 mL of 3-(2-aminoethylamino) propyldimethoxymethyl silane in 294 mL of acetone (*see* **Notes 6** and **7**).
4. Immerse the metal rack with the slides to be functionalized in the glass cuvette containing the functionalization solution (*see* **Note 8**).
5. Activate the surfaces with this solution for 30 min in orbital shaking at RT.
6. Discard or reuse the functionalization solution (*see* **Note 9**).
7. Wash the functionalized slides with enough acetone to cover the slides completely for 30 min in orbital shaking at RT.

3.4 Noncontact Printing

Once source sample plate is properly prepared for nanoinjection printer the printing design must be generated in Marathon ArrayJet software. In this step block distribution, number of spots printed, number of drops per spot, and number of replicates can be determined. With this software it is also possible to determine the number and the dimension of the subarrays. Several examples of array distribution are depicted in Fig. 7.

For noncontact printing with Marathon ArrayJet, follow these steps:

1. Turn on the nanoinjection printer and the temperature and humidity control station as well as the computer if it is turned off.
2. Perform daily maintenance of the equipment to check the nanoinjection system (*see* **Note 10**).

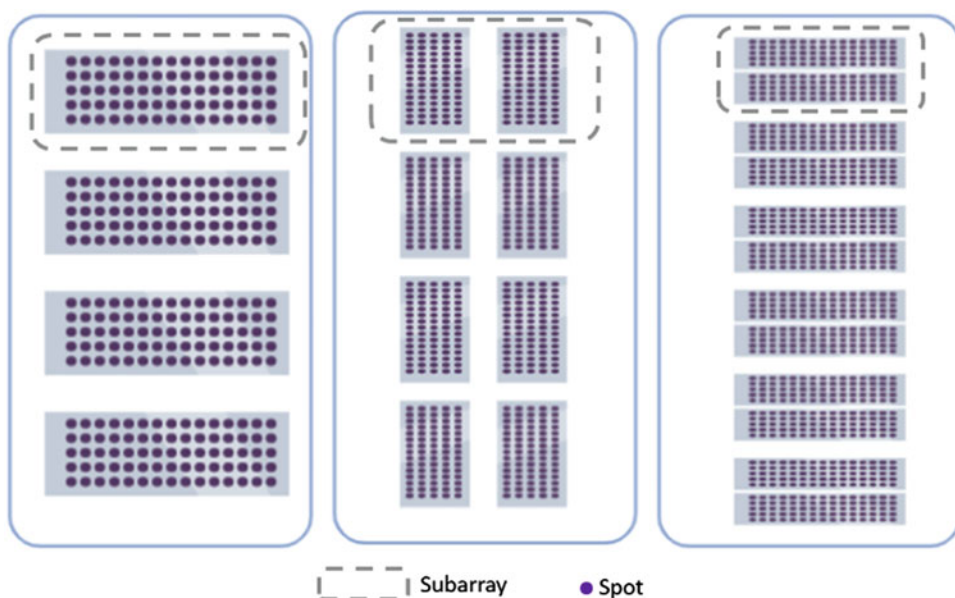


Fig. 7 Examples of distribution of subarrays and spots within the array

3. Introduce the plate with the samples and the microarrays into the equipment using the route: Marathon → Options → Load/unload microplates and slide (*see Note 11*).
4. Create a folder with the print registration number inside the Microarray Design folder where both the “run slide” experiment and the print results provided by the ArrayJet system will be saved (*see Note 12*).
5. Go to “*Print run Settings (slides)*” and enter the settings appropriate to your printing requirements.
 - (a) In the menu “Source microplate properties,” set microplate characteristic as plate type or number of samples (Fig. 8).
 - (b) Samples of JetSpyder can be deactivated in the menu “JetSpyder properties” as shown in Fig. 9.
 - (c) Measures and distribution of subarrays can be established in the menu “Slide properties.” In Fig. 10, an example of a slide with seven subarrays is shown.
 - (d) In the menu “Spot properties,” the distribution of the spots in the subarray can be determined as well as the technical repeats included in the assay (Fig. 11).
 - (e) Begin printing in the last menu of this tab, “Options (start).” Select the source of the sample (microplate in this case) and press the button “START RUN PRINT” (Fig. 12).

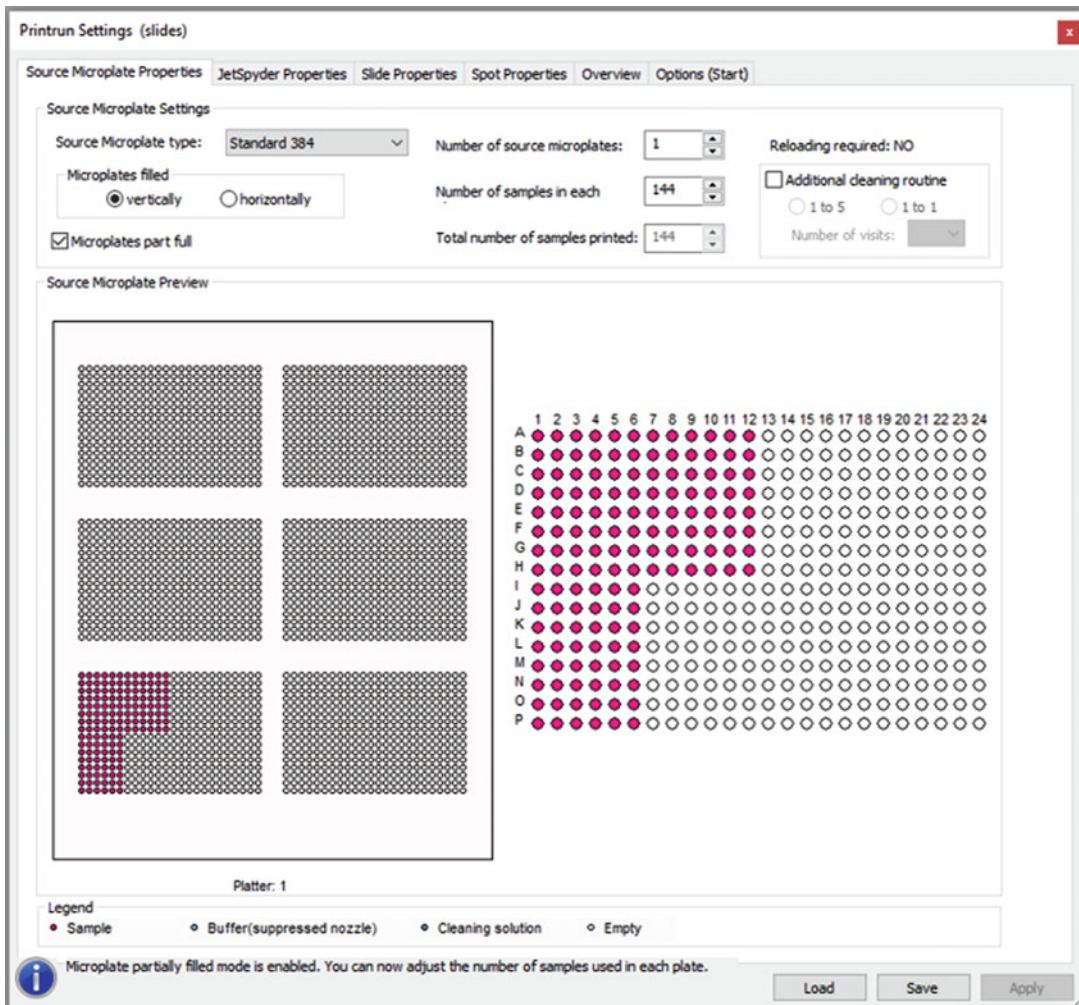


Fig. 8 Menu “source microplate properties”

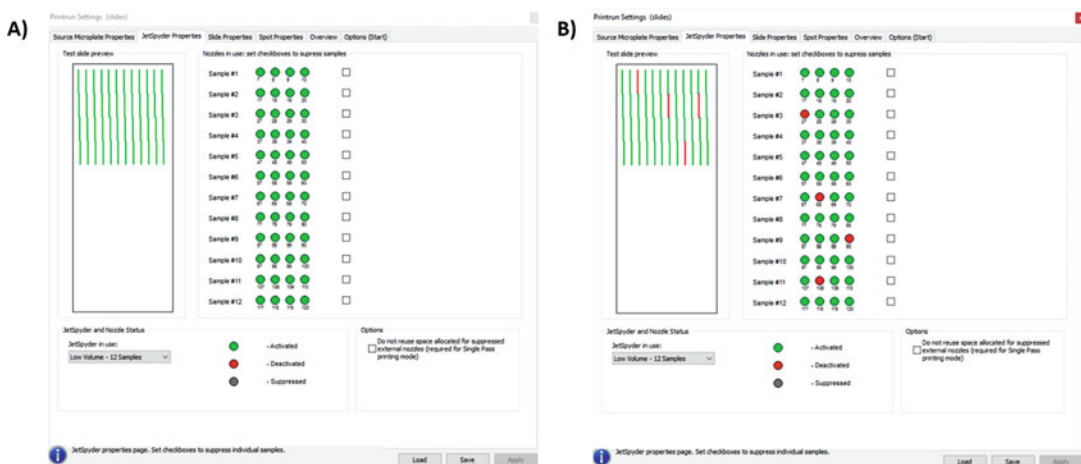


Fig. 9 Menu “JetSpyder properties”. (a) All samples activated. (b) JetSpyder with some samples deactivated

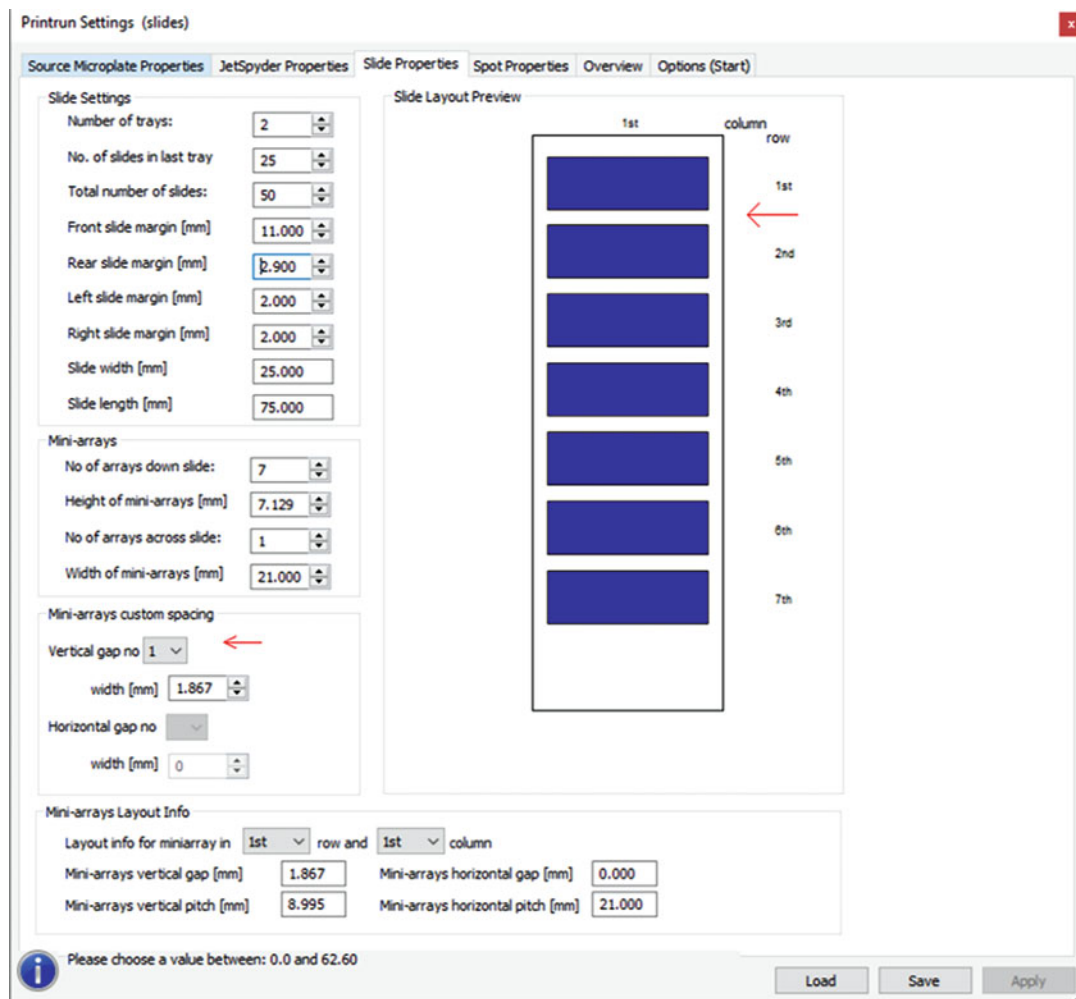


Fig. 10 Menu “Slide properties” with and example of subarray dispositions

6. At the end of printing, software informs us of the quality of the printed arrays provided by the system. Immediately, save all images and results generated by this system in the folder created for this assay.
7. Initialize system again.
8. Remove plates and store under conditions required by the samples.
9. Extract the printed microarrays and label them with array number and print request/registration number.
10. Dry microarrays in an oven at 37 °C with a moisture-absorbing agent.

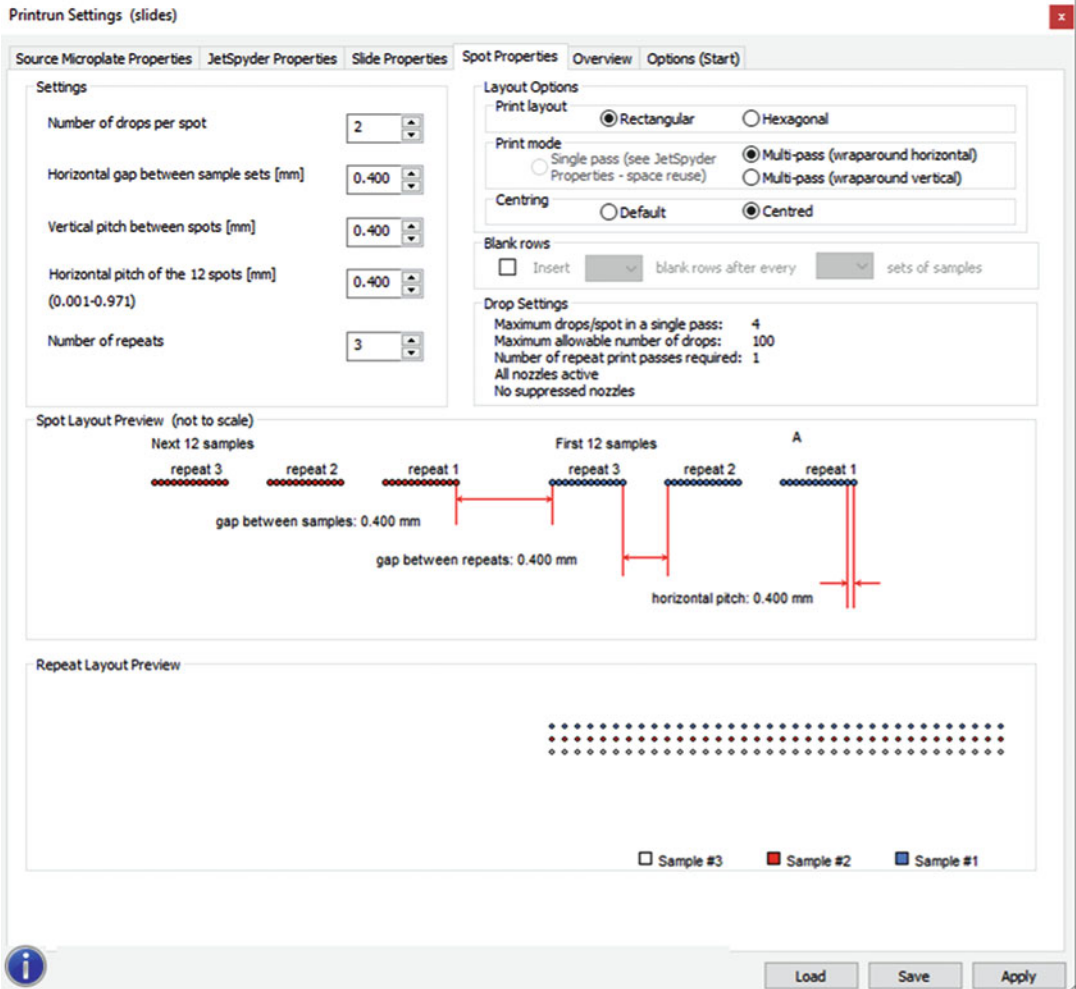


Fig. 11 Menu “Spot properties” to distribute spots in each subarray

11. Once microarrays are dry, label them with appropriate dimensions so that it does not interfere in the design of the microarray and in the design of experiments to be carried out.

3.5 Generate the File for Analysis

A GenePix Array List (.GAL) document is the file necessary to carry out the analysis of the microarrays results.

Follow these steps to generate the GAL of the printed arrays (Fig. 13):

1. Go to “*Print run Settings (slides)*,” tab “*Options (Start)*,” and section “Output Files.”
2. Select:
 - (a) “GenePix Array List file (GAL).”
 - (b) The folder to save the .gal file.

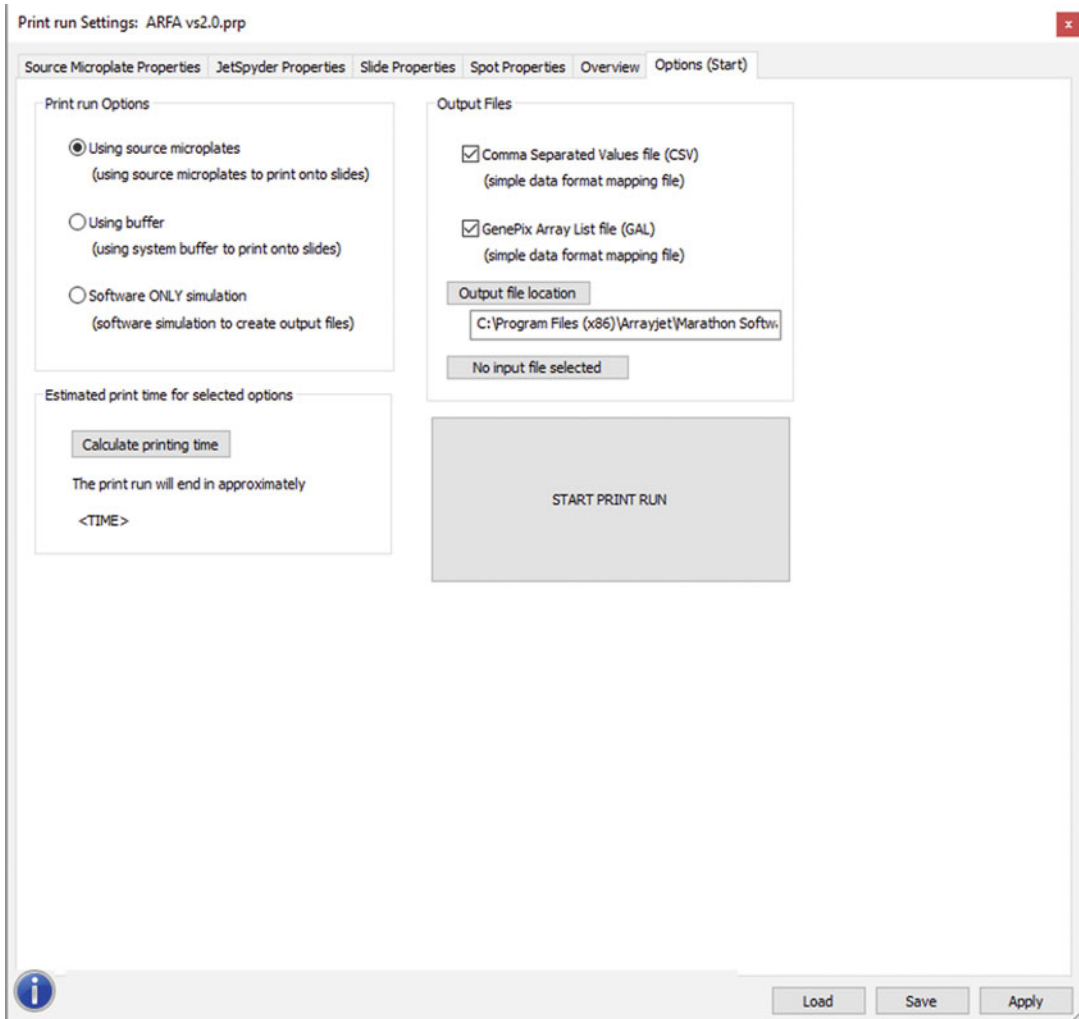


Fig. 12 Menu “Options (start)” to set up the final properties for the printing

3. To add the name of samples to the .gal file, export the input file (.txt):
 - (a) In “*Print run Settings (slides)*” → “*Options (Start)*” → “*Output Files*” → “*No input file selected*” button (Fig. 13).
 - (b) In the new tab “*Input File Options*,” click “*Browse*” button and select the corresponding file obtained in Subheading 3.2.1, step 8.
 - (c) Click on “*Open*” button.
 - (d) In the section “*Input File Options*,” select “*Automatic Detection Mode (TAB or COMMA)*.”
 - (e) Click on “*OK*” button.

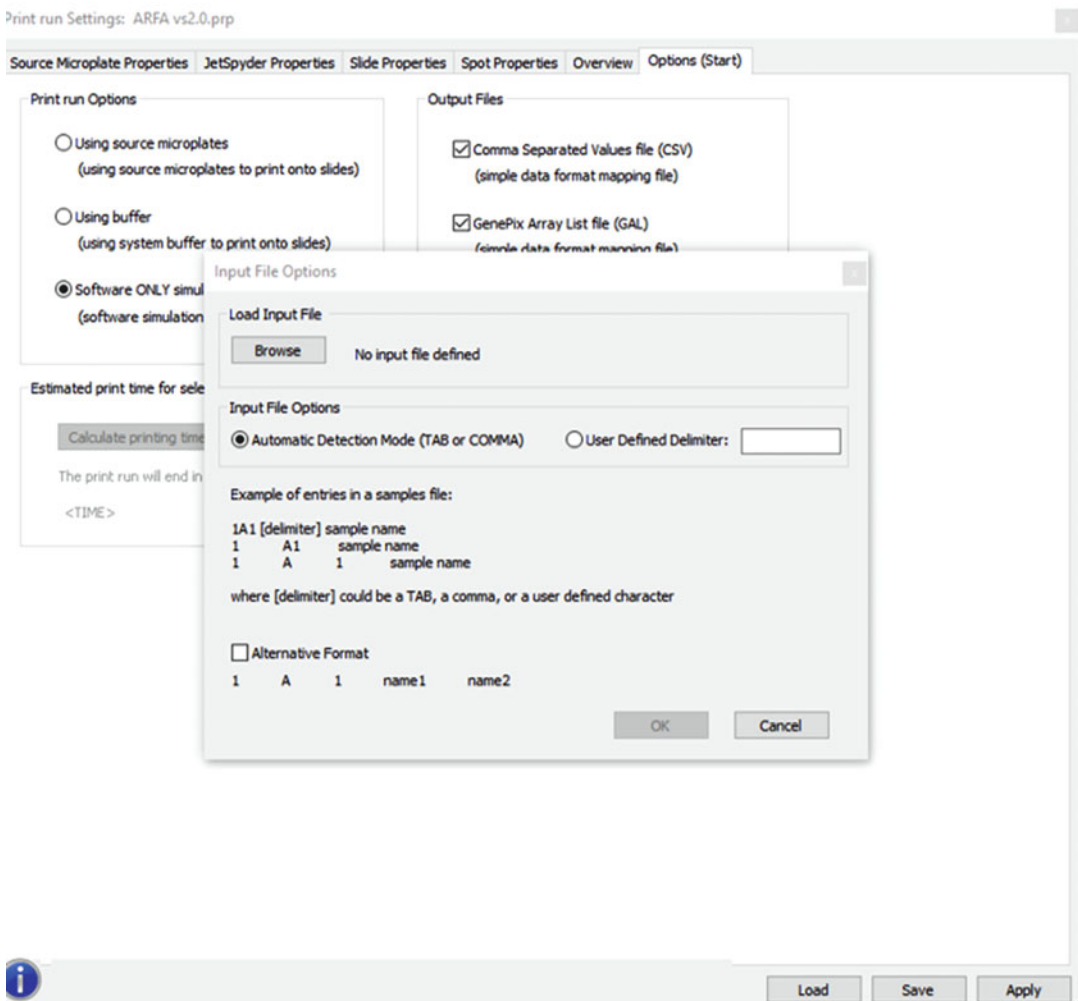


Fig. 13 Tab for GenePix Array List file (GAL) generation

3.6 Quality Control

Once the printing is finished, some quality criteria are obtained directly from the software. It evaluates the printing of each one of the microarrays thanks to the camera system providing not only the quality parameters but also the images of each of the microarrays.

Quality criteria:

1. Maximum % of missing spots: 5.
2. Maximum % of other defects: 5.

Furthermore, the quality criteria can be completed with the following indications:

Functionalization process: Once the functionalized slides have dried, no residue or mark on the surface should be observed with the naked eye.

Printing of microarrays:

Follow these steps to confirm that printing is correct:

1. Randomly select several microarrays from the printing (*see Note 13*).
2. Scan the microarray and export the file.
3. Open the .tif file obtained after scanning in GenePix and .gal file.
4. Check that the design of microarray is correct before starting any test.

3.7 Analysis of Results

Microarray technology allows obtaining a customized platform to make a high-throughput screening from your biological samples. Once the design and printing are carried out, test results must be processed and analyzed [13]. In this last section, highlights for data processing are briefly presented as shown in the following workflow (Fig. 14).

1. Prepare your biological samples for microarray incubation. Consider the great detection capacity of microarray technology and take advantage of the possibility of testing different conditions simply and quickly.
2. When you have processed the samples with microarrays, scan slides according to the detection method that you incorporated into the array design for this purpose. Use a Sensovation Fluorescent Array Imaging Reader or a similar instrument.
3. Use GenePix software to combine the images obtained from the scan with array sample information (.gal file) and obtain a GenePix Results file (.gpr) with individual spot information for each biological sample incubated.
4. Finally, compile all the information obtained from all your samples in a specialized software such as Python or R and perform the corresponding biostatistical analysis with consistent methods.

4 Results

In our previous studies, we evaluated the suitability of the experimental pipeline for serum screening in osteoarticular pathologies for biomarker discovery and validation using a different array printing technology in each phase: contact and noncontact printing, respectively [17].

In both types of printing, identical processing steps were followed to be able to evaluate functionality and detect differences in performance. The experimental design avoided introducing additional biases to the intrinsic variability of the printing process.

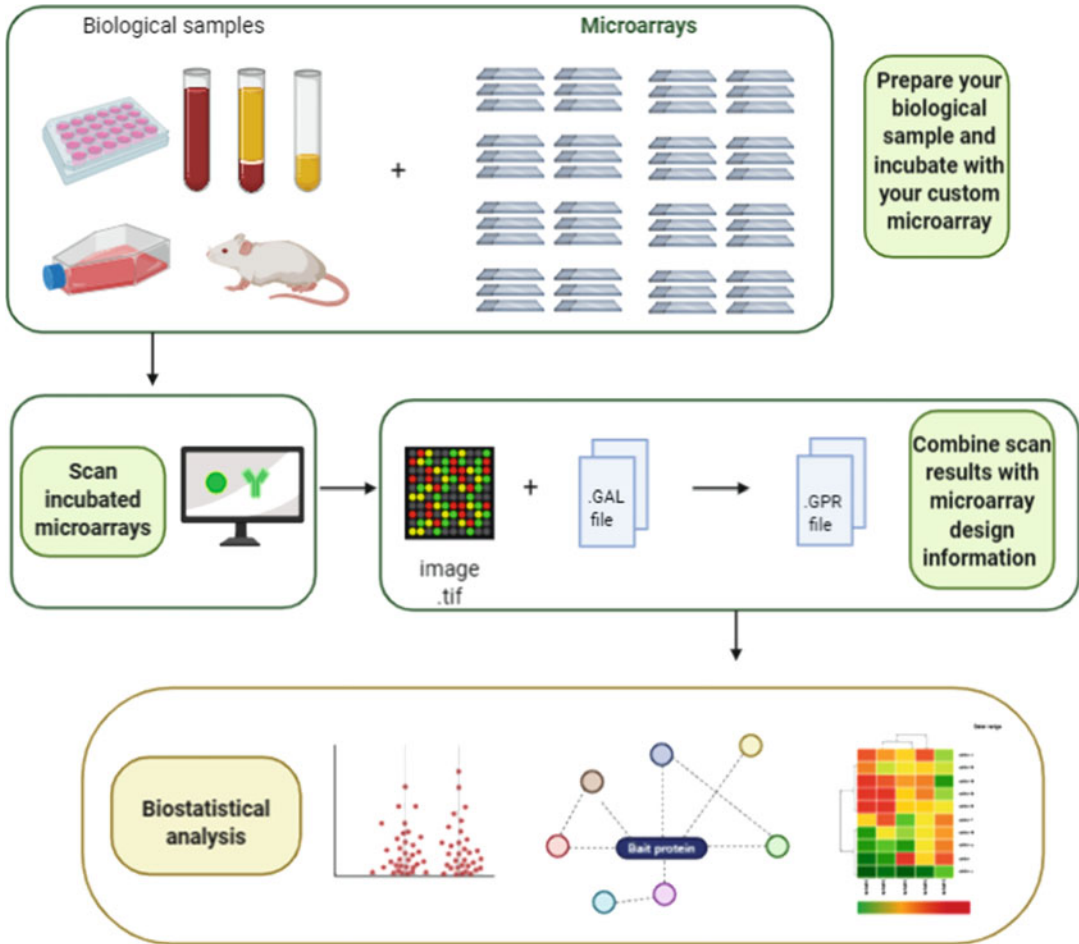


Fig. 14 Workflow for analysis results after biological assays

An identical quality control (QC) was performed to evaluate spot characteristics and to detect undesirable effects such as cross-talking or cross contamination between points in both types of arrays. Antibody arrays were incubated with HRP-conjugated anti-rabbit IgG and the expected values of each spot (i.e., antibody spot, empty spot, blank) were observed. In addition, it was determined that the variation of the signal could be related to the amount of immobilized antibodies and the saturation concentration.

Based on the quality control results, both printing methods showed high reliability for use in biomarker discovery. However, the contact arrays showed a larger shape and size of the spots, which may lead to a higher probability of cross contamination (Fig. 15). This limited the number of subarrays that can be had per array, and therefore the number of replicas and samples that could be analyzed. Furthermore, contact arrays required considerably larger

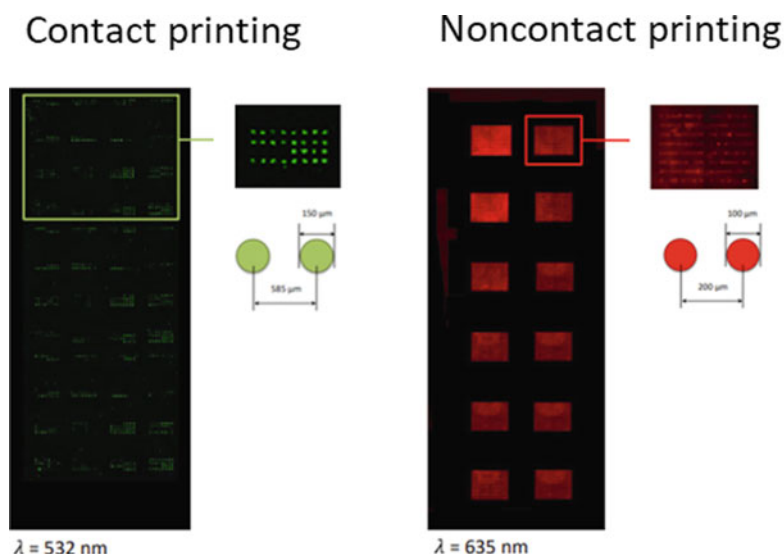


Fig. 15 Contact and noncontact printing results. (Adapted from ref. 17)

amounts of sample and reagents in printing than noncontact arrays, making it unsuitable for the analysis of large numbers of clinical samples.

Regarding the validation phase, noncontact antibody arrays were used in which more antibodies and more clinical samples were studied. This technology was selected due to the higher precision printing and the lower number of reagents and samples required. In addition, to evaluate the similarity in the performance of both technologies, the median signal normalized by antigen and the group of patients (osteoarthritis, rheumatoid arthritis, and controls) was calculated for each type of array. The correlation (r , Pearson) of these values between the different arrays for each antigen was obtained. Despite the multiple sources of variability (experimental procedures, different samples tested in each case, etc.), the correlations were positive for most of the antigens (71%), showing similar behaviors on both platforms. In fact, 31% of the antigens showed correlations higher than 0.8, which implied a measurement agreement between both technologies (Fig. 16).

5 Notes

1. It is important to print different controls (positive/negative for experimental assay) and quality controls, depending on the needs of the study. It is also interesting to print landmarks to make the final analysis easy.

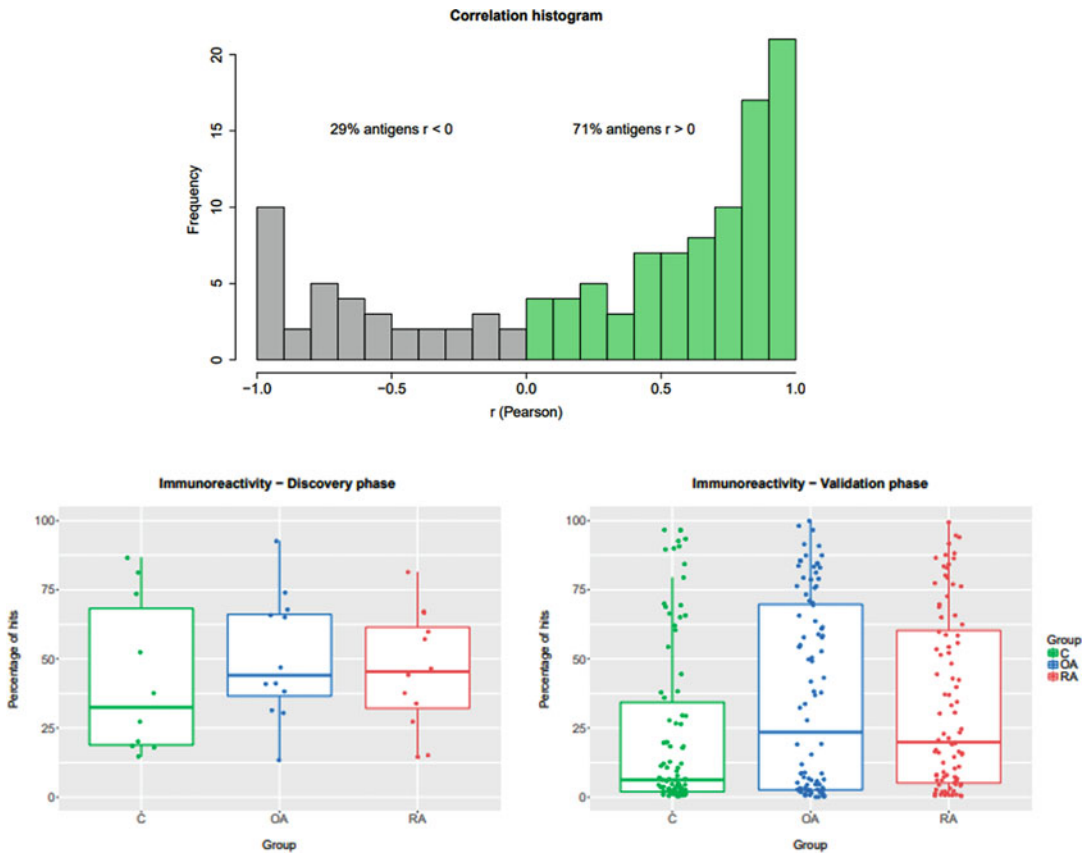


Fig. 16 Correlation of antigens by both printing technologies

2. It should be noted that the JetSpyder aspirates 12 samples at the same time but sampling is not linear. According to this fact, the map array will not correspond to the sample distribution and it is necessary to generate this sample map to distribute them properly in the plate.
3. Selecting “*Generate associated input file*” allows us to generate a file that we can combine with the GAL file.
4. The .txt and .Csv files are saved in the same folder as our microarray design.
5. Antibodies can be prepared in different concentrations (1:100, 1:200, 1:500 ... v/v) to establish a detection range within the array.
6. Use only glass material, avoiding plastic material in these steps and the further steps of microarray functionalization. Also avoid any alcoholic solvent for salinization process.
7. Use suitable protective equipment for the treatment of reagents.

8. Once the process begins, do not let the slides dry out at any time.
9. The functionalization solution can be reused twice. Be sure to discard the solution according to chemical waste management standards.
10. Check information on pressure measurements at the start and end of printing and the quality of the printed microarrays provided by the system.
11. Make sure that the plates are perfectly positioned in the correct orientation and that the microarrays are in the corresponding positions, paying special attention that none of them sticks out of the tray.
12. For a correct operation and design of the printing, include an additional wash between sampling the nanoinjection system during the printing of the microarrays.
13. Microarrays must be dried before scanning.

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References

1. Miller JC, Butler EB, Teh BS et al (2001) The application of protein microarrays to serum diagnostics: prostate cancer as a test case. *Dis Markers* 17(4):225–234
2. Gao W, Kuick R, Orzechowski RP et al (2005) Distinctive serum protein profiles involving abundant proteins in lung cancer patients based upon antibody microarray analysis. *BMC Cancer* 5(1):110
3. Tannapfel A, Anhalt K, Häusermann P et al (2003) Identification of novel proteins associated with hepatocellular carcinomas using protein microarrays. *J Pathol* 201(2):238–249
4. Díez P, Lorenzo S, Dégano RM et al (2016) Multipronged functional proteomics approaches for global identification of altered cell signalling pathways in B-cell chronic lymphocytic leukaemia. *Proteomics* 16(8):1193–1203
5. Molero C, Rodríguez-Escudero I, Aleman A et al (2009) Addressing the effects of Salmonella internalization in host cell signaling on a reverse-phase protein array. *Proteomics* 9(14):3652–3665
6. Li B, Jiang L, Song Q et al (2005) Protein microarray for profiling antibody responses to *Yersinia pestis* live vaccine. *Infect Immun* 73(6):3734–3739
7. Thanawastien A, Montor WR, LaBaer J et al (2009) *Vibrio cholerae* proteome-wide screen for immunostimulatory proteins identifies phosphatidylserine decarboxylase as a novel

- Toll-like receptor 4 agonist. *PLoS Pathog* 5(8): e1000556
8. Lourido L, Diez P, Dasilva N et al (2014) Protein microarrays: overview, applications and challenges. In: Anonymous genomics and proteomics for clinical discovery and development. Springer, New York, pp 147–173
 9. Anderson KS, Ramachandran N, Wong J et al (2008) Application of protein microarrays for multiplexed detection of antibodies to tumor antigens in breast cancer. *J Proteome Res* 7 (4):1490–1499
 10. Gibson DS, Qiu J, Mendoza EA et al (2012) Circulating and synovial antibody profiling of juvenile arthritis patients by nucleic acid programmable protein arrays. *Arthritis Res Ther* 14(2):R77
 11. Henjes F, Lourido L, Ruiz-Romero C et al (2014) Analysis of autoantibody profiles in osteoarthritis using comprehensive protein array concepts. *J Proteome Res* 13 (11):5218–5229
 12. Matarraz S, González-González M, Jara M et al (2011) New technologies in cancer. Protein microarrays for biomarker discovery. *Clin Transl Oncol* 13(3):156–161
 13. García-Valiente R, Fernández-García J, Carabias-Sánchez J et al (2019) A systematic analysis workflow for high-density customized protein microarrays in biomarker screening. In: Anonymous functional proteomics. Springer, New York, pp 107–122
 14. Juanes-Velasco P, Carabias-Sanchez J, Garcia-Valiente R et al (2018) Microarrays as platform for multiplex assays in biomarker and drug discovery. In: Rapid test-advances in design, format and diagnostic applications. IntechOpen, Rijeka
 15. Gonzalez-Gonzalez M, Jara-Acevedo R, Matarraz S et al (2012) Nanotechniques in proteomics: protein microarrays and novel detection platforms. *Eur J Pharm Sci* 45 (4):499–506
 16. Díez P, González-González M, Lourido L et al (2015) NAPPA as a real new method for protein microarray generation. *Microarrays* 4 (2):214–227
 17. Sierra-Sánchez A, Garrido-Martín D, Lourido L et al (2017) Screening and validation of novel biomarkers in osteoarticular pathologies by comprehensive combination of protein array technologies. *J Proteome Res* 16 (5):1890–1899
 18. Desmet C, Marquette CA (2016) Surface functionalization for immobilization of probes on microarrays. In: Anonymous microarray technology. Springer, New York, pp 7–23
 19. Xia Y, Whitesides GM (1998) Soft lithography. *Annu Rev Mater Sci* 28(1):153–184
 20. Marquette CA, Corgier BP, Heyries KA et al (2008) Biochips: non-conventional strategies for biosensing elements immobilization. *Front Biosci* 13(1):382–400
 21. Piner RD, Zhu J, Xu F et al (1999) Dip-pen nanolithography. *Science* 283(5402):661–663
 22. Ginger DS, Zhang H, Mirkin CA (2004) The evolution of dip-pen nanolithography. *Angew Chem Int Ed* 43(1):30–45
 23. Avseenko NV, Morozova TY, Ataullakhanov FI et al (2001) Immobilization of proteins in immunochemical microarrays fabricated by electrospray deposition. *Anal Chem* 73 (24):6047–6052
 24. González-González M, Bartolome R, Jara-Acevedo R et al (2014) Evaluation of homo- and hetero-functionally activated glass surfaces for optimized antibody arrays. *Anal Biochem* 450:37–45



Chapter 3

Phage Microarrays for Screening of Humoral Immune Responses

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Abstract

Chronic diseases are the leading cause of disability and responsible for about 63% of deaths worldwide. Among the noninfectious chronic diseases with the highest incidence are cancer and neurodegenerative diseases. Although they have been extensively studied in the last years, there is still an urgent need to find and elucidate the molecular mechanisms underlying their formation and progression to get an early diagnosis and find new therapeutic targets of intervention. Beyond other microarray-based proteomic techniques more extensively used because of their commercial availability, such as protein and antibody microarrays, phage microarrays are another kind of protein microarrays useful for the identification and characterization of disease-specific humoral immune responses and to get further insights into these devastating diseases. Here, we describe the integration and utilization of phage microarrays, which offer such a combination of sensitivity and cost-effective multiplexing capabilities that makes them an affordable strategy for the characterization of humoral immune responses in multiple diseases.

Key words Autoantibody, Biomarkers, Phage microarrays, Proteomics, Diagnostic/prognostic auto-antibody biomarkers

1 Introduction

The longer life expectancy and the progressive aging of the population are causing a marked increase in chronic diseases. According to the World Health Organization, chronic diseases are and will be, as population ages, the main economic burden on health systems. Chronic diseases are the leading cause of disability and responsible for about 63% of deaths worldwide. Cancer and neurodegenerative diseases are found among the noninfectious chronic diseases with the highest incidence.

Ana Montero-Calle and Pablo San Segundo-Acosta share authorship.

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For the identification of protein profiles and specific targets and molecular pathways, efforts have been mainly focused on using high-throughput molecular biology methods to understand the clinical behavior of cancer and neurodegenerative diseases [1–11], and other not so prevalent diseases [12–15]. The belief that the specific dysregulation of proteins and protein complexes and alterations of signature networks and pathways are associated with a particular disease is feeding proteomics. Defining specific protein expression patterns reflecting the disease would allow an early diagnosis and better prognosis, paving the way for new intervention targets and a better management of patients to offer them personalized therapeutic modalities.

Beyond mass spectrometry-based proteomics, another potential solution for identifying such protein profiles lies in the use of protein microarrays [16–21]. Protein microarray-based proteomics represents a valuable tool to improve our understanding of physiological and pathological mechanisms, for the identification of both dysregulated protein profiles and abnormalities, and the identification and characterization of disease-associated humoral immune responses. The most extensively used protein microarrays are based on high-density commercial recombinant protein microarrays and antibody microarrays. Indeed, in the market there are now available microarrays containing recombinant proteins, HuProt [22], or fragments of human proteins up to 150 amino acids in length (PrESTs) [23], bearing almost the whole human proteome (considering one protein per gene), or antibody microarrays containing several hundreds or thousands of antibodies [24]. On the other hand, phage microarrays are an alternative to study disease-specific alterations at protein level [25]. These arrays are printed with specific peptides and proteins, as produced in the pathological tissue of study due to specific mRNA alterations, displayed on the surface of M13 or T7 phages. This allows for identifying protein alterations related to the disease, such as aberrant peptides or proteins, alternative splicings, frameshifts, or point mutations, which otherwise would be missing by the screening of wild-type protein microarrays [25, 26]. Multiplexing capacities of phage microarrays allow for simultaneously surveying several hundreds or dozens of thousands of probes using small amounts of serum samples. The combination of phage display and protein microarrays enables to identify potential biomarkers and possible therapeutic targets as well as specific disease-related pathways.

In this context, we here report the protocols needed for printing and screening of phage microarrays from the phage display selection to the fabrication of the arrays, their probe with sera, data analysis, and validation for the characterization of disease-associated humoral immune responses.

2 Materials

This section outlines all common resources used in the different sections for phage microarray production and screening, followed by the specific materials and solutions used in subsequent indicated subheadings.

2.1 Common Reagents and Equipment

1. Phosphate-buffered saline pH 7.4: PBS, 137 mM NaCl, 2.7 mM KCl, 8 mM Na₂PO₄, and 1.5 mM KH₂PO₄.
2. Washing solution: 0.1% PBST, 999 mL PBS containing 1 mL Tween-20.
3. Bovine serum albumin: BSA.
4. Milli-Q water.
5. Double-distilled water: ddH₂O.
6. Microgrid II robot/sciFLEXARRAYER S3 (*see Note 1*).
7. Nitrocellulose microarrays (FAST slides, Merck).
8. Orbital shaker/oscillating rocker/roller.
9. Heating and drying oven.
10. Falcon centrifuge 5810 R.
11. Microtube centrifuge.
12. 1.5 mL Eppendorf tubes.
13. 15 mL Falcon tubes.
14. 50 mL Falcon tubes.
15. 384-Well plates.
16. 96-Well plates.
17. QuadriPERM cell culture vessel.
18. Slide hybridization polycarbonate chamber.
19. GenePix 4000B.
20. GenePix Pro 7.1.

2.2 Phage Display

1. Commercial phage libraries.
2. Control or patient serum samples.
3. Protein G Plus-Agarose beads.
4. PBS.
5. 1% BSA: 1 g BSA and 100 mL PBS.
6. 10% BSA: 10 g BSA and 100 mL PBS.
7. BLT5615 bacteria cell strain.
8. TB: 900 mL ddH₂O, 12 g Bacto tryptone, 24 g yeast extract, 4 mL glycerol, 100 mL sterile KPO₄, autoclave.

9. 20× M9 salts: 20 g NH_4Cl , 60 g KH_2PO_4 , 120 g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, pH 7.0, in 1 L ddH₂O, autoclave.
10. M9TB: 5 mL 20× M9 salts, 2 mL 20% glucose, 0.1 mL 1 M MgSO_4 in 100 mL TB.
11. Carbenicillin (*see* **Note 2**).
12. 1 M Isopropyl thio- β -D-galactoside: IPTG.
13. 100 mM Phenylmethylsulfonyl fluoride: PMSF.
14. Protease inhibitor cocktail.
15. Gelatin, from porcine skin.
16. 1% SDS.
17. 80% Glycerol.
18. Washing solution.
19. PCR Kit (TaKaRa).

2.3 Phage Microarrays

1. Blocking solution: 5% Skimmed milk in 0.1% PBST; 50 g skimmed milk powder and 1 L 0.1% PBST.
2. Centrifuge blocking solution at $1500 \times g$ for 15 min at room temperature.
3. PBS.
4. Washing solution.
5. 1 mg/mL BSA.
6. Control or patient serum samples.
7. Anti-T7 tag-antibody.
8. Anti-human IgG antibody.
9. Anti-mouse IgG antibody.
10. Alexa Fluor 647-labeled goat anti-human IgG.
11. Alexa Fluor 555-labeled goat anti-mouse IgG.

3 Methods

Phage microarrays are an economical and home-made alternative to commercial protein arrays that might be used for multiple-purpose protein-protein interaction studies, identification of ligands of small molecules, and identification of substrate kinases, among others [16, 17], probing in parallel thousands of proteins. Nevertheless, their main application has been related to the characterization of the humoral immune response [26–33], and consequently we here describe the workflow to use them appropriately with that aim.

Phage libraries, usually from T7 phages, are firstly constructed using cDNA libraries from the mRNA isolated from the target tissue. The peptides and proteins encoded by these cDNAs are subsequently displayed on the surface of the phages in frame with the C-terminal end of the 10B capsid protein. Then, the libraries are enriched in phages displaying peptides recognized by patient's sera in a process called biopanning. Finally, individual phages are printed onto nitrocellulose microarrays and hybridized with patients' and controls' sera to identify those phages displaying the most immunoreactive peptides (Fig. 1).

3.1 Phage Display

The use of phage microarrays involves multiple steps related to the enrichment of T7 phage display libraries on immunoreactive phages displaying disease-specific peptides and proteins by means of three or four rounds of selection in a procedure called biopanning, monoclonal phage amplification, printing of individual phages, and, finally, microarray hybridization. For a full description of the procedures related to T7 phage library construction, biopanning, and phage amplification *see* refs. 26–29.

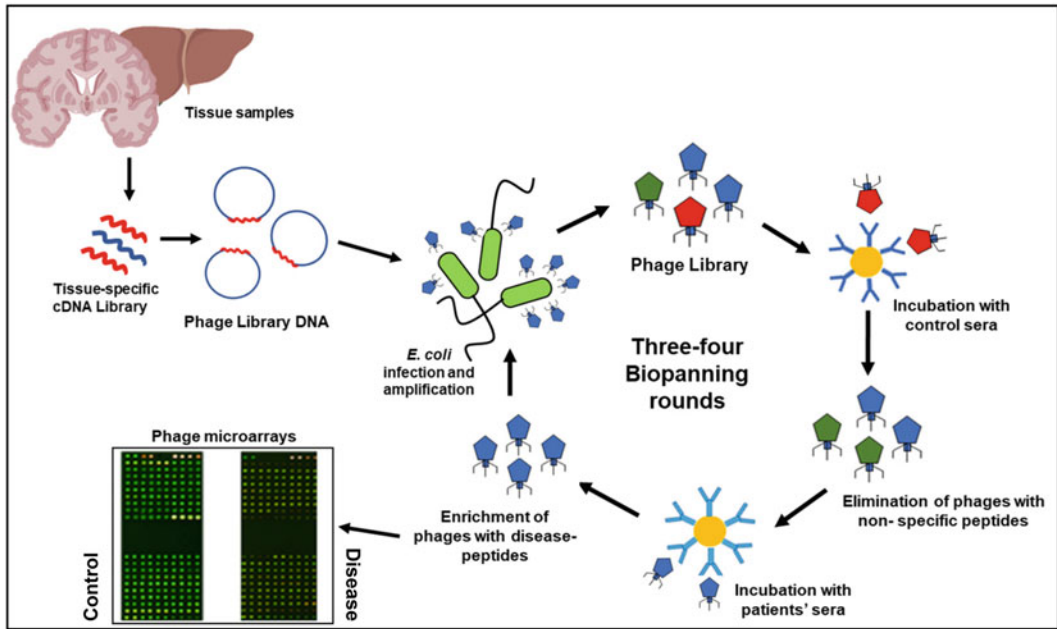
After T7 phage display library construction, biopanning allows the enrichment of the library in about 10^6 times in immunoreactive phages to IgG of the same disease whose libraries were constructed. The protocol involves a negative selection with controls' sera to remove unspecific phages, and thereafter a positive selection with patients' sera to enrich on specific phages, in a multistep procedure described below.

3.1.1 Patient and Control Serum IgG Isolation

1. Prepare the necessary Eppendorf tubes to perform the biopanning (one tube per patient or control sera) (*see* **Note 3**).
2. Transfer 25 μ L of Protein G Plus-Agarose beads per each 1.5 mL Eppendorf tube.
3. Equilibrate beads with 350 μ L PBS per tube.
4. Centrifuge at $2000 \times g$ for 1 min.
5. Discard the supernatant.
6. Repeat **steps 3–5** twice.
7. Block beads with 350 μ L 1% BSA for 1 h at 4 °C on a roller.
8. Centrifuge at $2000 \times g$ for 1 min.
9. Discard the supernatant.
10. Incubate beads with 250 μ L of control or patient serum 1:20 diluted in 1% BSA during 4 h at 4 °C on a roller.

3.1.2 Amplification of T7 Phage Display Libraries

1. Prepare an overnight BLT5615 bacterial culture on M9TB containing 50 μ g/mL carbenicillin.
2. The day after, propagate 1.5 mL overnight BLT5615 culture onto 29 mL M9TB containing 50 μ g/mL carbenicillin.



Validation

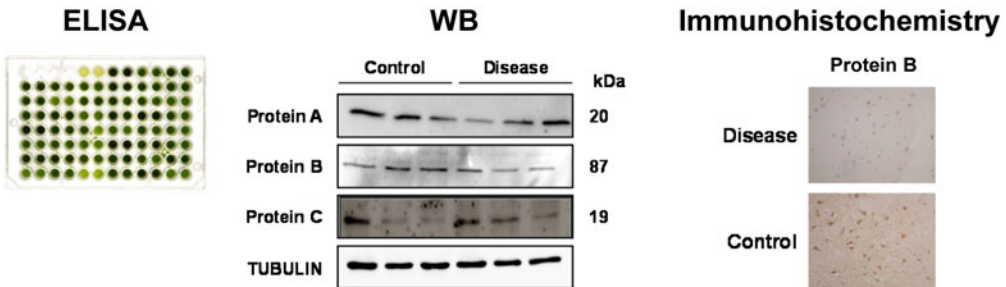


Fig. 1 Technical workflow for the construction of a T7 phage library and its subsequent biopanning process to get phage microarrays to be screened for the identification of potential biomarkers related to the humoral immune responses that patients develop against specific chronic diseases. Construction of T7 phage libraries is made from purified total mRNA from control and diseased tissue. Then, phage display libraries are enriched in specific immunoreactive phages to IgGs from diseased patients by means of a so-called biopanning procedure. By negative selection, immunoreactive phages against control antibodies are removed, and then phages binding to the antibodies of interest are recovered and re-amplified. The biopanning process can be repeated three or four times for a higher enrichment. Then, the enriched phage library is amplified and monoclonal phages are isolated, individually re-amplified, and printed on the microarrays. Subsequently, phage microarrays are screened with controls' and patients' sera to identify phages displaying disease-specific peptides or proteins. After image quantification, normalization, and data analysis, those phages significantly recognized by IgGs from diseased patients in comparison to controls are amplified for a further validation using orthogonal techniques (i.e., ELISA, WB, or IHC)

3. Incubate at 37 °C until the OD₆₀₀ reaches 0.4–0.5 shaking at 250 rpm. Subsequently, add IPTG (1 mM final concentration) and shake for 20–30 min more at 37 °C and 250 rpm.
4. Inoculate 1 µL of the phage display library into 5 mL IPTG-induced bacterial BLT5615 culture in a 15 mL Falcon tube. Use 50 µL of eluted phages for amplification after each round of biopanning. Incubate at 37 °C and 250 rpm until complete lysis is observed (*see* **Note 4**).
5. Mix each 5 mL bacterial lysate with 50 µL PMSF (1 mM final concentration), 50 µL protease inhibitor cocktail, and 50 µL 2% gelatin.
6. Remove cell debris by centrifugation at 4000 × *g* during 10 min.
7. Transfer the supernatant containing the amplified phage—ready for biopanning—to a new 15 mL Falcon tube (*see* **Note 5**).

**3.1.3 Removal of Phages
Displaying Unspecific
Peptides or Proteins
(Negative Selection)**

1. Wash beads incubated with controls' sera using 350 µL PBS.
2. Discard the supernatant by centrifugation at 2000 × *g* for 1 min (*see* **Note 6**).
3. Repeat **steps 1** and **2** twice. Perform centrifugations at 4 °C unless indicated.
4. Mix 35 µL of a 10% BSA with 315 µL out of the 5 mL amplified phage.
5. Incubate with controls' sera beads of **step 3** for 2 h at 4 °C on a roller.
6. Collect the supernatant (SP1) by centrifugation at 2000 × *g* for 1 min.

**3.1.4 Enrichment
of the T7 Phage Library
on Specific Phages
(Positive Selection)**

1. Wash beads incubated with patients' sera using 350 µL PBS.
2. Discard supernatant after centrifugation at 2000 × *g* for 1 min.
3. Repeat **steps 1** and **2** twice.
4. Incubate patients' sera beads with supernatant SP1 overnight at 4 °C on a roller.
5. Centrifuge at 2000 × *g* for 1 min and remove supernatant.
6. Wash with 350 µL PBS.
7. Repeat **steps 5** and **6** twice in the first round, five in the second round, and ten times in the third and fourth rounds of biopanning.
8. Elute phages by incubating with 100 µL 1% SDS during 10 min at room temperature on a roller.

9. Centrifuge for 5 min at $5000 \times g$. Collect the eluted phages: save 10 μL at -80°C for phage titration (*see* **Note 7**) [26] and 50 μL for phage amplification, and store the remaining phage containing glycerol (20% final concentration) at -80°C .

3.1.5 Monoclonal Phage Amplification

After biopanning, 1824 individual monoclonal phages from the third and fourth rounds of selection are individually amplified onto 96-well plates. After centrifugation to remove cell debris, amplified phages are diluted 1:3 in 0.1% PBST. Then, 20 μL of 1:3 PBS-diluted individual phages are transferred onto 384-well plates for microarray printing onto nitrocellulose slides.

Prior to transferring monoclonal phages to 384-well plates and printing the microarrays, it is needed to confirm the absence of phage cross-contamination between wells and analyze the diversity of the inserted cDNA sequences on phages by PCR (*see* **Notes 8 and 9**).

3.2 Phage Microarrays

The next steps after phage display involve the printing of individual phages onto nitrocellulose slides and their hybridization and screening to identify specific reactive phages of the pathology of interest.

3.2.1 Printing

The printing of the T7 phages and controls is usually made using solid pins and contact printing on nitrocellulose microarrays with a Microgrid II robot at a constant temperature of 20°C and a relative humidity of 50%. However, other automated piezo-driven robots with a noncontact dispensing system can be alternatively used for printing, as the sciFLEXARRAYER S3, maintaining the temperature at 20°C and the humidity at 50%.

In total, 1824 individual monoclonal amplified phages and 96 controls distributed into five 384-well plates are printed in duplicate onto nitrocellulose slides. A total of 3840 spots are printed at a recommended space of 400 μm using 24 tips (Microgrid II robot) or 8 capillaries (sci FLEXARRAYER S3) (*see* **Note 10**).

After printing, nitrocellulose slides are maintained in the robot at a constant relative humidity of 45% between 4 h and maximum time of 16 h. Then, microarrays are removed from the robot, dried, and stored at -20°C until use.

3.2.2 Hybridization

While phage microarrays can be used to identify protein-protein interactions, small-molecule binding, etc., where the hybridization should involve the use of labeled proteins or small molecules for a better detection of the interaction, the workflow for the screening of the humoral immune response of patients and controls involves the use, apart from the serum or plasma of patients and controls, of secondary antibodies labeled with fluorophores for the detection of disease-specific IgGs, IgMs, or IgAs.

1. Equilibrate the microarrays at 4 °C for 10 min and then at room temperature for 10 min prior to starting with subsequent steps.
2. Equilibrate the necessary microarrays to perform the experiment (one microarray per patient or control sera). Make subsequent steps on a quadriPERM cell culture vessel unless otherwise indicated.
3. Incubate the nitrocellulose phage microarrays with 4 mL of PBS for 5 min to completely rehydrate the slides.
4. Discard and remove the excess of PBS (*see Note 11*). Immediately incubate the microarrays with centrifuged blocking solution at $1500 \times g$ for 1 h at room temperature on a roller at 30 rpm (*see Note 12*).
5. Discard and remove the excess of blocking solution II. Immediately incubate the array with 1.6 mL of 1:300 dilution of serum and 1:200 dilution of anti-T7 tag antibody in a blocking solution for 90 min at room temperature on a roller at 30 rpm (*see Note 13*). Use a hybridization chamber for this incubation (*see Note 14*).
6. Wash each array with 4 mL of washing solution for 5 min at room temperature at 30 rpm.
7. Discard and remove the excess of washing solution.
8. Repeat **steps 6 and 7** five more times.
9. Immediately incubate the microarrays with 2 mL blocking solution containing 1:2000 diluted Alexa Fluor 647-labeled goat anti-human IgG antibody and 1:10,000 diluted Alexa Fluor 555-labeled goat anti-mouse IgG antibody at 30 rpm (*see Note 15*).
10. Repeat **steps 6–8**.
11. To remove detergent traces wash again twice with PBS for 5 min at room temperature at 30 rpm and then in Milli-Q water to remove salt traces.
12. Dry the phage microarrays by centrifugation at $260 \times g$ for 10 min in a 50 mL falcon tube containing absorbent paper at the bottom (*see Note 16*). If the microarray possesses a printed barcode put the barcode at the bottom of the falcon tube (*see Note 17*).

3.3 Scanning and Data Analysis

For microarray scanning, a specific microarray scanner compatible with all surfaces, including nitrocellulose, and containing at least 532 and 635 nm solid-state lasers is needed, such as the GenePix 4000B.

The 635 nm laser (red channel) is used for the detection of Alexa Fluor 647, Cy5, or any dye spectrally similar in signal, whereas the 532 nm laser (green channel) is used for Alexa Fluor 555, Cy3, or any dye spectrally similar.

For a better analysis of low- and high-reactive phages (spots), we recommend to scan phage microarrays at two laser powers (i.e., 100% and 10%). However, the ideal laser power percentages need to be adjusted during scanning. Scan all the microarrays at the same laser powers for proper subsequent data normalization.

The obtained images are next quantified using a microarray acquisition and analysis software allowing for intra-array normalization using the background signal around each spot (i.e., GenePix Pro 7.1 program). Then, data from the software for each spot in the microarray is inter-array normalized and processed. We recommend using the median intensity of each spot for data processing using the desired statistical tests from free available webpage-specific bioinformatics tools, such as babelomics suite for functional profiling analysis, Pomelo II, or MultiExperiment Viewer [34–36]. Babelomics allows intra-array normalization and subsequent data processing, whereas Pomelo II and MultiExperiment Viewer allow for data processing to obtain those statistically significant upregulated and downregulated seroreactive phages displaying disease-associated peptides or proteins (*see Note 18*).

3.4 Validation

Since the identification of false positives is a problem that can take place using high-throughput techniques, results derived from high-density microarray-based proteomic technologies must be validated by other approaches to ensure the accuracy of the results. Indeed, a recurring problem with regard to massive data analysis is the lack of validation of the derived results (either unaltered or altered proteins). Thus, this lack of validation and the actual possibility of finding erroneous identifications can pose problems due to the subsequent use of these proteins, which ultimately would produce a delay in the translation of results into clinical diagnosis and in the identification of new therapeutic targets.

Therefore, additional validation and functional analysis of the data obtained from phage microarrays should be mandatory to get available validated data by using alternative techniques including dot blot, WB, ELISA, or in-solution immunoassays [10, 11, 37, 38]. However, this strategy could be restricted due to the absence of adequate and affordable antibodies that recognize the peptides or proteins of interest. The acceptance criteria of the validation assay must take into account the objectives of the study, the nature of the methodology, and the biological variability of the biomarker. Taken together, these validation steps should result in high-quality validated bioanalytical data available to the scientific community.

4 Notes

1. Either a MicroGrid II robot using solid pins or a sciFLEXAR RAYER S3 piezo-driven robot with a noncontact dispensing system with the here-described printing buffer has been satisfactorily used for printing T7 phages in nitrocellulose slides. Alternatively, other microarray printers can be used. However, printing buffer and conditions should be tested prior to a high-density printing.
2. Carbenicillin is a semisynthetic ampicillin analog that is more stable than ampicillin in growth media because it has better tolerance for heat and acidity. However, if needed it can be substituted by ampicillin in the here-described experiments.
3. Either individual or pool of sera can be used. Most works report the use of sera pools during biopanning and individual serum for the screening of phage microarrays.
4. To facilitate the visualization of the lysis because of T7 phage usage, it is recommended to include as control a noninfected bacterial cell culture to monitor a correct lysis either during biopanning or during the growth of monoclonal phages in 96-well plates.
5. The phage library is amplified to be used only in the first round of selection during biopanning. Subsequent amplified phages are consecutively used in the next rounds of selection (i.e., amplified eluted phages from the first round is used in the second round of biopanning and so on).
6. Do not centrifuge at higher speeds than $2000 \times g$ to avoid beads from crashing, except during T7 phage elution to recover as much eluted phage as possible.
7. To determine the number of phages and confirm the enrichment of the library in specific phages, the eluted and amplified phages from each round of biopanning should be titrated [26]. An increase in the number of phages should be observed between the eluted phages from the first and the third and fourth rounds of biopanning if enrichment is observed.
8. The diversity of the DNAs inserted on the phages of the third and fourth rounds of biopanning should be analyzed, since a high diversity is needed in the monoclonal phages printed on nitrocellulose microarrays. The diversity is analyzed by PCR using specific primers (forward primer T7_up2: 5'-TGCTAAG GACAACGTTATCGG-3' and reverse primer T7_down2: 5'-TTGATACCGGACGTTTCAC-3') annealing in the genome of the phage upstream and downstream of the foreign inserted DNAs and visualized using 1.5% agarose gels containing GelRed [26–28].

9. T7 phage DNA isolation has to be performed for sequencing of foreign DNA. T7 phages are precipitated using PEG/NaCl [11], and then T7 phage DNA can be purified and directly used for PCR amplification and sequencing using the described specific primers in **Note 8**.
10. BSA at a concentration of 1 mg/mL, empty T7 phages (or a T7 phage displaying a known sequence), and printing buffer (0.1% PBST) are the recommended negative controls to be included in the array. Human and mouse IgG at 0.1 mg/mL and three tenfold serial dilutions, and 0.1 mg/mL Alexa Fluor 647-labeled goat anti-human IgG and 0.1 mg/mL Alexa Fluor 555-labeled goat anti-mouse IgG, are recommended as positive controls. It is recommended to distribute positive and negative controls in the upper and lower corners of each sub-array for a good alignment of the grid for image quantification.
11. Be careful to avoid the nitrocellulose slide to be dried after any incubation or during washes to avoid unspecific signal or an increase in the background signal of the microarrays.
12. If any of the subsequent steps involves the use of biotinylated secondary antibodies to further amplify the signal with fluorophore-labeled streptavidin do not block the membrane with skimmed milk (use 5% BSA as blocking agent). The presence of biotin in milk could abrogate the specific signal of subsequent steps or increase the background signal of the microarray.
13. A 1:300 dilution of serum is the standard dilution in most experiments. However, the optimal serum dilution to be used in the phage microarrays should be tested between 1:50 and 1:600 prior to incubation with a large set of samples. Plasma instead of serum could also be used.
14. It is recommended to hybridize a phage microarray only with an anti-T7 antibody followed by the appropriate secondary antibody as quality control of printing. In addition, it is also recommended to probe a phage microarray with only the secondary labeled antibodies to identify potential unspecific reactive phages recognized by the secondary antibodies.
15. Keep the fluorophores away from light using aluminum foil during any step involving fluorophore-labeled antibodies. If IgM or IgA autoantibodies are the purpose of the study, use appropriate secondary fluorescently labeled antibodies to detect them instead of Alexa Fluor 647-labeled goat anti-human IgG.
16. Prior to microarray scanning it is recommended to apply filtered air to remove any particle from the surface and completely dry the slides. It is also recommended to scan the

nitrocellulose slides the day after hybridization to allow the matrices to dry completely and to avoid the presence of traces of moisture that can interfere during scanning.

17. Put the microarray with the barcode on the bottom of the falcon tube; otherwise during the centrifugations the barcode glue can potentially affect the signal and significantly increase the background on the microarray.
18. Since webpage-specific bioinformatics tools use different algorithms to obtain significantly dysregulated proteins, it is recommended to use different programs and validate those reactive phages in common since they are more prone to be actual targets of autoantibodies. In addition, validate as much top hits identified by the different programs as possible.

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References

1. Moya-Alvarado G, Gershoni-Emek N, Perlson E, Bronfman FC (2016) Neurodegeneration and Alzheimer's disease (AD). What can proteomics tell us about the Alzheimer's brain? *Mol Cell Proteomics* 15(2):409–425. <https://doi.org/10.1074/mcp.R115.053330>
2. Lovestone S, Guntert A, Hye A, Lynham S, Thambisetty M, Ward M (2007) Proteomics of Alzheimer's disease: understanding mechanisms and seeking biomarkers. *Expert Rev Proteomics* 4(2):227–238. <https://doi.org/10.1586/14789450.4.2.227>
3. Aebersold R, Mann M (2016) Mass-spectrometric exploration of proteome structure and function. *Nature* 537(7620):347–355. <https://doi.org/10.1038/nature19949>
4. Jaeger PA, Lucin KM, Britschgi M, Vardarajan B, Huang RP, Kirby ED, Abbey R, Boeve BF, Boxer AL, Farrer LA, Finch N, Graff-Radford NR, Head E, Hoffree M, Huang R, Johns H, Karydas A, Knopman DS, Loboda A, Masliah E, Narasimhan R, Petersen RC, Podtelezchnikov A, Pradhan S, Rademakers R, Sun CH, Younkin SG, Miller BL, Ideker T, Wyss-Coray T (2016) Network-driven plasma proteomics expose molecular changes in the Alzheimer's brain. *Mol Neurodegener* 11:31. <https://doi.org/10.1186/s13024-016-0095-2>
5. Mallick P, Kuster B (2010) Proteomics: a pragmatic perspective. *Nat Biotechnol* 28(7):695–709. <https://doi.org/10.1038/nbt.1658>
6. Zelaya MV, Perez-Valderrama E, de Morentin XM, Tunon T, Ferrer I, Luquin MR, Fernandez-Irigoyen J, Santamaria E (2015) Olfactory bulb proteome dynamics during the

- progression of sporadic Alzheimer's disease: identification of common and distinct olfactory targets across Alzheimer-related co-pathologies. *Oncotarget* 6(37):39437–39456. <https://doi.org/10.18632/oncotarget.6254>
7. Garranzo-Asensio M, San Segundo-Acosta P, Martinez-Useros J, Montero-Calle A, Fernandez-Acenero MJ, Haggmark-Manberg-A, Pelaez-Garcia A, Villalba M, Rabano A, Nilsson P, Barderas R (2018) Identification of prefrontal cortex protein alterations in Alzheimer's disease. *Oncotarget* 9 (13):10847–10867. <https://doi.org/10.18632/oncotarget.24303>
 8. Garranzo-Asensio M, San Segundo-Acosta P, Poves C, Fernandez-Acenero MJ, Martinez-Useros J, Montero-Calle A, Solis-Fernandez G, Sanchez-Martinez M, Rodriguez N, Ceron MA, Fernandez-Diez S, Dominguez G, de Los Rios V, Pelaez-Garcia A, Guzman-Aranguez A, Barderas R (2020) Identification of tumor-associated antigens with diagnostic ability of colorectal cancer by in-depth immunomic and seroproteomic analysis. *J Proteome* 214:103635. <https://doi.org/10.1016/j.jprot.2020.103635>
 9. Katchman BA, Barderas R, Alam R, Chowell D, Field MS, Esserman LJ, Wallstrom G, LaBaer J, Cramer DW, Hollingsworth MA, Anderson KS (2016) Proteomic mapping of p53 immunogenicity in pancreatic, ovarian, and breast cancers. *Proteomics Clin Appl* 10(7):720–731. <https://doi.org/10.1002/prca.201500096>
 10. Montero-Calle A, San Segundo-Acosta P, Garranzo-Asensio M, Rabano A, Barderas R (2020) The molecular misreading of APP and UBB induces a humoral immune response in Alzheimer's disease patients with diagnostic ability. *Mol Neurobiol* 57(2):1009–1020. <https://doi.org/10.1007/s12035-019-01809-0>
 11. San Segundo-Acosta P, Montero-Calle A, Fuentes M, Rabano A, Villalba M, Barderas R (2019) Identification of Alzheimer's disease autoantibodies and their target biomarkers by phage microarrays. *J Proteome Res* 18 (7):2940–2953. <https://doi.org/10.1021/acs.jproteome.9b00258>
 12. San Segundo-Acosta P, Garranzo-Asensio M, Oeo-Santos C, Montero-Calle A, Quiralte J, Cuesta-Herranz J, Villalba M, Barderas R (2018) High-throughput screening of T7 phage display and protein microarrays as a methodological approach for the identification of IgE-reactive components. *J Immunol Methods* 456:44–53. <https://doi.org/10.1016/j.jim.2018.02.011>
 13. San Segundo-Acosta P, Oeo-Santos C, Benede S, de Los Rios V, Navas A, Ruiz-Leon B, Moreno C, Pastor-Vargas C, Jurado A, Villalba M, Barderas R (2019) Delin-eation of the olive pollen proteome and its allergenome unmasks cyclophilin as a relevant cross-reactive allergen. *J Proteome Res* 18 (8):3052–3066. <https://doi.org/10.1021/acs.jproteome.9b00167>
 14. Henjes F, Lourido L, Ruiz-Romero C, Fernandez-Tajes J, Schwenk JM, Gonzalez-Gonzalez M, Blanco FJ, Nilsson P, Fuentes M (2014) Analysis of autoantibody profiles in osteoarthritis using comprehensive protein array concepts. *J Proteome Res* 13 (11):5218–5229. <https://doi.org/10.1021/pr500775a>
 15. Lourido L, Ayoglu B, Fernandez-Tajes J, Oreiro N, Henjes F, Hellstrom C, Schwenk JM, Ruiz-Romero C, Nilsson P, Blanco FJ (2017) Discovery of circulating proteins associated to knee radiographic osteoarthritis. *Sci Rep* 7(1):137. <https://doi.org/10.1038/s41598-017-00195-8>
 16. Fasolo J, Im H, Snyder MP (2015) Probing high-density functional protein microarrays to detect protein-protein interactions. *J Vis Exp* 102:e51872. <https://doi.org/10.3791/51872>
 17. LaBaer J, Ramachandran N (2005) Protein microarrays as tools for functional proteomics. *Curr Opin Chem Biol* 9(1):14–19. <https://doi.org/10.1016/j.cbpa.2004.12.006>
 18. Mattoon DR, Schweitzer B (2009) Profiling protein interaction networks with functional protein microarrays. *Methods Mol Biol* 563:63–74. https://doi.org/10.1007/978-1-60761-175-2_4
 19. Moore CD, Ajala OZ, Zhu H (2016) Applications in high-content functional protein microarrays. *Curr Opin Chem Biol* 30:21–27. <https://doi.org/10.1016/j.cbpa.2015.10.013>
 20. Barderas R, Villar-Vázquez R, Casal JI (2014) Colorectal cancer circulating biomarkers. In: Preedy VR, Patel VB (eds) *Biomarkers in cancer*. Springer Netherlands, Dordrecht, pp 1–21. https://doi.org/10.1007/978-94-007-7744-6_29-1
 21. Barderas R, Babel I, Casal JI (2010) Colorectal cancer proteomics, molecular characterization and biomarker discovery. *Proteomics Clin Appl* 4(2):159–178. <https://doi.org/10.1002/prca.200900131>
 22. Hu C, Huang W, Chen H, Song G, Li P, Shan Q, Zhang X, Zhang F, Zhu H, Wu L, Li Y (2015) Autoantibody profiling on human

- proteome microarray for biomarker discovery in cerebrospinal fluid and sera of neuropsychiatric lupus. *PLoS One* 10(5):e0126643. <https://doi.org/10.1371/journal.pone.0126643>
23. Sjoberg R, Mattsson C, Andersson E, Hellstrom C, Uhlen M, Schwenk JM, Ayoglu B, Nilsson P (2016) Exploration of high-density protein microarrays for antibody validation and autoimmunity profiling. *N Biotechnol* 33(5 Pt A):582–592. <https://doi.org/10.1016/j.nbt.2015.09.002>
 24. Huang W, Whittaker K, Zhang H, Wu J, Zhu SW, Huang RP (2018) Integration of antibody array technology into drug discovery and development. *Assay Drug Dev Technol* 16(2):74–95. <https://doi.org/10.1089/adt.2017.808>
 25. Freckleton G, Lippman SI, Broach JR, Tavaoie S (2009) Microarray profiling of phage-display selections for rapid mapping of transcription factor-DNA interactions. *PLoS Genet* 5(4):e1000449. <https://doi.org/10.1371/journal.pgen.1000449>
 26. Babel I, Barderas R, Diaz-Uriarte R, Moreno V, Suarez A, Fernandez-Acenero MJ, Salazar R, Capella G, Casal JI (2011) Identification of MST1/STK4 and SULF1 proteins as autoantibody targets for the diagnosis of colorectal cancer by using phage microarrays. *Mol Cell Proteomics* 10(3):M110 001784. <https://doi.org/10.1074/mcp.M110.001784>
 27. Chatterjee M, Ionan A, Draghici S, Tainsky MA (2006) Epitomics: global profiling of immune response to disease using protein microarrays. *OMICS* 10(4):499–506. <https://doi.org/10.1089/omi.2006.10.499>
 28. Chatterjee M, Mohapatra S, Ionan A, Bawa G, Ali-Fehmi R, Wang X, Nowak J, Ye B, Nahhas FA, Lu K, Witkin SS, Fishman D, Munkarah A, Morris R, Levin NK, Shirley NN, Tromp G, Abrams J, Draghici S, Tainsky MA (2006) Diagnostic markers of ovarian cancer by high-throughput antigen cloning and detection on arrays. *Cancer Res* 66(2):1181–1190. <https://doi.org/10.1158/0008-5472.CAN-04-2962>
 29. Wang X, Yu J, Sreekumar A, Varambally S, Shen R, Giacherio D, Mehra R, Montie JE, Pienta KJ, Sanda MG, Kantoff PW, Rubin MA, Wei JT, Ghosh D, Chinnaiyan AM (2005) Autoantibody signatures in prostate cancer. *N Engl J Med* 353(12):1224–1235. <https://doi.org/10.1056/NEJMoa051931>
 30. Nagele E, Han M, Demarshall C, Belinka B, Nagele R (2011) Diagnosis of Alzheimer's disease based on disease-specific autoantibody profiles in human sera. *PLoS One* 6(8):e23112. <https://doi.org/10.1371/journal.pone.0023112>
 31. Nagele EP, Han M, Acharya NK, DeMarshall C, Kosciuk MC, Nagele RG (2013) Natural IgG autoantibodies are abundant and ubiquitous in human sera, and their number is influenced by age, gender, and disease. *PLoS One* 8(4):e60726. <https://doi.org/10.1371/journal.pone.0060726>
 32. DeMarshall C, Sarkar A, Nagele EP, Goldwaser E, Godsey G, Acharya NK, Nagele RG (2015) Utility of autoantibodies as biomarkers for diagnosis and staging of neurodegenerative diseases. *Int Rev Neurobiol* 122:1–51. <https://doi.org/10.1016/bs.irn.2015.05.005>
 33. Han M, Nagele E, DeMarshall C, Acharya N, Nagele R (2012) Diagnosis of Parkinson's disease based on disease-specific autoantibody profiles in human sera. *PLoS One* 7(2):e32383. <https://doi.org/10.1371/journal.pone.0032383>
 34. Morrissey ER, Diaz-Uriarte R (2009) Pomelo II: finding differentially expressed genes. *Nucleic Acids Res* 37(Web Server issue):W581–W586. <https://doi.org/10.1093/nar/gkp366>
 35. Alonso R, Salavert F, Garcia-Garcia F, Carbonell-Caballero J, Bleda M, Garcia-Alonso L, Sanchis-Juan A, Perez-Gil D, Marin-Garcia P, Sanchez R, Cubuk C, Hidalgo MR, Amadoz A, Hernansaiz-Ballesteros RD, Aleman A, Tarraga J, Montaner D, Medina I, Dopazo J (2015) Babelomics 5.0: functional interpretation for new generations of genomic data. *Nucleic Acids Res* 43(W1):W117–W121. <https://doi.org/10.1093/nar/gkv384>
 36. Howe EA, Sinha R, Schlauch D, Quackenbush J (2011) RNA-Seq analysis in MeV. *Bioinformatics* 27(22):3209–3210. <https://doi.org/10.1093/bioinformatics/btr490>
 37. Garranzo-Asensio M, Guzman-Aranguez A, Povedano E, Ruiz-Valdepenas Montiel V, Poves C, Fernandez-Acenero MJ, Montero-Calle A, Solis-Fernandez G, Fernandez-Diez S, Camps J, Arenas M, Rodriguez-Tomas E, Joven J, Sanchez-Martinez M, Rodriguez N, Dominguez G, Yanez-Sedeno P, Pingarron JM, Campuzano S, Barderas R (2020) Multiplexed monitoring of a novel autoantibody diagnostic signature of colorectal cancer using HaloTag technology-based electrochemical immunosensing platform. *Theranostics* 10(7):3022–3034. <https://doi.org/10.7150/thno.42507>
 38. Garranzo-Asensio M, Guzman-Aranguez A, Poves C, Fernandez-Acenero MJ, Torrente-

Rodriguez RM, Ruiz-Valdepenas Montiel V, Dominguez G, Frutos LS, Rodriguez N, Villalba M, Pingarron JM, Campuzano S, Barderas R (2016) Toward liquid biopsy: determination of the humoral immune response in

cancer patients using HaloTag fusion protein-modified electrochemical bioplatfroms. *Anal Chem* 88(24):12339–12345. <https://doi.org/10.1021/acs.analchem.6b03526>



Chapter 4

Identification of Antibody Biomarker Using High-Density Nucleic Acid Programmable Protein Array

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Abstract

A novel protein microarray technology, called high-density nucleic acid programmable protein array (HD-NAPPA), enables the serological screening of thousands of proteins at one time. HD-NAPPA extends the capabilities of NAPPA, which produces protein microarrays on a conventional glass microscope slide. By comparison, HD-NAPPA displays proteins in over 10,000 nanowells etched in a silicon slide. Proteins on HD-NAPPA are expressed in the individual isolated nanowells, via in vitro transcription and translation (IVTT), without any diffusion during incubation. Here we describe the method for antibody biomarker identification using HD-NAPPA, including four main steps: (1) HD-NAPPA array protein expression, (2) primary antibodies (serum/plasma) probing, (3) secondary antibody visualization, and (4) image scanning and data processing.

Key words Nucleic acid programmable protein array, HD-NAPPA, Biomarker, Antibody biomarker discovery, Silicon nanowells

1 Introduction

Protein microarrays are typically printed on a microscope slide, which can be used as immunoassays that identify multiple antibody biomarkers. Conventional purified protein microarrays require laborious protein transformation/transfection, extraction or purification, and then spotting on the array. By comparison, nucleic acid programmable protein array (NAPPA) [1, 2] expresses proteins in situ directly on the array. The processing steps for NAPPA are (1) spot cDNA plasmid and a capturing reagent, like fusion tag antibody, in an array format onto a glass slide; (2) express the protein, with a fusion tag, using in vitro transcription and translation (IVTT) reagent; and (3) in situ capture the expressed proteins through the binding of the fusion tag and the fusion tag antibody. Compared to purified protein microarrays, NAPPA allows for the

programmable layout of the proteins on the array and prolongs the array's shelf life and makes proteins freshly at the time of immunoassay.

High-density NAPPAs (HD-NAPPAs) are silicon nanowell versions of NAPPAs, which spot the cDNA plasmid and capture agents into individual nanowells. The individual nanowells are sealed during protein expression, minimizing proteins' diffusion during the *in vitro* transcription and translation incubation process. Compared to NAPPAs, HD-NAPPAs reach a higher throughput of more than 10,000 proteins per array. So far, HD-NAPPAs have been used for antibody biomarker identification of *Mycobacterium tuberculosis* (Mtb), type 1 diabetes (T1D), and Borrelia disease [3–5]. In this chapter, we describe antibody biomarker identification using HD-NAPPAs in four steps: (1) protein array expression, (2) primary antibody (serum/plasma) probing, (3) secondary antibody visualization, and (4) image scanning and data processing [5, 6].

2 Materials

Prepare all solutions using 18 MΩ cm ultrapure water (at 25 °C), prepared by purifying deionized water. All reagents are at an analytical grade level. Prepare and store reagents as listed conditions (temperature and moisture). Process and dispose of reagents according to standard material safety data sheets (MSDS).

2.1 HD-NAPPA Protein Array Expression Materials

1. Stocking assay running buffer: 10× PBS, pH 6.8. Add about 100 mL water to a 1 L glass beaker (*see Note 1*). Weight 25.6 g Na₂HPO₄·7H₂O (or 13.6 g Na₂HPO₄), 80 g NaCl, 2 g KCl, and 2 g KH₂PO₄ and transfer to the beaker. Add water to a volume of 900 mL. Mix and adjust pH to 6.8 with HCl (*see Note 2*). Add water to a volume of 1 L. Store at room temperature.
2. Assay running buffer: 0.2% PBST, pH 7.2. Take 100 mL of 10× PBS (pH 6.8) in a graduated cylinder. Add 800 mL water to the cylinder. Take 200 μL Tween 20 (*see Note 3*) and add to the cylinder. Make up to 1 L with water. Store at 4 °C.
3. HD-NAPPA pre-expression blocking buffer: SuperBlock™ (PBS) Blocking Buffer (Thermo Fisher) (*see Note 4*). Store at 4 °C.
4. Blocking and dilution buffer: 5% Skim milk in 0.2% PBST, pH 7.2. Take 400 mL of 0.2% PBST (pH 7.2) in a graduated cylinder. Weight 25 g of skim milk powder. Add to the cylinder and mix with a magnetic stir bar for 1 h at room temperature. Make up to 500 mL with 0.2% PBST (pH 7.2). Store at 4 °C (*see Note 5*).
5. Cold water bath trough: 400 mL of water in a square glass trough. Store at 4 °C (*see Note 6*).

6. In vitro transcription and translation (IVTT)-coupled protein expression reagent solution: 1-Step Human Coupled IVT Kit—DNA (Thermo Fisher). One kit includes 500 μ L HeLa lysate, 100 μ L accessory proteins, 200 μ L reaction mix, and 1.5 mL nuclease-free water. There are also two control DNAs, 10 μ g pCFE-GFP and 10 μ g pT7CFE1-cHis (*see Note 7*).
7. IVTT expression solution: A mixture of 450 μ L 80% IVTT per silicon slide (freshly prepared). Add 175 μ L HeLa lysate to a 1.5 mL new Eppendorf centrifuge tube. Add 50 μ L accessory proteins to the centrifuge tube and mix with pipette tips. Let the tube sit on ice and wait for 1 min. Add 100 μ L reaction mix to the centrifuge tube and mix with pipette tips. Add 125 μ L nuclease-free water to the centrifuge tube and mix with pipette tips. Centrifuge the tube at 10,000 rpm ($9,500 \times g$) at 4 °C for 2 min. Take the clear supernatant and add to a new 1.5 mL Eppendorf centrifuge tube. Let it sit on ice. Put the 1.5 mL tube on ice in a vacuum chamber and apply vacuum of -28 mmHg for 5 min (*see Note 8*).
8. Corn syrup: A bottle of Karo corn syrup stored at room temperature.
9. HD-NAPPA silicon slides: HD-NAPPA silicon slides printed with 8 DNA replicate subarrays.
10. Four-chamber tray: A plastic tray with four chambers, in which each one fits in one 25 mm, 75 mm standard slide.
11. Plastic tray: A 120 mm, 80 mm plastic tray with three spacers evenly distributed every 30 mm. It is used for slide rinsing and blocking procedures.
12. Water baths with temperature controllers: Two water baths for IVTT expression temperature control, one set at 30 °C and the other set at 15 °C.

2.2 HD-NAPPA Protein Array Expression Setup

Expression fill-n-seal apparatus and accessory materials: Apparatus and accessory materials are used for IVTT filling into nanowells, sealing with a polymer film, and expressing proteins on the HD-NAPPA array. The fill-n-seal apparatus includes two parts, a window part (top) and a base part (bottom), that sandwich a polymer film and silicon slide in the middle (Fig. 1).

1. Fill-n-seal apparatus window part (top): A customized transparent plastic window with an aluminum holder (Fig. 1a). There are one inlet port and one outlet port through the window at its two ends. The inlet port accommodates a copper tube for injecting a sealing liquid used for sealing the polymer film. The outlet port attaches to a two-way valve (Fig. 1a). The aluminum metal holder has six screws, at four corners and middle of long sides, aligned with the base part, for clamping the top and bottom sandwich structure of the fill-n-seal apparatus.

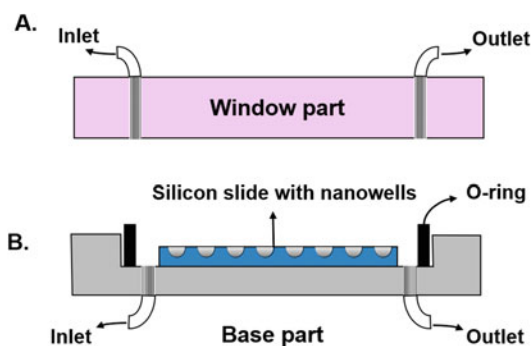


Fig. 1 Apparatus of HD-NAPPA expression system. (a) A customized transparent plastic window with an aluminum holder (not shown). (b) A customized aluminum metal base with etched slots for silicon slide and O-ring, respectively

2. Fill-n-seal apparatus base part (bottom): A customized aluminum metal base with etched slots for silicon slide and O-ring, respectively (Fig. 1b). The slots are a rectangle in the middle and two half-circle ends. The rectangle accommodates the silicon slide, and the two half circles accommodate the O-ring. There are one inlet port and one outlet port through the base at each end. The inlet port allows the injecting of IVTT liquid. The outlet port attaches to a tube for collecting and recovering excess IVTT (Fig. 1b). The aluminum metal base has six screw holes, at four corners and middle of long sides, aligned with the window part for clamping the sandwich structure.
3. Polymer film: A bi-polymer transparent film with polystyrene (PS) on one side and polyvinylidene fluoride (PVDF) on the other side. Cut the polymer film into a size of 4.5 cm, 10.0 cm. Label the PS side with “Up” with a marker. Spray both sides of the film with RNaseZap™ RNase decontamination solution (Thermo Fisher) (*see Note 9*). Use Kimwipes soaked with RNaseZap solution to wipe both sides of the film. Rinse the film with flowing water. Gently blow-dry the film with compressed air. Store at room temperature in a lens paper book.
4. O-ring along with silicon slide: An O-ring that fits in the slot and around the silicon slide on the base part.
5. Red burst disk: A disk film for temporarily holding corn syrup (sealing liquid). The burst disk is composed of a plastic annulus (7.15 mm OD, 3 mm ID, 0.075 thick) bonded to a 7.15 mm OD wax paper disk. Cut the plastic annulus and wax paper disk with a laser cutter and bond them together. Place the burst disk in the bottom of the inlet port of the fill-n-seal window.
6. 007 N90 O-ring: This goes onto the top of the burst disk before attaching the copper tube filled with sealing liquid.
7. Window inlet sealing plug: A black plug that fits the inlet port of the window part.

8. Two-way valve: A valve that attaches, at one end, to the outlet port of the window part and, at the other end, attaches to a pressure or vacuum supply line.
9. Copper tube: An L-shaped copper tube that holds the sealing liquid and attaches to the window part's inlet port. The copper tube is filled with corn syrup (sealing liquid) for sealing the nanowells with the polymer film. One end of the tube attaches to the inlet port of the window part, and the other end attaches to the nitrogen (N_2) pressure supply line.
10. Tightening tool: This is to tighten or untighten the attachment of the copper tube to the inlet port of the window part.
11. 50% Glycerol. Add 25 mL water to a 50 mL conical tube. Add 25 mL glycerol to the tube and mix well with a vortex. Store at room temperature. 50% Glycerol is used to hold down the silicon slide in the base part of the fill-n-seal apparatus.
12. Nitrogen (N_2): Provide the pressure for sealing liquid injection and maintaining the pressure at 200 psi during protein expression incubation to seal the nanowells. A tank of N_2 is attached to a pressure supply line with a valve.
13. Vacuum pump: A vacuum pump that produces a vacuum of -28 mmHg.
14. White burst disk: A disk film for temporarily holding IVTT reagent. The burst disk is a 5.55 mm OD, 0.03 mm thick circle that is punched out of a sheet of waxed paper.
15. Window inlet port sealing plug: A screw plug that fits the window part inlet port.
16. Check valve: A check valve allows only one-way air and liquid flow and fits the window part outlet port.
17. 20 mL Syringe with a Luer adaptor to the check valve.
18. Collection tube: A collection tube attached to the check valve for IVTT recovery.
19. Injection setup: An injection setup assembled with a glass syringe and an injector, attached to a pressure supply line.
20. 500 μ L Syringe: A 500 μ L Hamilton glass syringe, attached to the injector with a screw.
21. Injector: A pressure-powered injector attached to the pressure supply line through a valve.
22. Pressure and vacuum control knobs and switches: To control the pressure and vacuum during IVTT injection.
23. Clamps: Two clamps, to clamp the window and base parts of the fill-n-seal apparatus during disassembly.
24. Water baths with temperature controllers: Two water baths for IVTT expression temperature control, one set at 30°C and the other set at 15°C .

2.3 Primary Antibodies (Serum/ Plasma) Probing

1. Prepare 1:300 diluted mouse anti-GST solution: Add 2990 μL 5% skim milk to a 15 mL conical tube and add 10 μL monoclonal mouse anti-GST (Cell Signaling) to the tube. Mix well with vortex and let it sit on ice until use.
2. Prepare 1:500 diluted rabbit anti-p53 solution: Add 2994 μL 5% skim milk to a 15 mL conical tube and add 6 μL rabbit anti-p53 (Cell Signaling) to the tube. Mix well with vortex and let it sit on ice until use.
3. Serum/plasma sample: A human pooled sample.
4. Prepare 1:125, 1:250, 1:500, 1:1000, 1:2000, 1:4000, 1:8000, and 1:16,000 diluted pool serum solution.
 - (a) Add 620 μL 5% skim milk to a 1.5 mL Eppendorf centrifuge tube and add 5 μL pool serum to the tube. Mix well with vortex and let it sit on ice as 1:125 dilution pool serum solution.
 - (b) Take 300 μL 5% skim milk to a new 1.5 mL Eppendorf centrifuge tube, add 300 μL 1:125 dilution pool serum, mix well, and let it sit on ice as 1:250 dilution pool serum solution.
 - (c) Take 300 μL 5% skim milk to a new 1.5 mL Eppendorf centrifuge tube, add 300 μL 1:250 dilution pool serum, mix well, and let it sit on ice as 1:500 dilution pool serum solution.
 - (d) Take 300 μL 5% skim milk to a new 1.5 mL Eppendorf centrifuge tube, add 300 μL 1:500 dilution pool serum, mix well, and let it sit on ice as 1:1000 dilution pool serum solution.
 - (e) Take 300 μL 5% skim milk to a new 1.5 mL Eppendorf centrifuge tube, add 300 μL 1:1000 dilution pool serum, mix well, and let it sit on ice as 1:2000 dilution pool serum solution.
 - (f) Take 300 μL 5% skim milk to a new 1.5 mL Eppendorf centrifuge tube, add 300 μL 1:2000 dilution pool serum, mix well, and let it sit on ice as 1:4000 dilution pool serum solution.
 - (g) Take 300 μL 5% skim milk to a new 1.5 mL Eppendorf centrifuge tube, add 300 μL 1:4000 dilution pool serum, mix well, and let it sit on ice as 1:8000 dilution pool serum solution.
 - (h) Take 300 μL 5% skim milk to a new 1.5 mL Eppendorf centrifuge tube, add 300 μL 1:8000 dilution pool serum, mix well, and let it sit on ice as 1:16,000 dilution pool serum solution.
5. 8-Chamber gasket: A proplate 8-chamber gasket.

6. Aluminum foil seal film: An aluminum foil seal film cut to 25 mm, 75 mm rectangle.
7. Glass slides: Standard Fisher brand 25 mm, 75 mm microscope glass slide.
8. Binder clips: Medium, 1–1/4" Wide, 5/8" Capacity, Black, Pack of 24.
9. Laboratory rocker.

2.4 Secondary Antibody Visualization and Image Scanning

1. Prepare 1:500 diluted Alex Fluor[®] 647-labeled goat anti-mouse IgG and 1:500 diluted Alex Fluor[®] 555 goat anti-rabbit IgG solution: Add 2988 μ L 5% skim milk to a 15 mL conical tube. Add 6 μ L Alex Fluor[®] 647-labeled goat anti-mouse IgG (Thermo Fisher) and 6 μ L Alex Fluor[®] 555 goat anti-rabbit IgG (Thermo Fisher) to the tube. Mix well with vortex and let it sit on ice until use.
2. Prepare 1:200 diluted Alex Fluor[®] 647-labeled goat anti-human IgG: Add 2985 μ L 5% skim milk to a 15 mL conical tube and add 15 μ L Alexa Fluor[®] 647-labeled goat anti-human IgG (Jackson Immunology) to the tube. Mix well with vortex and let it sit on ice until use.
3. Backing film: A 0.3 mm thick film, with pressure-sensitive adhesive, to bring the silicon slide thickness from 0.7 to 1 mm.
4. Tecan scanner: Two lasers for scanning both Alex Fluor 647 and Alex Fluor 555/Cy3.
5. Array Pro microarray image analysis software: To extract the fluorescence intensities from Tecan scanner-generated images.

3 Methods

All procedures are performed under room temperature unless otherwise specified.

3.1 HD-NAPPA Protein Array Expression

3.1.1 Materials and Preparation

1. Preheat water baths: Set two water baths to 30 °C and 15 °C, respectively.
2. Turn on N₂ tank pressure to 200 psi.
3. Take one HD-NAPPA slide from an argon gas-filled storage tank.
4. Place the slide in a four-chamber plastic tray and prepare a dummy slide in another four-chamber plastic tray. Load 9 mL cold superblock into the slide chamber and a dummy slide tray, respectively.
5. Centrifuge at 4000 rpm ($3,750 \times g$) for 1 min, to dislodge air bubbles from the nanowells.

Table 1
The IVTT mixture for 1–6 slides

80% IVTT mixture for HD-NAPPA expression					
Slide no.	HeLa lysate (μL)	Accessory protein (μL)	Reaction mixture (μL)	Nuclease-free water (μL)	Total volume (μL)
1	200	50	100	100	450
2	400	100	200	200	900
3	600	150	300	300	1350
4	800	200	400	400	1800
5	1000	250	500	500	2250
6	1200	300	600	600	2700

6. Take the slide out and load slide in a slide rack. Quickly immerse the rack into a cold-water trough and lift it up. Repeat ten times (~10 s total).
7. Centrifuge the slide dry at 1200 rpm ($338 \times g$), room temperature, for 20 min.
8. Prepare 80% IVTT solution (Table 1) (*see* **Notes 10** and **11**).

3.1.2 *Fill-n-Seal*
Apparatus Assembling
and HD-NAPPA Expression

1. Prepare an expression fill-n-seal apparatus and separate the window part and base part (Fig. 2a, b).
2. Insert a white burst disk into the base part’s inlet port, screw in a black plug, and tighten it (Fig. 2a, steps 1 and 2).
3. Lay the base part on a flat surface, fixed to the lab bench. Add three drops of 30 μL 50% glycerol spaced evenly along the rectangular slot’s middle length. Load the HD-NAPPA slide onto the slot and lay an O-ring along with the slide (*see* **Note 12**) (Fig. 2a, steps 3 and 4).
4. Let one polymer film sit on top of the slide with the PVDF side facing down toward the slide (Fig. 2a, step 5).
5. Screw an inlet sealing plug into the inlet port of the window part and tighten it (Fig. 2b, steps 1 and 2).
6. Attach the two-way valve into the outlet port of the window part. Tighten the valve and turn counterclockwise (release) for two rounds (Fig. 2b, step 3).
7. Assemble the window part and the base part using the six screws attached (image not shown). At first, finger tighten the screws. Attach an N₂ pressure supply line to the two-way valve at the window part’s outlet port and turn on the pressure (200 psi). This step pushes down on the silicon slide and spreads out the 50% glycerol to adhere to the bottom of the

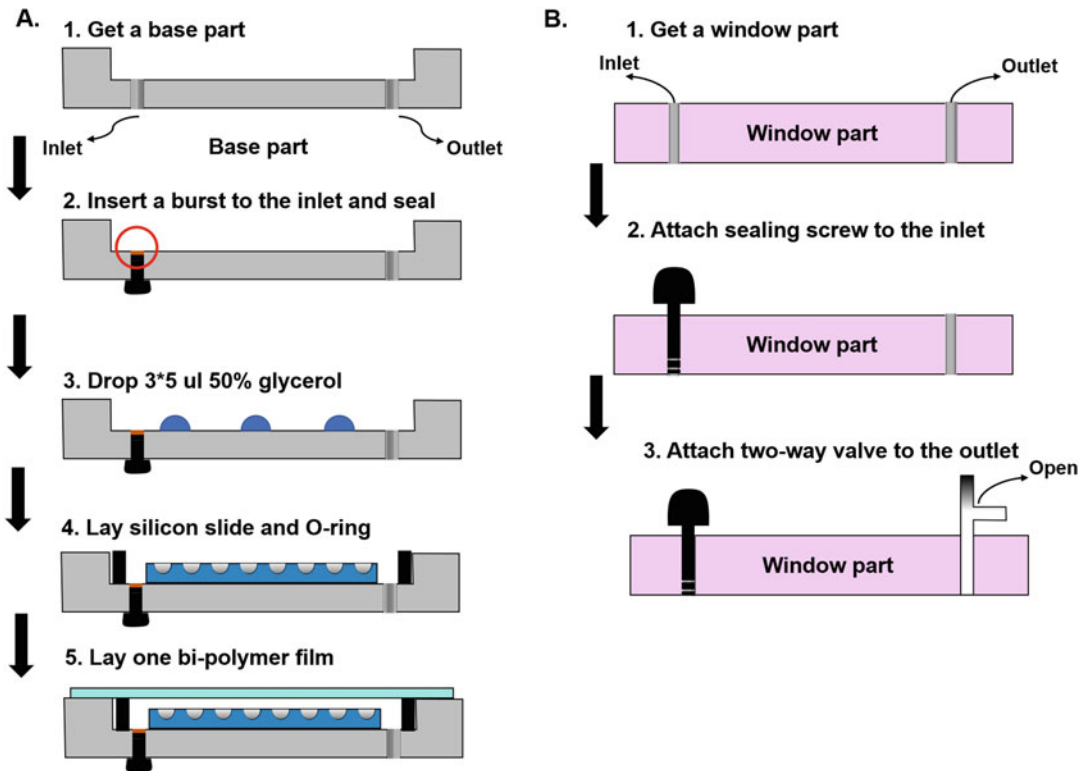


Fig. 2 IVTT incubation procedure for HD-NAPPA expression system. (a) Prepare base part. (b) Prepare window part. (c) Assemble the window part and base part. (d) IVTT injection

silicon slide to the base part. Wait for 5 s and then turn off the pressure supply line (Fig. 2c, steps 1 and 2). Detach the pressure supply line and attach it to the outlet port of the base part. Turn on the pressure (200 psi) to flatten the sealing film across the bottom of the window part. Tighten the six screws clamping the window and base parts of the fill-n-seal apparatus. Sequentially tighten each screw a little at a time to apply even clamping pressure between the window and base parts. Use a torque wrench to prevent overtightening of the screws. Turn off the pressure and detach the outlet port's pressure supply line (Fig. 2c, step 3).

8. Detach the inlet sealing plug from the inlet port of the window part. Load a red burst into the window part's inlet port and add a 007 N90 O-ring on top of the burst. Use a syringe to add corn syrup to a copper tube from one end until it starts to flow out from the other end. Attach the copper tube to the window part's inlet port and tighten it using the tightening tool. Attach and tighten the other end of the copper tube to the N₂ pressure supply line. Attach the two-way valve to a vacuum and pressure switch supply line (Fig. 2c, steps 4 and 5).

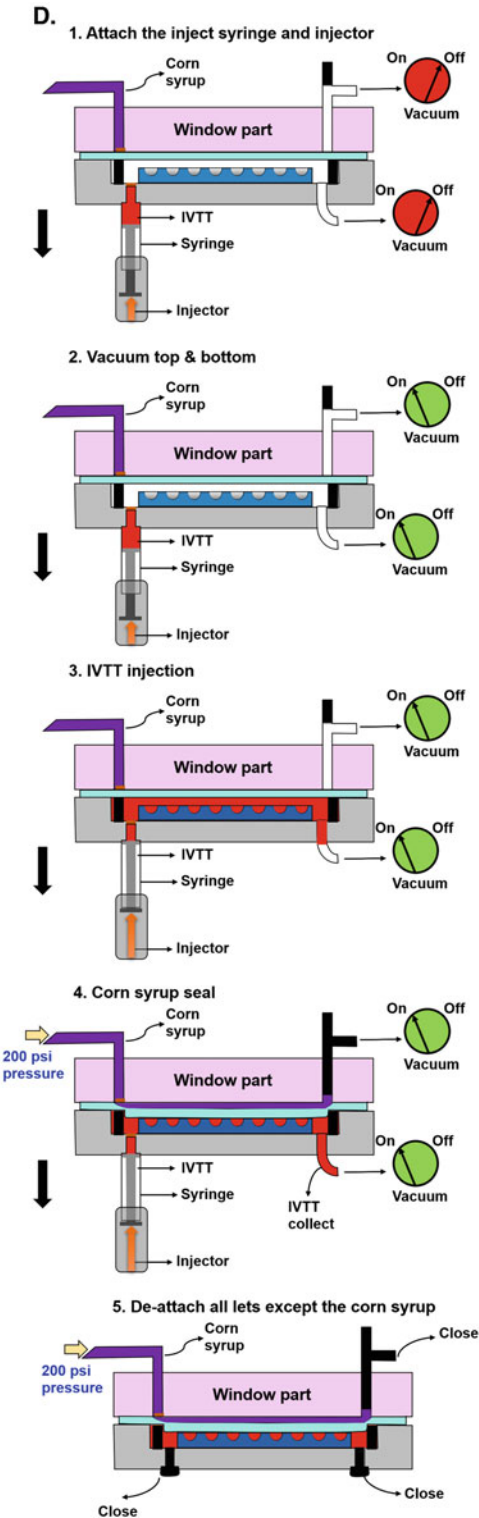
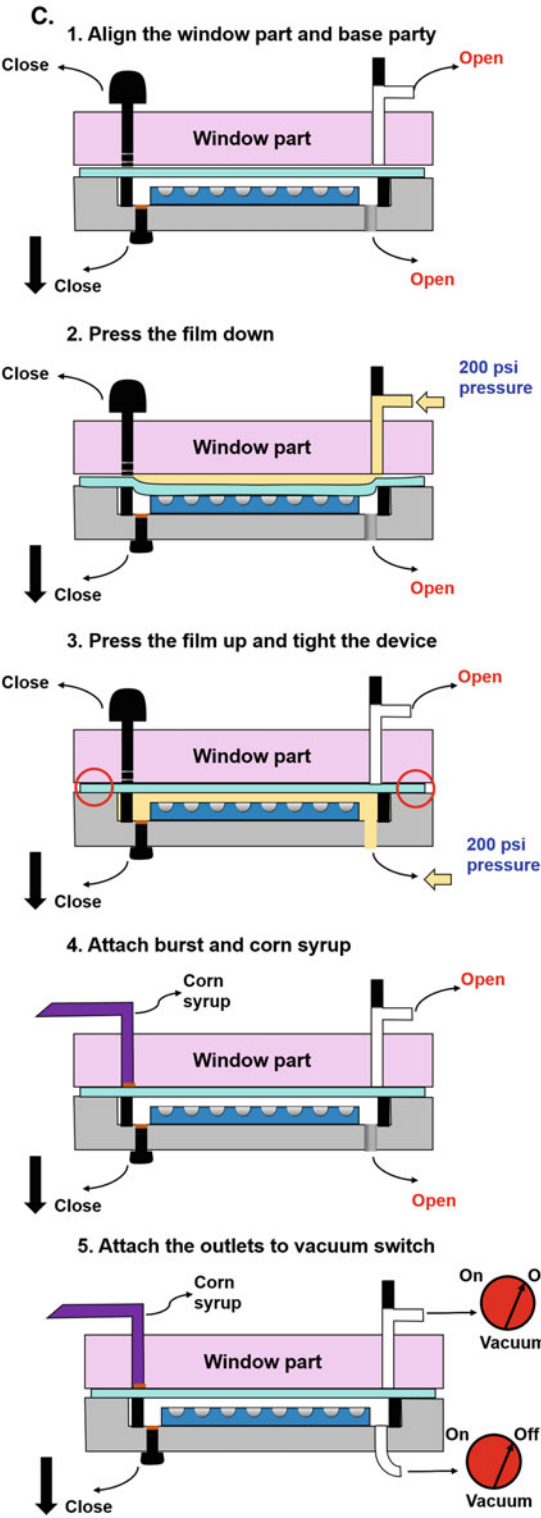


Fig. 2 (continued)

9. Detach the black plug from the base part's inlet port. Attach and tighten a check valve to the base part's outlet port. Attach and tighten the check valve to a collection tube for IVTT recovery (*see* **Note 13**). The other end of the collection tube is attached and tightened to a switched vacuum and pressure supply line (Fig. 2d, step 1).
10. Load a 500 μL Hamilton syringe with 300 μL prepared IVTT reagent. Attach the syringe to a pressure-powered injector and tighten the syringe to the inlet port of the base part (Fig. 2d, step 1).
11. Turn on the vacuum at the outlet port of the window part and then switch the vacuum on the outlet port of the base part (Fig. 2d, step 2). Turn on the pressure to the pressure-powered injector to inject the IVTT in the space between the polymer film and silicon slide (*see* **Note 14**) (Fig. 2d, step 3). Turn on the pressure at the inlet of the window part to inject the corn syrup to seal the nanowells onto the slides. Watch closely until the corn syrup gets close to the outlet and close the two-way valve (Fig. 2d, step 4). Turn off the vacuum and pressure for all supply lines except for the window part's inlet supply line. Collect the recovery IVTT in the collection tube. Detach the check valve and the injection syringe from the base part, and detach the pressure/vacuum supply line from the window part's outlet. Insert plugs at the inlet and outlet ports of the base part and tighten them (Fig. 2d, step 5).
12. Transfer the whole setup to a 30 °C water bath (preheated) and incubate for 2 h.
13. Transfer the whole setup to a 15 °C water bath (pre-cooled) and incubate for 30 min.

3.1.3 Fill-n-Seal Apparatus De-assembling

1. After 2.5 h, transfer the setup back to the lab bench on a flat surface.
2. Turn off the pressure at the inlet of the window part. Detach the copper tube from the inlet port and two-way valve from the window part's outlet port. Load a syringe filled with 10 mL deionized water.
3. Detach the plug from the base part's inlet port, load a syringe filled with 1 mL of 5% skim milk, and attach it to the inlet port of the base part.
4. Inject 10 mL deionized water into the inlet port of the window part. This step helps to lift the sealing film from the silicon slide. Sequentially unscrew the six screws, starting from the inlet end of the window part.
5. When all screws are loose, inject 1 mL 5% skim milk into the base part's inlet port. This step separates the sealing film from the silicon slide.

6. Take off the window part and polymer film, exposing the silicon slide.
7. Quickly squirt ~10 mL 5% skim milk onto the silicon slide to prevent it from drying out.
8. Gently remove the silicon slide from the base part and quickly immerse it in a plastic tray filled with 30 mL 5% skim milk. Do not allow the slide to dry out.
9. Wash the slide with 5% skim milk in the plastic tray three times, 5 min each on a laboratory rocker, at room temperature.
10. Block the slide in 30 mL 5% skim milk for 1 h at 4 °C.

3.2 Primary (Antibody and Serum) Probing

1. Prepare a 1:300 diluted mouse anti-GST solution.
2. Prepare a 1:500 diluted rabbit anti-p53 solution.
3. Prepare 1:125, 1:250, 1:500, 1:1000, 1:2000, 1:4000, 1:8000, and 1:16,000 diluted pool serum solution.
4. Attach a 25 mm, 75 mm rectangle aluminum foil seal film to one side of an eight-chamber gasket and seal tight (*see Note 15*).
5. Load 250 µL primary antibody sample to the designed chamber. Align one dummy glass slide with the HD-NAPPA slide and align them together with the eight-chamber gasket. Tighten the chamber and the slide with four clamp clips (two clips on each side). Flip the setup over to allow the chamber's solution to come into contact with the silicon slide.
6. Incubate the whole device on a laboratory rocker, at speed setting 2, overnight (*see Note 16*) at 4 °C.

3.3 Secondary Antibody Probing and Image Scanning

1. Prepare 1:500 diluted Alex Fluor[®] 647-labeled goat anti-mouse IgG and 1:500 diluted Alex Fluor[®] 555-labeled goat anti-rabbit IgG solution.
2. Prepare 1:200 diluted Alex Fluor[®] 647-labeled goat anti-human IgG.
3. Take the whole device with a primary sample out of 4 °C. De-assemble the device and release the HD-NAPPA slide to a plastic tray filled with a 30 mL 5% skim milk solution.
4. Rinse the slide with 30 mL 5% skim milk three times, 5 min each, rocking at speed 2 at room temperature.
5. Post-rinse with 5% skim milk. Load 3 mL 1:500 diluted Alex Fluor[®] 647-labeled goat anti-mouse IgG and 1:500 diluted Alex Fluor[®] 555-labeled goat anti-rabbit IgG solution to a four-chamber tray. Immerse the rinsed HD-NAPPA slide for expression quality control in the solution. Incubate the slide by rocking at speed 2 on a laboratory rocker at room temperature for 1 h.

6. Post-rinse with 5% skim milk. Load 3 mL 1:200 diluted Alex Fluor[®] 647-labeled goat anti-human IgG solution to another four-chamber tray. Immerse the rinsed HD-NAPPA slide for serology study in the solution. Incubate the slide by rocking at speed 2 on a laboratory rocker at room temperature for 1 h.
7. After 1 h, take the slides to a plastic tray filled with a 30 mL 0.2% PBST solution. Rinse the slide with 30 mL 0.2% PBST three times, 5 min each, rocking at speed 2 on a laboratory rocker at room temperature.
8. Prepare a trough with 400 mL cold (4 °C) deionized water (*see Note 17*). Load the HD-NAPPA slides, rinsed with 0.2% PBST, into a slide rack. Immerse the rack with slides in the cold-water solution and lift up after 2 s; repeat this step ten times.
9. Centrifuge the rack with slide at a speed of 1200 rpm at room temperature (25 °C) for 20 min.
10. Remove the dried HD-NAPPA silicon slide from the centrifuge and attach a 25 mm, 75 mm backing film to bring the silicon slide thickness to 1 mm.
11. Load the slide into a Tecan scanner and set up the scanning setting as below:
 - (a) Turn on the warming step for both red laser (635 nm) and green laser (532 nm).
 - (b) Set both laser's scanning intensity at 25% and 25% gain.
 - (c) Use a customized slide type: tune the focus at the bottom of the nanowell (~70 μ m deep).
 - (d) Start scanning to generate two images at 635 nm and 532 nm, respectively.
12. Load the 25% intensity, 25% gain images to an Array Pro microarray image analysis software, and extract the fluorescence intensity saved as .txt file. The extracted data includes the spotlist information (Spot No., Grid, Row, Column, Protein name, Protein symbol, Organism, Spot category) and each array's median intensity.

3.4 Data Analysis

3.4.1 Expression Quality Control Analysis (Fig. 3a)

1. The expression quality control includes two aspects: an overall expression level of all proteins compare to no-DNA (printing mixture only, PM) controls and a linear regression correlation between different arrays.
2. The overall expression level is calculated as how many proteins (N_{Proteins}) out of all proteins ($N_{\text{All Proteins}}$) have expression level (Alex Fluor[®] 647-labeled goat anti-mouse IgG @mouse anti-GST probing intensity) that is higher than a cutoff. The cutoff is calculated as the average of no-DNA spot (PM) intensity plus

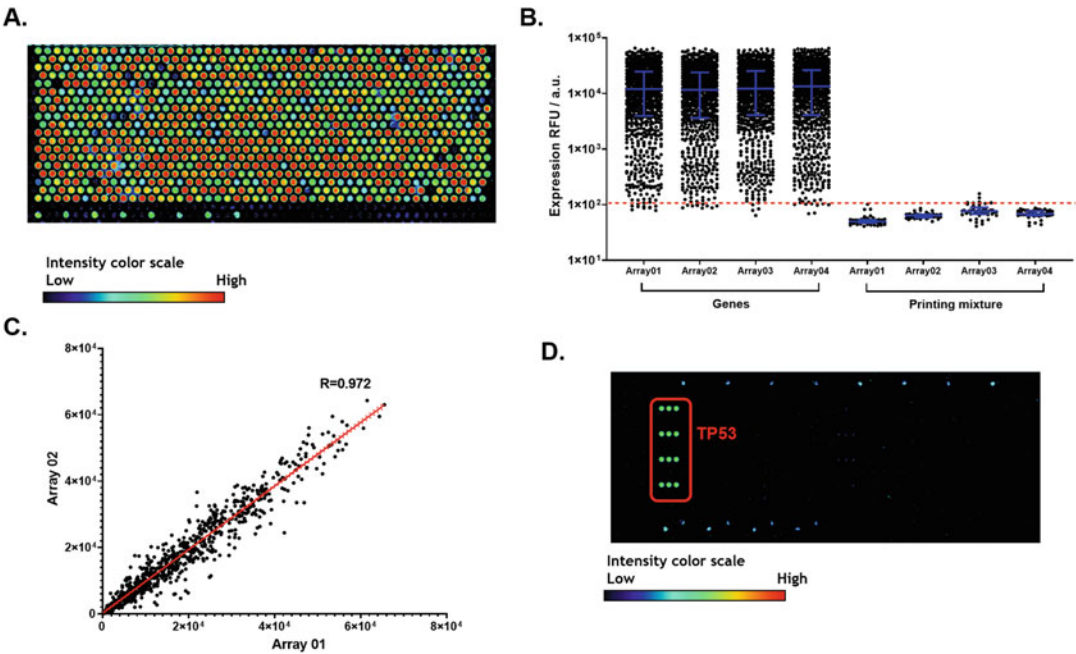


Fig. 3 HD-NAPPA results. (a) Mouse anti-GST result image. (b) The expression level of proteins on NAPPA array. A cutoff (red dotted line) at the average of PM spots plus three-times standard deviation. (c) Inter-correlation between two arrays. (d) Rabbit anti-p53 result image

three times of standard deviations ($\text{Mean}_{\text{PM}} + 3\text{Sd}_{\text{PM}}$). Figure 3a presents a mouse anti-GST probing result on an HD-NAPPA array with 907 proteins and 48 PM spots for each array (one grid). An HD-NAPPA expression level is $P = N_{\text{Proteins}}/N_{\text{All Proteins}}$, and a qualified array requires $P \geq 95\%$. In the case of Fig. 3b, four subarrays of 907 proteins and 48 PM spots showed expression levels of 98.5–99.5%.

3. The linear regression correlation is calculated between all proteins from array 1 and array 2. The array could be on the same slide (intra-slide correlation) or between two slides (inter-slide correlation). A linear regression correlation $R \geq 0.9$ is a passing criterion. Figure 3c shows an example of the eight-subarray HD-NAPPA correlation between array 1 and array 2 with $R = 0.972$.
4. Specific protein analysis is shown in Fig. 3d. The highlighted spots are TP53 protein spots.

3.4.2 Serology Result Analysis

1. The serology antibody response will be analyzed separately for IgG antibodies (Fig. 4a).

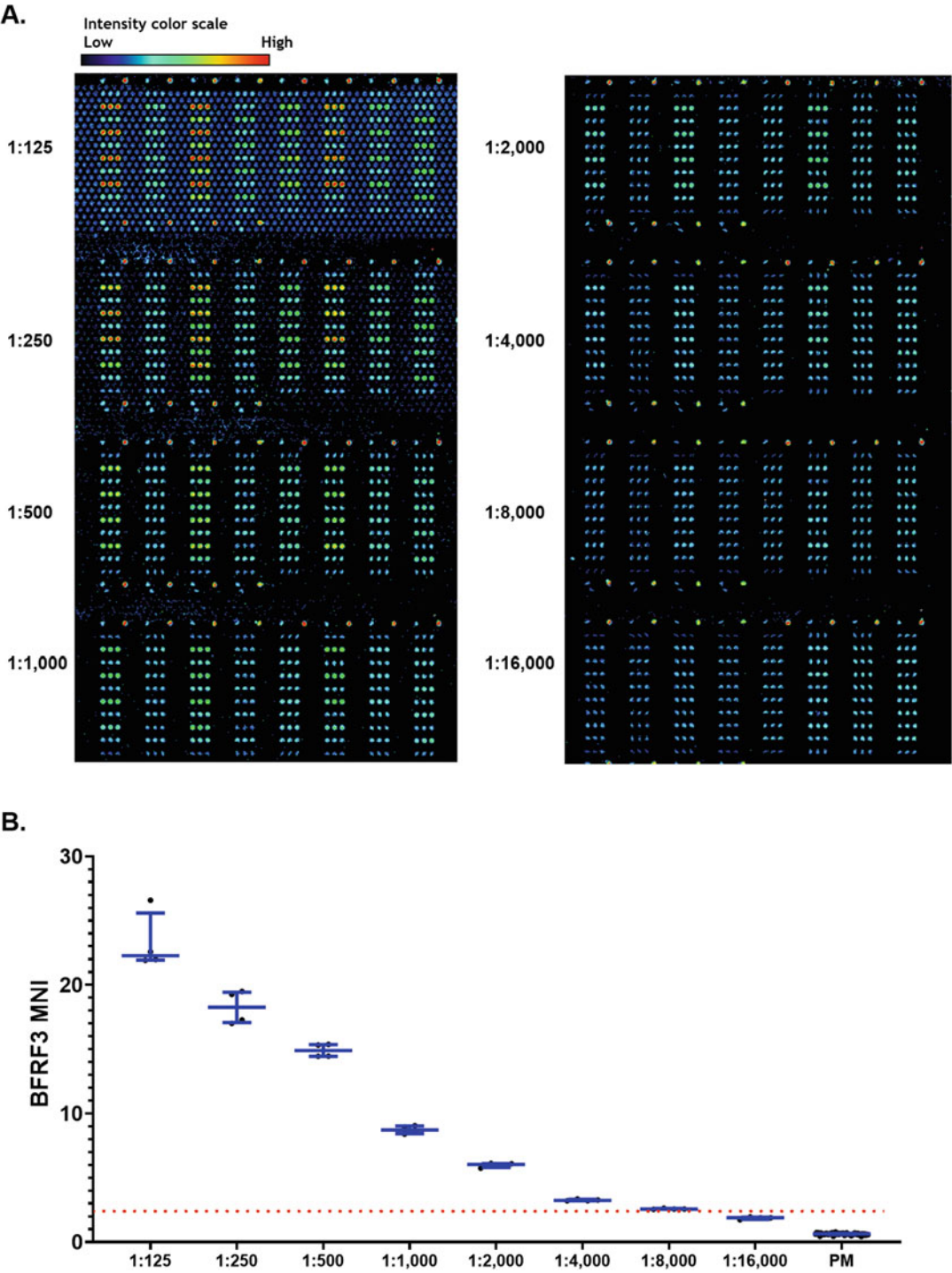


Fig. 4 Serology results. (a) Serial dilution serum probing result images (IgG). (b) Selected biomarker BFRF3 MNI through 1:125 to 1:16,000 dilution. The median and interquartile are shown as blue line

2. Load the median intensity of a serology IgG profiling of a whole slide to Microsoft Excel and separate the subarrays. Calculate the median intensity value (I_{Median}) of all proteins in each subarray. Divide the raw intensity of each protein by I_{Median} to generate a new set of median normalized intensity (MNI). $\text{MNI} \geq 2.0$ is an experienced seropositive cutoff. Seropositive response proteins will be selected as possible candidate biomarkers (Fig. 4b).
3. Further statistical analysis can be performed based on needs.

4 Notes

1. Having water in the glass beaker helps dissolve the PBS salts relatively easily, allowing the magnetic stir bar to work immediately.
2. HCl is a strong corrosive acid, so wear extra personal protection equipment. The pH of the PBS is close to what you want. You can choose to use serial diluted HCl (6 N or 1 N) to avoid a sudden pH drop. The pH of 10× PBS is around 6.8, and a final pH of the 1× PBS from this 10× PBS will be around 7.2.
3. Tween 20 is a sticky surfactant. Use a 1 mL pipette tip, trimmed with a blade, and pipette slowly. Pipette up and down multiple times until all Tween 20 solution is added to the cylinder.
4. According to the vendor Thermo Fisher, a superbloc solution is a nonprotein base blocking buffer, hence use as it is.
5. 5% Skim milk is typically required when de-assembling the HD-NAPPA expression apparatus, blocking, and sample probing. Prepare the solution ahead of time and allow it to stir with a magnetic bar for at least 2 h ahead of usage. Do not use 5% skim milk older than 3 days. The blocking buffer could vary based on needs. 1–2.5% BSA could also be taken as a blocking and dilution buffer.
6. The temperature of the cold-water bath is a critical factor. Allow enough time for the water to cool down to 4 °C. A higher temperature affects rinsing quality.
7. These plasmids are not required for the HD-NAPPA experiment. However, they are a standard kit accessory for testing the IVTT solution expression efficiency.
8. The centrifuge and de-gas steps are critical for the resulting HD-NAPPA image quality. IVTT mixture has cloudy aggregates while mixing the solution. A centrifuge at 10,000 rpm is recommended by the vendor (Thermo Fisher), and it helps to remove those aggregates. If they are not removed ahead of usage, the resulting image has many high signal patches. The

vacuum de-gas step is also critical. The HD-NAPPA expression apparatus is under vacuum before IVTT injection to prevent air bubbles from being trapped in the nanowells on the HD-NAPPA slides. Genes in wells with trapped air bubbles are not expressed.

9. This step requires an RNase-free environment to maintain expression efficiency. RNaseZap treatment is critical for the polymer film since the PVDF polymer directly touches the IVTT during protein expression in each nanowell.
10. The IVTT kits are stored separately at -80°C . A thawing step on ice takes typically around 30–45 min for a full kit (500 μL HeLa lysate, 100 μL accessory protein, and 200 μL reaction mix). Try to plan the experiment ahead of time and maintain the minimum freeze-thaw cycle for the IVTT kits. A maximum of two freeze-thaw cycles is recommended.
11. Before usage, it is suggested to keep the IVTT mixture on ice. Also, it is suggested to monitor the vacuum step to avoid overflow of bubbles carefully.
12. The O-ring helps to seal the whole apparatus for successful expression of HD-NAPPA slides. Carefully check the O-ring elasticity strength and avoid using an O-ring with cracks. Lay the O-ring down into its slot in the base part and make sure that it does not pop out.
13. The check valve and collection tube will allow the collection of IVTT. They will help to keep the vacuum status during IVTT injection.
14. Since the chamber is in a vacuum state and the IVTT injection tool's pressure is high, the injection step will be speedy. Look closely during IVTT injection to avoid bubbles. An evenly distributed IVTT will go smoothly along the long axis.
15. The multiple chambers will allow the probing of the different regions of the same array. Sealing is essential to avoid contamination.
16. An overnight incubation usually is equal to 16 h. It could also vary based on needs.
17. The last step before drying is critical to maximize the signal while minimizing the background. A quick wash in cold water (4°C) is needed.

References

1. Ramachandran N, Hainsworth E, Bhullar B et al (2004) Self-assembling protein microarrays. *Science* 305:86–90
2. Ramachandran N, Raphael JV, Hainsworth E et al (2008) Next-generation high-density self-assembling functional protein arrays. *Nat Methods* 5:535–538
3. Bian XF, Wiktor P, Kahn P et al (2015) Antiviral antibody profiling by high-density protein arrays. *Proteomics* 15:2136–2145

4. Song L, Wallstrom G, Yu X et al (2017) Identification of antibody targets for tuberculosis serology using high-density nucleic acid programmable protein arrays. *Mol Cell Proteomics* 16:S277–S289
5. Wiktor P, Brunner A, Kahn P et al (2015) Microreactor array device. *Sci Rep* 5:8736
6. Takulapalli BR, Qiu J, Magee DM et al (2012) High density diffusion-free nanowell arrays. *J Proteome Res* 11:4382–4391



Bead-Based Assays for Validating Proteomic Profiles in Body Fluids

Annika Bendes, Matilda Dale, Cecilia Mattsson, Tea Dodig-Crnković, Maria Jesus Iglesias, Jochen M. Schwenk, and Claudia Fredolini

Abstract

Protein biomarkers in biological fluids represent an important resource for improving the clinical management of diseases. Current proteomics technologies are capable of performing high-throughput and multiplex profiling in different types of fluids, often leading to the shortlisting of tens of candidate biomarkers per study. However, before reaching any clinical setting, these discoveries require thorough validation and an assay that would be suitable for routine analyses. In the path from biomarker discovery to validation, the performance of the assay implemented for the intended protein quantification is extremely critical toward achieving reliable and reproducible results. Development of robust sandwich immunoassays for individual candidates is challenging and labor and resource intensive, and multiplies when evaluating a panel of interesting candidates at the same time. Here we describe a versatile pipeline that facilitates the systematic and parallel development of multiple sandwich immunoassays using a bead-based technology.

Key words Suspension bead array (SBA), Single-binder immunoassays, Sandwich immunoassay (SIA), Antibody, Protein standard, Mass spectrometry (MS)

1 Introduction

Proteins in human biological fluids offer a clinically useful window into human health, and play a central role in clinical decision-making [1, 2]. In particular, the simultaneous analysis of multiple proteins has become an attractive approach to provide better insights into the complexity of human health and pathophysiological conditions [3, 4]. Mass spectrometry (MS) is regarded as the most commonly used workhorse of proteomic applied to biomarker discovery, since it allows the analysis of hundreds to thousands of proteins, as well as the characterization of their structures and modifications [5]. On the other hand, affinity-based methods such as suspension bead arrays (SBA) [6–8] or proximity extension assays [9, 10] have shown to be extremely powerful for

the profiling of biofluids at a higher sample throughput with medium-high multiplexing capacity and consuming only minimal amounts of samples.

A further argument in favor of affinity-based profiling methods is that, despite the tremendous advances in clinical MS with its potential in complementing some of immunoassay's shortcomings, affinity assays are still the most versatile and broadly applied methods in research and clinical laboratories.

In recent years, the field of biomarkers has shifted the attention from pure discovery to verification and efforts on clinical translation and validation.

Relevant aspects in biomarker validation include: a focused clinical question; the availability of high-quality samples from large and clinically representative population; and robust and high-throughput assays for accurate and reproducible quantification of candidate protein biomarkers [11].

The need to accelerate preclinical research and translation of biomarkers “from bench to clinic” have posed a growing demand on strategies evaluating the quality and suitability of the reagents used [12, 13], and the reproducibility of the discovery using orthogonal approaches [9, 14, 15].

Verification and validation of large panels of candidate biomarkers generated by proteomic profiling of body fluids call for appropriate pipelines that can cope with a systematic development of high-quality assays for robust quantification of several proteins.

Antibodies coupled to color-coded beads used to profile biotin-labeled body fluids such as serum, plasma, and CSF have been extensively described [7, 16, 17]. This single-binder immunoassay has provided the foundation to establish a protocol for the development of sandwich immunoassays (SIA) for several candidate biomarkers in parallel (Fig. 1).

Using this microtiter plate procedure, we recently screened 2170 antibody pairs targeting a total of 209 unique proteins from the human secretome. Out of these, 22 sandwich immunoassays were developed and validated with samples collected in a longitudinal manner [18]. Here we describe the details for performing such assays.

2 Materials

Prepare all solutions using Milli-Q ultrapure water (deionized water purified, to attain a sensitivity of 18 MΩ cm at 25 °C) and analytical grade reagents.

2.1 Bead Coupling

1. Carboxylated color-coded paramagnetic beads (MagPlex-C, Luminex Corp).

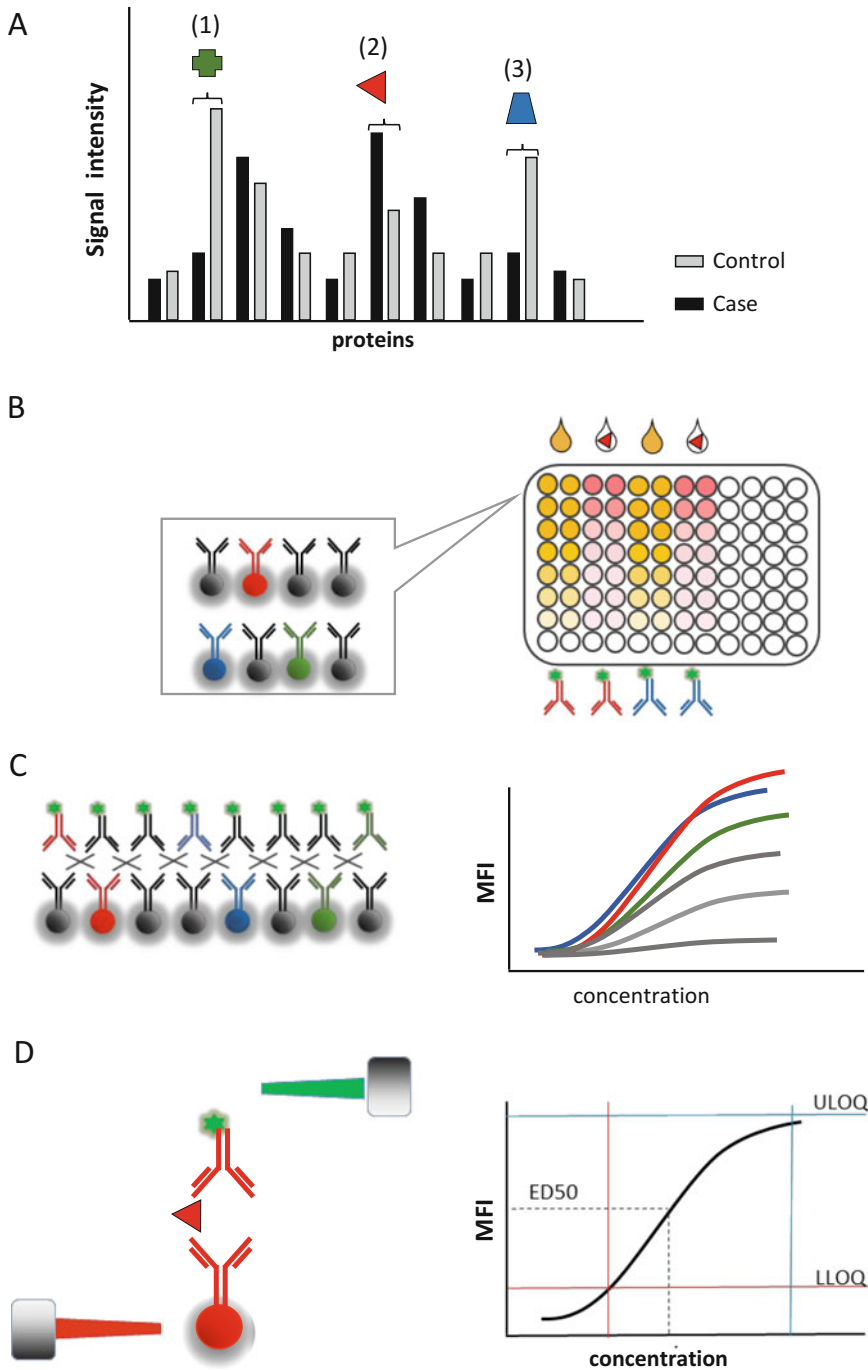


Fig. 1 Pipeline for bead-based development and validation of sandwich immunoassays. (a) Multiplexed proteomic profile of biofluids leads to the discovery of multiple candidate biomarkers (example of a case-control study scenario). (b) A bead array containing capture antibodies raised against the candidate biomarkers is prepared and (c) tested in combination with multiple detection antibodies in dilution curves of protein standards and plasma. (d) Best performing pair is selected for assay validation. Signal detection on each bead ID is detected by dual laser

2. MES buffer: 0.1 M 2-(*N*-morpholino) ethanesulfonic acid, pH 4.5. Add about 150 mL ultrapure water to 2.93 mg. Adjust pH to 4.5 using NaOH. Filtrate the buffer using a 0.45 μ M filter. Store at 4 °C for up to 1 month.
3. Activation buffer (AB): 0.1 M NaH₂PO₄, pH 6.0–6.2. Dissolve NaH₂PO₄ in 150 mL ultrapure water. Adjust the pH with NaOH, and filtrate the solution using a 0.45 μ M filter. Store at 4 °C for up to 1 month.
4. Washing buffer (WB): Phosphate-buffered saline prepared by dissolving PBS tablet in deionized (DI) filtered H₂O supplemented with 0.05% Tween 20. Store at room temperature.
5. Storage buffer: Blocking reagent for ELISA supplemented with ProClin™ 0.003% v/v. Store at 4 °C for up to 1 month.
6. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC): 0.5 mg per antibody dissolved to 10 mg/mL in AB.
7. Sulfo-*N*-hydroxysulfosuccinimide (Sulfo-NHS): 0.5 mg per antibody dissolved to 10 mg/mL in AB.
8. Antibodies to be coupled to beads: Dilute 1.75 μ g antibody in MES buffer to a final volume of 100 μ L and final concentration of 17.5 ng/ μ L. Antibodies can be conveniently distributed in 96-well plates (antibody selection is further discussed in **Note 1**).
9. For coupling test: Anti-species IgG conjugated to R-phycoerythrin (R-PE).
10. Equipment: Protein low-binding tubes; half-area flat-bottom 96 and 384 plates; PCR plates; magnet for plates; magnetic tube holder; Luminex instrument for readout (Luminex Corp.).

2.2 Selection of Antibody Pairs and Assay Validation

1. Capture bead population previously prepared (*see* Subheading 3.1) at a working concentration of 40,000 beads/mL.
2. Washing buffer (WB) (*see* Subheading 2.1).
3. Biotinylated detection antibodies [19]: Working concentration of 10^{−3} mg/mL (*see* details described in the Chapter 12).
4. Protein standard (when available): In order to run nine dilution curves in duplicate, approximately 1.5 μ g of protein is needed. The preferred standard is purified and lyophilized recombinant protein reconstituted at a concentration of 1–0.1 μ g/ μ L in 0.2 μ m filtered PBS, supplemented with 1% BSA. Reconstituted proteins should be stored in working aliquots at −70 °C.
5. Sample pools: A pool of the body fluid, ideally from the cohort that was analyzed in the discovery phase. Samples should be pooled in order to have a “high pool” (the protein of interest was detected at high levels) and “low pool” (protein was

detected at low level). Commercial pool of samples from healthy donors are available for most types of body fluids (e.g., serum, plasma, cerebrospinal fluids (CSF), saliva) at <https://bioivt.com/>.

6. Plasma from other species (e.g., chicken, donkey, horse).
7. Surrogate of body fluid of interest (e.g., artificial CSF [20]).
8. Assay buffer 1: PBS with 0.5% (w/v) polyvinyl alcohol, 0.8% (w/v) polyvinylpyrrolidone, 0.1% casein, supplemented with 0.5 mg/mL rabbit IgG.
9. Assay buffer 2: PBS with 1% BSA.
10. Assay buffer 3: Low-cross buffer (*see Note 9*).
11. R-PE-conjugated streptavidin (SAPE): Dilute to 1:750 to 0.4 $\mu\text{g/mL}$.

3 Methods

All procedures should be carried out at room temperature unless otherwise specified.

3.1 Bead Coupling

A suspension bead array is prepared by adding diluted antibodies to chemically activated beads, each featuring a different color code (ID) for identification. The antibodies are covalently bound to the carboxylated beads by carbodiimide crosslinker chemistry, and any excess of antibody is washed away. The different bead IDs are then pooled together to create a capture array.

1. Gently vortex the antibodies and dilute them in MES buffer each to a final concentration of 17.5 ng/ μL and final volume of 100 μL . For the first antibody pair screening, the bead array may include up to 100 different capture antibodies (indicatively to evaluate assays for 20–50 target proteins in parallel). The array must contain at least two capture antibodies per target and a couple of negative controls (bare beads, same species IgG) and positive controls such as antibody against a protein with long residence in the biofluid analyzed (e.g., albumin or IgG in plasma) (*see Notes 1 and 2*).
2. Vortex the bottles containing each specific color-coded magnetic bead ID and distribute 40 μL beads, one bead ID per well, into a flat-bottom low-binding 96-well plate.
3. Wash the beads in AB. Place the flat-bottom plate on the magnet and wait for 30 s to pull beads to the bottom of the plate. Remove the liquid carefully without touching the beads and then add 80 μL AB per well. Wait for another 30 s, decant the supernatant, remove the plate from the magnet, and add 50 μL of AB.

4. Prepare the activation solution (AS) by dissolving EDC and sulfo-NHS in AB at a concentration of 10 mg/mL. The solution needs to be prepared fresh and used immediately. Timing is critical for this step since the intermediate generated by EDC may hydrolyze and lose its reactivity toward primary amines (*see Note 3*). Add 50 μ L activation solution to each well in the flat-bottom plate. Seal the plate, place it on a plate shaker, and incubate it for 20 min at 650 rpm in the dark.
5. After incubation, wash the plate two times with 100 μ L MES buffer (*see step 3*). Place the plate on the plate magnet and wait for 30 s. Remove the supernatant and add 100 μ L MES buffer. Repeat the washing steps once. After MES buffer has been discarded for the second time, remove the plate from the magnet and add 100 μ L of diluted antibody to the beads. Seal the plate, vortex it gently, place it on a plate shaker, and incubate for 2 h at 650 rpm in the dark.
6. After incubation, wash the plate two times with WB (*see steps 3 and 5*). After WB has been discarded for the second time, remove the plate from the magnet and add 50 μ L of storage buffer to each well. Seal the plate, vortex it carefully, and incubate it at 4 °C overnight in order to block the beads.
7. After overnight blocking at 4 °C, individual bead IDs are pooled together creating a bead array containing beads coupled to all the different antibodies, at a concentration of 40,000 beads/mL.
8. The coupling efficiency is tested by incubating the beads with an R-phycoerythrin-conjugated (RPE) antibody targeting the host species in which the antibody coupled to the bead was raised. The RPE-conjugated antibody is diluted to 0.5 μ g/mL in WB. 100 μ L diluted antibody is added to 5 μ L coupled beads and incubated for 20 min at 650 rpm at room temperature. After incubation, wells are washed three times with 100 μ L WB before being analyzed on a Luminex instrument. Readout signals are reported in terms of median fluorescence intensities (MFI). Coupling test for a bead ID is regarded as failed if the signals obtained are lower than $2 \times$ standard deviation (SD) of the mean value for the bead array or if the value obtained does not exceed the mean value for the bare bead (bead not coupled to any antibody). In case of a failed coupling, a new coupling is performed only for the specific bead identity. During coupling test, the bead counts are also evaluated. In each well, bead count per ID should be of 100–200.

3.2 Selection of Antibody Pairs

Desirable characteristics for antibody pair combinations are:

- Recognition of their target both in the format of a recombinant protein standard and in the native form in the sample matrix of interest.

- Low interaction with other proteins.
- Low background and therefore a high signal-to-background ratio.

Each sandwich assay requires its own development and optimization in terms of sample dilution, assay buffers, incubation time, need for heat treatment, and concentration of detection antibody. However, the starting point for the development should be the selection of an antibody pair working in a dilution series of a protein standard (if available) (*see Note 4*) and in a dilution series of a sample pool (*see Note 5*). Below we will describe the screening of antibody pairs for a protein in plasma and with the recombinant protein where all antibodies are tested as capture and as detection as described in Fig. 1.

1. Prepare a plate layout following the representation in Fig. 1b.
2. Thaw sample pool and recombinant protein on ice. Dilute the plasma in assay buffer 1 to cover a dilution range of 1:4 to 1:3000 in seven steps (threefold serial dilutions). Dilute the standard protein in assay buffer 1 covering a concentration range from 1 $\mu\text{g/mL}$ to 1 ng/mL . A blank sample (containing only the specific assay buffer) is prepared for both the dilution curves. Each dilution curve should be prepared in duplicate (*see Note 6*). To optimize the number of antibody pairs and eventually protein standard tested, the format of 96-well plate described in Fig. 1 can be extended to a 384-well plate by combining four 96-well plates (*see Notes 7 and 8*). However, to simplify, in the following steps volumes have been calculated for a 96-well plate format.
3. Calculate the volume of diluted standard and plasma to be enough for 50 μL diluted sample per well. Start by making the highest concentration and go stepwise through the series by mixing previous concentration with buffer, excluding the blank.
4. Prepare the bead array of capture antibodies by vortexing and sonicating the tube. Dispense 5 μL beads into the wells of a 96-well Greiner assay plate. Vortex the tube containing the bead array every other column since the beads will sediment. Transfer 45 μL of each point in the dilution series for both recombinant protein and plasma to the corresponding wells in the Greiner plate with beads. Seal, vortex, and incubate overnight at room temperature, dark, and 650 rpm.
5. Spin down the plate and wash $3 \times 100 \mu\text{L}$ WB. Dilute detection antibodies to a concentration of 1 $\mu\text{g/mL}$. Add 50 μL detection antibody into the wells according to the plate layout. Seal, vortex, and incubate for 1.5 h at room temperature, dark, and 650 rpm.

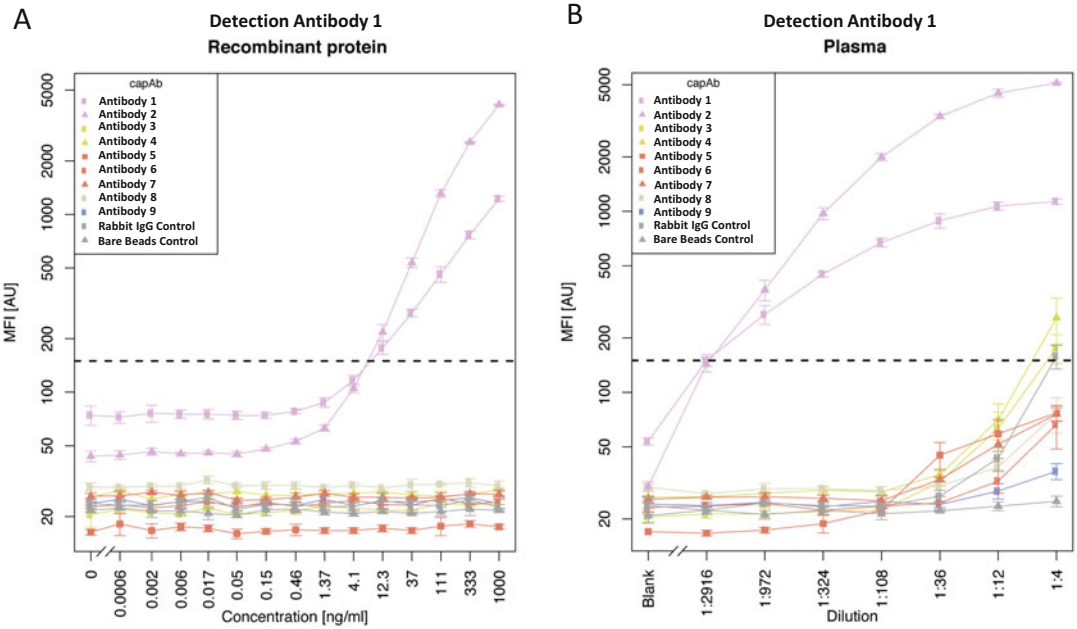


Fig. 2 Selection of antibody pairs. Combinations of multiple capture antibodies (CapAb) coupled to beads and detection antibodies were tested in a dilution curve of recombinant protein (a) and plasma (b)

6. Spin down the plate and wash $3 \times 100 \mu\text{L}$ WB. Dispense $50 \mu\text{L}$ SAPE diluted 1:750 in each well. Seal, vortex, and incubate for 30 min at room temperature, dark, and 650 rpm.
7. Spin down the plate and wash $3 \times 100 \mu\text{L}$ of WB. After the last wash add $100 \mu\text{L}$ of WB to each well and start measurement in Luminex instrument.
8. In the instrument, set sample volume ($100 \mu\text{L}$), bead type, and bead IDs included in the array. The signal detected from each bead ID is reported as “median fluorescence intensity.” A minimum count of 50 beads per ID is required.
9. To evaluate the performance of antibody pairs during the selection phase, data are log-transformed and visualized by plotting the dilution series of each capture antibody with each detection antibody for both plasma and recombinant protein (Fig. 2).
10. Antibody pairs can be grouped into four categories according to their performance: (1) dilution-dependent curves with protein and plasma, (2) dilution-dependent curves with protein only, (3) dilution-dependent curves with plasma only, and (4) no dilution-dependent curves with either sample type.
11. Antibody pairs that are selected for further assay validation should be assigned to group 1 or group 3 (if a recombinant standard protein is not available) and they report only signals

above an average background. When more than one promising pair is available, priority should be given to the pair showing (a) the widest dynamic range of detectable concentrations of recombinant protein and plasma, (b) the lowest background level in antigen-free samples, and (c) the lowest interferences or off-target recognition of other captured proteins.

Antibody pairs should be excluded when a dilution-dependent curve for its own target was observed in combination with a dilution-dependent curve for the same detection antibody paired with another capture antibody toward a different target or for a control bead (*see Note 9*).

3.3 Immunoassay Optimization and Validation

When working antibody pairs have been identified, validation should proceed for each single assay by evaluating parameters such as linearity, dynamic range, reproducibility, robustness (*see Note 10*), and matrix effect (*see Note 11*). Below we describe how the upper limit of quantification (ULOQ), lower limit of quantification (LLOQ), limit of detection (LOD), and 50% effective dose (ED50) are calculated.

1. Prepare multiple SBAs to target few proteins at the time. Each SBA may contain up to 20 capture antibodies including (a) antibodies binding the protein target of interest; (b) antibodies raised against proteins different from those targets for which the assay is under evaluation in order to exclude off-target interactions and interference; and (c) negative control such as bare beads as described in Subheading 3.1, **step 1**.
2. Prepare a plate layout according to an experimental design that aims to test 14-point dilution curves for standard proteins and 7-point dilution curves for plasma excluding blanks. Each dilution curve should be prepared in triplicate, and could be repeated under different experimental conditions. Parameters that may be investigated are (a) different dilution buffers (*see Note 11*), (b) effect of sample preheat (*see Note 12*), (c) different concentration of detection antibody and SAPE, and (d) different incubation times and temperatures. For validation, the assay can be upscaled to a 384-well format (*see Subheading 3.2, step 2 and Note 7*).
3. Thaw sample pool and recombinant protein on ice.
4. Prepare 14 vials with decreasing concentration of recombinant protein covering a range of 1 $\mu\text{g/mL}$ to 1 pg/mL (14-point dilution curve). Dilute the plasma starting from a dilution 1:2 to a dilution 1:1500 in plasma dilution buffer (seven steps, threefold serial dilution). Blanks of both assay buffer and plasma dilution buffer are included. Each point of the dilution curves in plasma and for standard proteins must be prepared in triplicates.

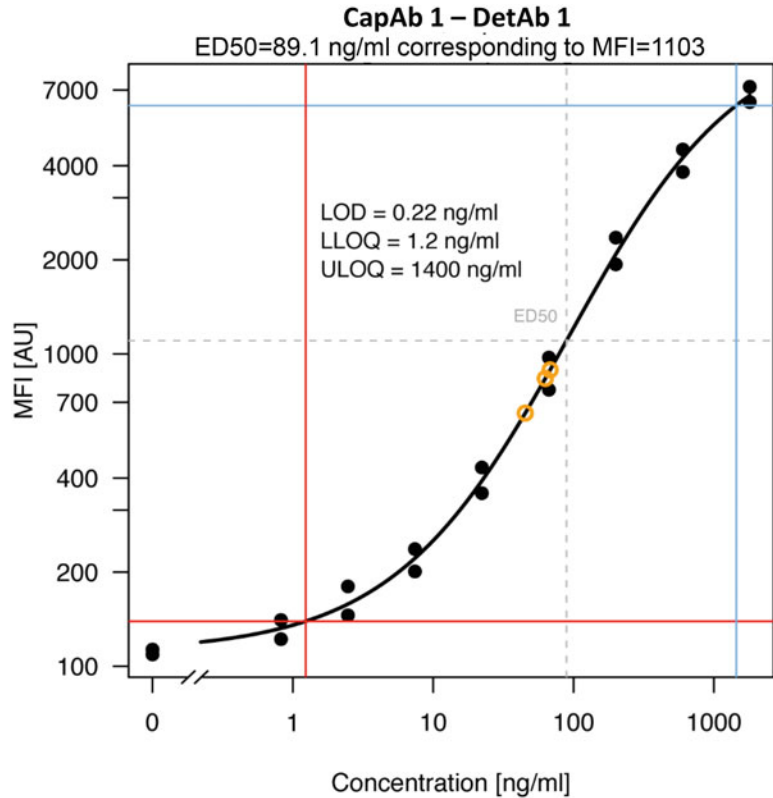


Fig. 3 Dilution curve of recombinant proteins. An antibody pair was tested in a dilution curve of AFM. A 5-parametric log-logistic model was applied to fit the data. LLOQ, ULOQ, and ED50 are represented, respectively, by red, blue, and gray dashed lines. Values obtained for a plasma pool are plotted onto the curve (orange dots)

5. Calculate the volume of diluted protein standard and plasma to be 50 μ L per assay well. Start by making the highest concentration and go stepwise through the series by mixing previous concentration with buffer, excluding the blank. Mix each dilution thoroughly before the next transfer.
6. Sample and bead incubation, washes, and detection steps are performed according to **steps 3–7** from the previous section (Subheading 3.2).
7. To evaluate the performance of antibody pairs during the selection phase, data are $\log_{10}$ transformed and visualized by plotting the dilution series of each capture antibody with each detection antibody for both plasma and recombinant protein.
8. Determine the CV for each dilution point averaging it across all dilution steps. Acceptable CVs are below 10% (Fig. 3).
9. Calculate the limits of quantification as follows: (a) LOD: average blank signal + $3 \times$ SD of the blank sample; (b) LLOQ:

average blank signal + $10 \times$ SD of the blank sample;
(c) ULOQ: average signal obtained for the highest protein concentration point subtracted by the SD at that point; and
(d) ED50 (50% effective dose): defined as the concentration (dose) at which 50% of the maximum intensity is observed.

10. The standard curves are used to extrapolate the concentration for each sample by log10 transforming the measured MFI signal intensities according to the equation obtained from the five-parametric log-logistic model and by multiplying it with the applied dilution factor of the sample (Fig. 3).

4 Notes

1. Antibodies: To create a sandwich assay, at least two different antibodies targeting the same protein are preferred. Optimally a list of antibodies targeting different epitopes should be screened. Pairs composed by a monoclonal (capture) and a polyclonal (detection) are more common than other combinations. However, multiple antibodies are often not available, and in that case one polyclonal antibody can be tested as both capture and detection reagent.
2. At least two types of negative controls in the array are recommended: a bare bead (blocked bead without coupled antibody) and an affinity-purified IgG for each host species in which the capture antibodies were generated (e.g., affinity-purified rabbit IgG, affinity-purified mouse IgG). Control beads assess background binding to IgG molecules or to the bead itself.
3. During the activation step, EDC reacts with carboxylic acid groups on the beads to form an active *O*-acylisourea intermediate that reacts with proteins' primary amino groups. The *O*-acylisourea intermediate is unstable in aqueous solutions; for this reason sulfo-*N*-hydroxysuccinimide (NHS) is included in the coupling procedure, to improve the efficiency of the reaction. The *O*-acylisourea reacts with sulfo-NHS, producing a more stable amine-reactive sulfo-NHS ester allowing for efficient conjugation to antibodies at physiologic pH. Since EDC is moisture sensitive, we found it convenient to buy it in a pre-aliquoted format to prevent loss of reactivity and contamination.
4. When the recombinant protein is not available, the performance of an antibody pair can be evaluated using an over-expressed lysate spiked in a buffer resembling the sample of interest or a pool of samples where the target proteins were detected at high levels with another method.

5. It is very important to use a sample pool which is expected to contain the target protein. If the discovery of the candidate biomarker was performed in a specific sample cohort, then samples in that cohort, where the protein level was high, should be pooled. Different plasma pools might give different results.
6. For an optimal evaluation, any test should be performed at least in duplicate. However, for the first screening of antibody pairs we found it convenient to run each condition as a single point to optimize the number of antibody pairs tested.
7. Assay development in the 384-well format is convenient not only to increase the number of tested conditions, but also to reduce reagent and sample consumption. In the 384-well format the volumes for each reaction can be reduced to the half.
8. If the assay under development will be applied in a large set of samples, the 384-well format may be more suitable to increase sample throughput. A method developed in a 96-well format will always require some optimization while upscaling to the 384-well format; therefore, we found it more convenient to start the development in a 384-well plate directly.
9. A common source of interference when developing sandwich immunoassays is represented by HAMA = human anti-mouse antibodies. HAMA circulates in the human blood and can introduce false-positive results if either one or both of the capture antibodies bound to the beads and the detection antibody were raised in mouse. Other anti-animal antibodies (rat, rabbit, goat, etc.) that are common for example in animal workers may interfere with the assay. We have found LowCross-Buffer and other supplements effective for reducing HAMA interference.
10. Robustness is per definition the ability of a method to remain unaffected by small variations. To evaluate robustness, it is necessary to identify critical parameters in the procedure such as temperature and duration of incubation, and perform the assay while systematically changing these parameters when testing the same sample.
11. During antibody pair selection, the assay is often performed with a relatively simple dilution buffer (e.g., 1% BSA) which does not fully represent the complexity of the samples. During assay validation it is advisable to use higher complexity matrices to dilute protein standard such as plasma from other species or surrogates of biofluids such as artificial CSF (*see* Subheading 2). Eventually also the dilution of sample pool should be performed diluting a sample containing high level of the protein of interest, with a sample containing nondetectable levels of it.

12. We have observed that sample preheat treatment at 56 °C for 30 min, followed by 10 min at RT, may improve an antibody pair performance due to a partial protein denaturation and better exposure of the epitope.

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References

1. Lamb JR, Jennings LL, Gudmundsdottir V, Gudnason V, Emilsson V (2020) It's in our blood: a glimpse of personalized medicine. *Trends Mol Med*. <https://doi.org/10.1016/j.molmed.2020.09.003>
2. Williams SA, Kivimäki M, Langenberg C, Hingorani AD, Casas JP, Bouchard C, Jonasson C, Sarzynski MA, Shipley MJ, Alexander L, Ash J, Bauer T, Chadwick J, Datta G, DeLisle RK, Hagar Y, Hinterberg M, Ostroff R, Weiss S, Ganz P, Wareham NJ (2019) Plasma protein patterns as comprehensive indicators of health. *Nat Med* 25(12):1851–1857. <https://doi.org/10.1038/s41591-019-0665-2>
3. Uhlén M, Karlsson MJ, Hober A, Svensson AS, Scheffel J, Kotol D, Zhong W, Tebani A, Strandberg L, Edfors F, Sjöstedt E, Mulder J, Mardinoglu A, Berling A, Ekblad S, Dannemeyer M, Kanje S, Rockberg J, Lundqvist M, Malm M, Volk AL, Nilsson P, Månberg A, Dodig-Crnkovic T, Pin E, Zwaalen M, Oksvold P, von Feilitzen K, Häussler RS, Hong MG, Lindskog C, Ponten F, Katona B, Vuu J, Lindström E, Nielsen J, Robinson J, Ayoglu B, Mahdessian D, Sullivan D, Thul P, Danielsson F, Stadler C, Lundberg E, Bergström G, Gummesson A, Voldborg BG, Tegel H, Hober S, Forsström B, Schwenk JM, Fagerberg L, Sivertsson Å (2019) The human secretome. *Sci Signal* 12(609). <https://doi.org/10.1126/scisignal.aaz0274>
4. Geyer PE, Holdt LM, Teupser D, Mann M (2017) Revisiting biomarker discovery by plasma proteomics. *Mol Syst Biol* 13(9):942. <https://doi.org/10.15252/msb.20156297>
5. Wright I, Van Eyk JE (2017) A roadmap to successful clinical proteomics. *Clin Chem* 63(1):245–247. <https://doi.org/10.1373/clinchem.2016.254664>
6. Bachmann J, Burté F, Pramana S, Conte I, Brown BJ, Otimadegun AE, Ajetunmbi WA, Afolabi NK, Akinkunmi F, Omokhodion S, Akinbami FO, Shokunbi WA, Kampf C, Pawitan Y, Uhlén M, Sodeinde O, Schwenk JM, Wahlgren M, Fernandez-Reyes D, Nilsson P (2014) Affinity proteomics reveals elevated muscle proteins in plasma of children with cerebral malaria. *PLoS Pathog* 10(4):e1004038. <https://doi.org/10.1371/journal.ppat.1004038>
7. Häggmark A, Byström S, Ayoglu B, Qundos U, Uhlén M, Khademi M, Olsson T, Schwenk JM, Nilsson P (2013) Antibody-based profiling of cerebrospinal fluid within multiple sclerosis. *Proteomics* 13(15):2256–2267. <https://doi.org/10.1002/pmic.201200580>
8. Remnestrål J, Just D, Mitsios N, Fredolini C, Mulder J, Schwenk JM, Uhlén M, Kultima K, Ingelsson M, Kilander L, Lannfelt L, Svenningsson P, Nellgård B, Zetterberg H, Blennow K, Nilsson P, Häggmark-Månberg A (2016) CSF profiling of the human brain enriched proteome reveals associations of neuromodulin and neurogranin to Alzheimer's disease. *Proteomics Clin Appl* 10(12):1242–1253. <https://doi.org/10.1002/prca.201500150>
9. Tebani A, Gummesson A, Zhong W, Koistinen IS, Lakshmikanth T, Olsson LM, Boulund F, Neiman M, Stenlund H, Hellström C, Karlsson MJ, Arif M, Dodig-Crnković T, Mardinoglu A, Lee S, Zhang C, Chen Y, Olin A, Mikes J, Danielsson H, von Feilitzen K, Jansson PA, Angerås O, Huss M, Kjellqvist S, Odeberg J, Edfors F, Tremaroli V, Forsström B, Schwenk JM, Nilsson P, Moritz T, Bäckhed F, Engstrand L, Brodin P, Bergström G, Uhlén M, Fagerberg L (2020) Integration of

- molecular profiles in a longitudinal wellness profiling cohort. *Nat Commun* 11(1):4487. <https://doi.org/10.1038/s41467-020-18148-7>
10. Dodig-Crnković T, Hong MG, Thomas CE, Häussler RS, Bendes A, Dale M, Edfors F, Forsström B, Magnusson PKE, Schuppe-Koistinen I, Odeberg J, Fagerberg L, Gummeson A, Bergström G, Uhlén M, Schwenk JM (2020) Facets of individual-specific health signatures determined from longitudinal plasma proteome profiling. *EBioMedicine* 57:102854. <https://doi.org/10.1016/j.ebiom.2020.102854>
 11. Anderson NL, Ptolemy AS, Rifai N (2013) The riddle of protein diagnostics: future bleak or bright? *Clin Chem* 59(1):194–197. <https://doi.org/10.1373/clinchem.2012.184705>
 12. Fredolini C, Byström S, Sanchez-Rivera L, Ioannou M, Tamburro D, Pontén F, Branca RM, Nilsson P, Lehtiö J, Schwenk JM (2019) Systematic assessment of antibody selectivity in plasma based on a resource of enrichment profiles. *Sci Rep* 9(1):8324. <https://doi.org/10.1038/s41598-019-43552-5>
 13. Uhlen M, Bandrowski A, Carr S, Edwards A, Ellenberg J, Lundberg E, Rimm DL, Rodriguez H, Hiltke T, Snyder M, Yamamoto T (2016) A proposal for validation of antibodies. *Nat Methods* 13(10):823–827. <https://doi.org/10.1038/nmeth.3995>
 14. Andersson A, Remnestrål J, Nellgård B, Vunk H, Kotol D, Edfors F, Uhlén M, Schwenk JM, Ilag LL, Zetterberg H, Blennow K, Månberg A, Nilsson P, Fredolini C (2019) Development of parallel reaction monitoring assays for cerebrospinal fluid proteins associated with Alzheimer's disease. *Clin Chim Acta* 494:79–93. <https://doi.org/10.1016/j.cca.2019.03.243>
 15. Walker MJ, Zhou C, Backen A, Pernemalm M, Williamson AJ, Priest LJ, Koh P, Faivre-Finn C, Blackhall FH, Dive C, Whetton AD (2015) Discovery and validation of predictive biomarkers of survival for non-small cell lung cancer patients undergoing radical radiotherapy: two proteins with predictive value. *EBioMedicine* 2(8):841–850. <https://doi.org/10.1016/j.ebiom.2015.06.013>
 16. Schwenk JM, Nilsson P (2011) Antibody suspension bead arrays. *Methods Mol Biol* 723:29–36. https://doi.org/10.1007/978-1-61779-043-0_3
 17. Drobin K, Nilsson P, Schwenk JM (2013) Highly multiplexed antibody suspension bead arrays for plasma protein profiling. *Methods Mol Biol* 1023:137–145. https://doi.org/10.1007/978-1-4614-7209-4_8
 18. Häussler RS, Bendes A, Iglesias M, Sanchez-Rivera L, Dodig-Crnković T, Byström S, Fredolini C, Birgersson E, Dale M, Edfors F, Fagerberg L, Rockberg J, Tegel H, Uhlén M, Qundos U, Schwenk JM (2019) Systematic development of sandwich immunoassays for the plasma secretome. *Proteomics* 19(15):e1900008. <https://doi.org/10.1002/pmic.201900008>
 19. Dezfouli M, Vickovic S, Iglesias MJ, Nilsson P, Schwenk JM, Ahmadian A (2014) Magnetic bead assisted labeling of antibodies at nanogram scale. *Proteomics* 14(1):14–18. <https://doi.org/10.1002/pmic.201300283>
 20. Artificial cerebrospinal fluid (ACSF) (2011) Cold Spring Harbor Protoc 2011(9):pdb.rec065730. <https://doi.org/10.1101/pdb.rec065730>

Part III

Applications of Protein Microarrays



Analysis of Protein-Protein Interactions by Protein Microarrays

Ana Montero-Calle and Rodrigo Barderas

Abstract

The analysis of the proteome and the interactome would be useful for a better understanding of the pathophysiology of several disorders, allowing the identification of potential specific markers for early diagnosis and prognosis, as well as potential targets of intervention. Among different proteomic approaches, high-density protein microarrays have become an interesting tool for the screening of protein-protein interactions and the interactome definition of disease-associated dysregulated proteins. This information might contribute to the identification of altered signaling pathways and protein functions involved in the pathogenesis of a disease. Remarkably, protein microarrays have been already satisfactorily employed for the study of protein-protein interactions in cancer, allergy, or neurodegenerative diseases. Here, we describe the utilization of recombinant protein microarrays for the identification of protein-protein interactions to help in the definition of disease-specific dysregulated interactomes.

Key words Protein-protein interactions, Protein microarrays, Proteomics, Human disease

1 Introduction

Proteins are key effectors of all human cells, organs, and tissues. These biomolecules are composed of a linear sequence of amino acids (AAs) covalently bound by peptide bonds between the carboxyl group of the first AA and the amino group of the subsequent AA. After folding, their secondary and tertiary structures give rise to the different protein domains, which are the basic fundamental functional units of proteins [1]. Through these domains, proteins can interact with other biomolecules as DNA, lipids, or proteins, forming homo- or hetero-dimers, trimers, or tetramers, modifying their structure and applying an effect over them, which some proteins need to be functional or to mediate the formation of protein complexes and posttranslational modifications (PTMs) [2].

Protein-protein interactions are needed in all cell compartments for all cellular processes, such as transport, metabolic pathways, or signal transduction, among others [3]. Therefore, the characterization of the proteome and the interactome of disease-specific dysregulated proteins would be useful to get further insights into the pathobiology of devastating diseases, allowing the identification of not only potential markers for early diagnosis and prognosis but also new potential targets of intervention, and, thus, improving the clinical management of patients [4–6].

Proteome characterization of all predicted protein-coding genes is a very complex task that beyond the Human Proteome Project is far from being achieved to generate a map of the protein-based molecular architecture to help elucidate biological and molecular function [7–9], due to the presence of multiple isoforms and PTMs that convert each protein in 10–50 different proteoforms [10–12]. In addition, changes in protein expression levels and their localization over time increase the difficulty of protein studies. Currently, besides mass spectrometry methods and other classical methods as yeast two-hybrid screening or phage display for the identification of protein-protein interactions and for the characterization of a cell, tissue, or disease-specific interactome, high-density protein microarrays have become a valuable tool useful for the identification of protein-protein interactions, as well as for the identification of new biomarkers for patients' monitoring and for personalized medicine.

For the analysis of protein-protein interactions different types of protein microarrays might be used, according to the nature of the printed biomolecule:

1. *Recombinant protein microarrays*: These arrays containing thousands of proteins encoded by an organism directly printed onto the slide are useful for the characterization of protein functions, such as protein-protein binding, biochemical activity, enzyme-substrate relationships, and immune responses [13–15]. Currently, there are commercial high-density recombinant protein microarrays (HuProt) printed with up to 15,889 out of 19,613 canonical human protein-coding genes described in the Human Protein Atlas (*see* Subheading 3).
2. *PrESTs*: In these protein microarrays, fragments of proteins (protein-epitope signature tag antigens, PrESTs) up to 150 AA in length are printed on the slides [8, 16]. This approach developed by the Human Protein Atlas (HPA) initiative has synthesized PrESTs for almost each human protein-coding gene, without including isoforms and PTMs.
3. *NAPPA (Nucleic Acid-Programmable Protein Arrays)*: These arrays are printed with plasmid DNA containing the cDNA codifying the protein of interest fused to epitope tags on the

N- or C-terminal end of the protein, so that they can be captured by a high-affinity capture reagent immobilized along with the cDNA. This approach minimizes protein manipulation, inducing their expression using cell-free protein expression methods just at the time of the experiment, avoiding problems with protein purification and stability [17–20].

4. *Antibody microarrays*: Antibodies or fragment of antibodies (scFvs, Fabs ...) are printed onto these protein microarrays, which are mainly used for the identification of pathology-associated altered proteins [21–23]. There are commercial antibody microarrays (RayBiotech) composed of thousands of antibodies against human proteins [24].
5. *Phage arrays*: These arrays are an economic alternative to commercial protein microarrays, and are useful for the identification of antigens or autoantigens specific of a disease [25–27]. This approach requires the use of phage display libraries displaying on their surface peptides or proteins, which after enrichment are later printed onto nitrocellulose slides for subsequent analysis.

In this context, in this chapter we report the protocols needed for the identification of protein-protein interactions using different biological samples and recombinant protein microarrays, which are the most widely used protein microarrays together with NAPPa for such a purpose.

2 Materials

This section resumes common materials and reagents used for the identification of protein-protein interactions using commercial protein microarrays followed by the specific materials and solutions used for the specific procedure in subsequent indicated subheadings.

2.1 Common Reagents

1. Phosphate-buffered saline pH 7.4: PBS, 137 mM NaCl, 2.7 mM KCl, 8 mM Na₂PO₄, and 1.5 mM KH₂PO₄.
2. Bovine serum albumin: BSA.
3. 1,4-Dithiothreitol: DTT (*see Note 1*).
4. Probe buffer: 1% BSA, 0.05% Triton X-100, 5% glycerol, 5 mM MgCl₂, 0.5 mM DTT in PBS, 10 g BSA, 500 µL Triton X-100, 50 µL glycerol 100%, 1.02 g MgCl₂, 0.078 g DTT, and 950 mL PBS (*see Note 2*).
5. Blocking solution: 1% BSA and 0.1% Tween-20 in PBS; 10 g BSA, 1 mL Tween-20, and 999 mL PBS (*see Note 2*).
6. Milli-Q water.

7. Primary antibodies against the protein of interest (*see Note 3*).
8. Primary antibody against the tag fused to proteins printed onto the microarrays (positive control) (*see Note 3*).
9. Biotinylated Cy3/Cy5 pairs or Alexa Fluor 555/Alexa Fluor 647-paired secondary antibodies.
10. Cy3-, Cy5-conjugated streptavidin, or Alexa Fluor 555 or 647 fluorescent streptavidin.

2.2 Equipment

1. Orbital shaker/oscillating rocker/roller.
2. 0.22 μ m pore filters.
3. Slide hybridization polycarbonate chamber.
4. Falcon centrifuge 5810 R.
5. Microtube centrifuge.
6. Nanodrop 2000C.
7. Microarray scanner: GenePix 4000B.
8. Microarray acquisition and analysis software: GenePix Pro 7.1.
9. Bioinformatic tools (Pomelo II, MultiExperiment Viewer, or Protoarray Prospector, among others).

2.3 Protein Microarrays

High-density commercial protein microarrays (i.e., HuProt (CDI Labs)) contain thousands of recombinant human proteins whose open reading frames (ORF) are expressed with an N-terminal or a C-terminal tag.

Fusion proteins are printed on nitrocellulose slides along with positive and negative control proteins, like IgG, BSA, or GST. Each array is composed of several subarrays with about 100 μ m diameter spots, each probe being printed in duplicate or triplicate onto the slide. Customized protein microarrays are also available attending to researchers' needs and provided from different biotech (i.e., RayBiotech).

2.4 Proteins

Proteins to be used for the identification of protein-protein interactions with protein microarrays could be (1) purified proteins; (2) serum samples (i.e., detection of a disease-specific humoral immune response—antibody-antigen interaction); (3) cell or tissue protein extracts (i.e., pathological and healthy tissues to identify disease-specific interactions); or (4) conditioned medium from cell cultures (i.e., cancer cells with different metastatic abilities to identify protein-protein interactions related with metastasis, or production of a protein directly released into the media to be used for identification of their interacting partners).

If neither primary nor secondary antibodies are used, protein samples have to be labeled, either with biotin (indirect assay) or with a compatible pair of fluorophores (direct assay).

2.4.1 Protein Extraction

1. 4 mM EDTA PBS.
2. Lysis buffer: RIPA.
3. Protease inhibitor cocktail.
4. Phosphatase inhibitor cocktail.
5. 16- and 18-G needle syringes.
6. Coomassie brilliant blue R-250.
7. Nitrocellulose membrane.
8. Washing solution: 0.1% PBST; 999 mL PBS containing 1 mL Tween-20.
9. PBST supplemented with 3% skimmed milk (*see Note 4*).
10. Primary antibodies (anti-tag or anti-protein of interests).
11. HRP-labeled Anti-Mouse IgG secondary antibody.
12. HRP-labeled Anti-Rabbit IgG secondary antibody.
13. ECL Pico Plus chemiluminescent reagent.

2.4.2 Biotin Labeling

1. Sterile-filtered PBS.
2. Labeling buffer: PBS, DMSO, EZ-Link NHS-Biotin.
3. Dimethyl sulfoxide: DMSO.
4. Stop solution: 1 M Tris-HCl pH 8.0.
5. HRP-streptavidin.
6. Floating dialysis rack.

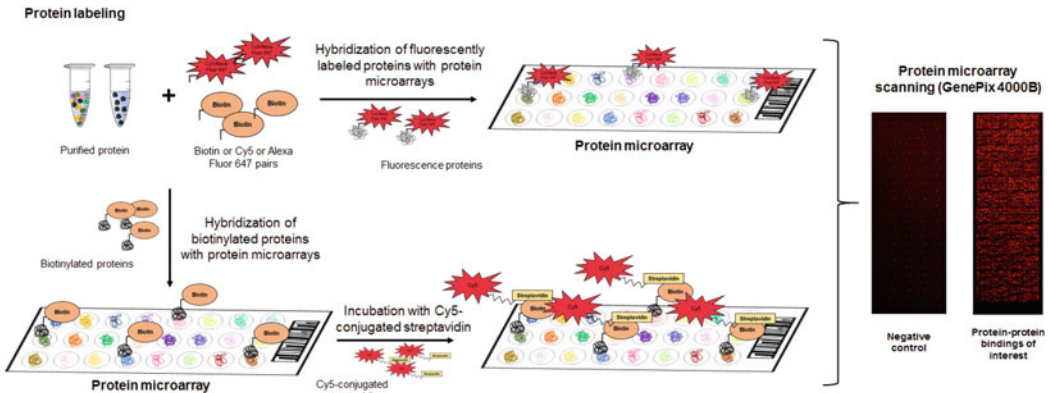
2.4.3 Fluorophore Labeling

1. Cy3 and Cy5 NHS ester.
2. Extraction/labeling solution (E0655-30ML, Sigma-Aldrich).
3. SigmaSpin column.

2.5 Detection

The detection of protein-protein interactions is mainly performed by using fluorophores (Fig. 1). If the proteins of interest have been labeled with a fluorophore, such as Bodipy, cyanine (Cy), or Alexa Fluor dyes, additional reagents or any steps prior to be probed in the arrays are not necessary, since the fluorescence signal is directly detected after the screening of the protein microarrays (Fig. 1a). However, when the proteins of interest are unlabeled, tagged, or untagged, a secondary incubation with fluorescence or biotinylated primary or secondary antibodies is necessary to detect their protein interactors (Fig. 1b). In addition, when biotinylated proteins or biotinylated antibodies are used, a subsequent incubation with Cy5-conjugated streptavidin (or other compatible fluorophores) is necessary prior to scanning.

A



B

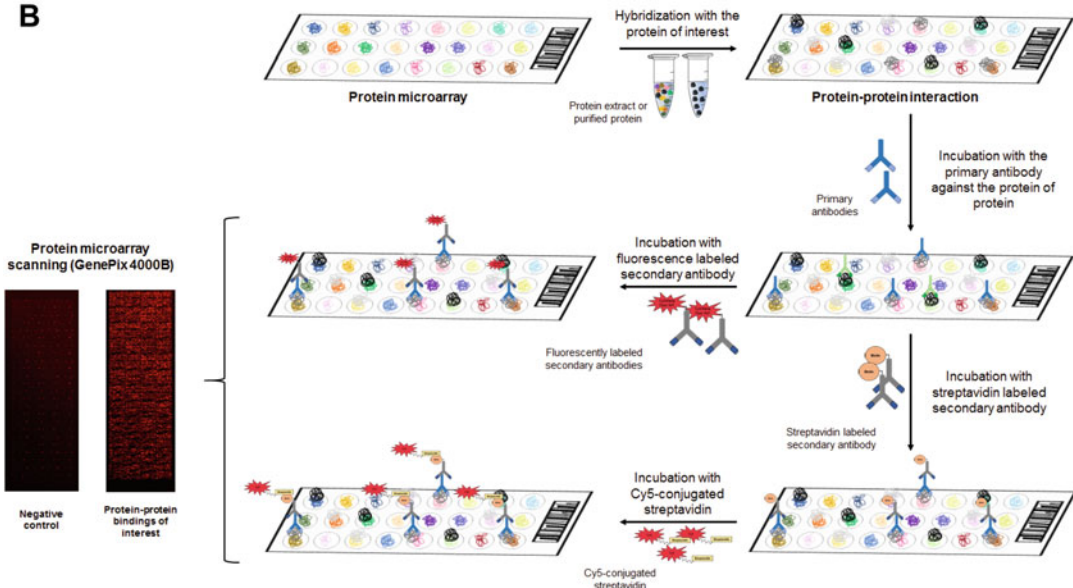


Fig. 1 Screening of human protein microarrays for the identification of protein-protein interactions. **(a)** Protein microarrays hybridized with the labeled protein of interest would be directly scanned after hybridization. Protein samples might be labeled with fluorescence (upper panel) or biotinylated (bottom panel). **(b)** Protein microarrays incubated with unlabeled protein samples will require subsequent hybridization steps for incubation with the primary or secondary labeled antibodies prior to scanning. These antibodies might be labeled with the selected fluorophore (middle panel) or with biotin (bottom panel). Biotinylated proteins and antibodies will require a subsequent incubation with fluorescently labeled streptavidin (i.e., Cy5- or Alexa Fluor 647-streptavidin) for signal development. Incubation of protein microarrays with only the secondary labeled antibody is required to identify unspecific reactive proteins (negative control). **(c)** Validation of protein-protein interactions identified by protein microarrays is recommended and might be performed by different orthogonal techniques, such as dot blot, western blot, ELISA, surface plasmon resonance (Biacore), phage display, or immunoprecipitation, among other techniques

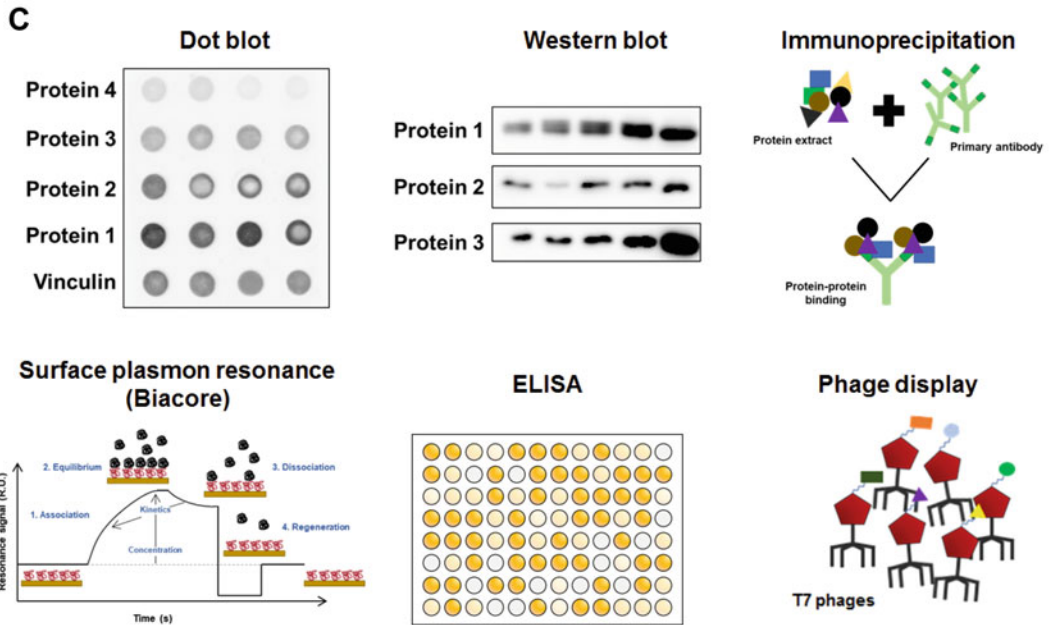


Fig. 1 (continued)

3 Methods

Protein microarrays have been mostly used for investigation of protein-protein interactions of proteins related to autoimmune diseases, chronic diseases such as cancer or neurodegenerative diseases, and allergy.

A schematic workflow for the screening of human protein microarrays for detection of protein-protein interactions is depicted in Fig. 1.

3.1 Protein Extraction and Quantification

For the analysis of protein-protein interactions using biological fluids, such as serum samples or cell culture-conditioned medium, protein concentration should be determined prior to hybridization. When using tissue or cell protein samples for these studies a previous step of protein extraction is necessary.

For cell protein extracts, adherent cells are resuspended using PBS containing 4 mM EDTA, whereas cells growing in suspension are harvested in growing culture medium. Cells are then harvested by centrifugation at $260 \times g$ during 5 min at room temperature. Then, cell pellets are resuspended in RIPA supplemented with $1 \times$ protease and phosphatase inhibitors and mechanically disaggregated using 16 G and 18 G needle syringes until homogeneity is observed [28]. Alternatively, for tissue protein extracts, frozen tissue samples are cut into small pieces on dry ice, and lysed with

RIPA supplemented as above [21]. Finally, samples are centrifuged at $10,000 \times g$ and 4°C during 10 min and protein extracts (supernatants) transferred to a new tube [28].

Protein quantification prior to protein labeling and hybridization is necessary and can be performed by different methods, such as tryptophan method [29]. In addition, quality of protein samples should be confirmed by Coomassie blue staining and western blot (WB) (*see Note 5*).

3.2 Labeling of Protein Samples

For protein-protein interaction analysis using protein samples without primary or secondary antibodies, protein labeling is necessary, either with biotin (indirect assay) or with a fluorophore (direct assay) (Fig. 1a), as previously described [30].

3.2.1 Biotin Labeling

Prior to biotinylation, any traces of amines and azides should be removed as they quench the biotinylation reaction. Dialysis and gel filtration are the preferred methodologies for such a purpose.

1. For biotinylation, 100–200 μL of purified protein samples at a concentration of 0.1–0.2 mg/mL are separately dialyzed on tubes incubated onto a floating dialysis rack inside a beaker containing approximately 1000 times more volume of PBS than the volume of the sample. Buffer should be continuously on stirring, and samples should be dialyzed three times for 2 h at 4°C each time, except the third one that could be left overnight (*see Note 6*).
2. Then, transfer the samples into microfuge tubes and centrifuge them for 5 min at $100 \times g$ at room temperature to remove any precipitate. Collect the supernatant and store at -20°C .
3. Immediately before labeling, prepare the labeling reagent. Add 100 μL of sterile-filtered PBS to the label reagent tube resuspended in DMSO (i.e., EZ-Link NHS-Biotin (Thermo Fisher Scientific)). Dissolve it by pipetting up and down.
4. Add 30 μg of the individual protein samples to the labeling reagent tube to get ≥ 20 -fold molar excess of NHS-Biotin. Perform the reaction in a final volume of 300 μL during 30 min at room temperature.
5. To stop the reaction, add 3 μL of stop solution.
6. Dialyze the samples as previously done to remove unbound biotin and any salt traces.
7. Centrifuge the samples for 5 min at $9500 \times g$ to remove floating particles and precipitates. Annotate the final volume, and measure again the protein concentration prior to hybridization of the protein microarrays (*see Note 7*).

3.2.2 Fluorophore Labeling

Although Cy3/Cy5 pairs or, alternatively, Alexa Fluor 555/Alexa Fluor 647 pairs can be used, for protein microarrays it is usually recommended to perform the hybridization with far-red fluorescent dyes (Cy5 or Alexa Fluor 647) to avoid the background observed in these arrays in the green channel (Fig. 1a). For fluorophore labeling, use 100 μ L of sample at a concentration of 1.25 μ g/ μ L (*see Note 8*):

1. To prepare the labeling reagent, add 50 μ L of the labeling solution to the lyophilized Cy5 tube. The buffer (and the sample) must not contain any azide or primary amine groups to avoid any interference in the reaction.
2. Add 6.25 μ L of the reagent to the sample tubes (20 μ L of Cy5 for each 400 μ g of protein). Incubate the labeling reaction for 30 min at room temperature at 600 rpm. Then, add 50 μ L of labeling reagent.
3. Prepare one SigmaSpin column to remove unbound dye for each labeling reaction.
4. Centrifuge during 2 min at 4 °C and $750 \times g$ to remove any resin excess.
5. Add the total volume of each labeling reaction onto the center of SigmaSpin columns. Use one SigmaSpin per sample. Do not reuse columns. Centrifuge for 4 min at 4 °C and $750 \times g$.
6. Collect the eluate and store 130 μ L at -20 °C and the remaining volume at 4 °C.
7. Calculate dye/protein ratio and protein concentration with the eluate stored at 4 °C (*see Note 9*).

3.3 Hybridization

Commercial or custom-made protein microarrays are normally stored at -20 °C. Before use, protein microarrays have to be equilibrated at 4 °C during 15 min. Then, microarrays are placed in the incubation chambers with the bar code facing up and incubations are made at room temperature unless indicated.

1. To minimize nonspecific binding, block each protein microarray with 3 mL of blocking solution during 1 h at 4 °C and 50 rpm.
2. Discard and remove the excess of blocking solution by pipetting (*see Note 10*).
3. Immediately incubate each protein microarray using a hybridization chamber with 1.6 mL of protein samples diluted in the probe buffer overnight at 4 °C and 30 rpm (*see Notes 11 and 12*).
4. Discard and remove the incubated protein sample by pipetting.
5. Wash each microarray with 3 mL of probe buffer during 5 min at 4 °C and 50 rpm.

6. Discard and remove the excess of probe buffer by pipetting.
7. Repeat **steps 5** and **6** three more times.
8. Incubate each protein microarray with 3 mL of the primary antibody at the dilution recommended by the manufacturers in probe buffer during 1 h at 4 °C and 50 rpm (*see Notes 13 and 14*).
9. Discard and remove the excess of primary antibody by pipetting.
10. Repeat **steps 5–7**.
11. Incubate each protein microarray with 3 mL of the corresponding fluorescently labeled secondary antibody at the dilution recommended by the manufacturers diluted in probe buffer during 90 min at 4 °C and 50 rpm (*see Notes 8 and 15*).
12. Discard and remove the excess of secondary antibody by pipetting.
13. Repeat **steps 5–7**.
14. Wash each protein microarray with 3 mL of PBS during 5 min at 4 °C and 50 rpm to remove any detergent traces.
15. Wash each microarray with 50 mL of Milli-Q H₂O to remove any salt traces (*see Note 16*).
16. Dry protein microarrays by centrifuging at $300 \times g$ for 10 min at room temperature in a 50 mL falcon tube containing absorbent paper at the bottom with the barcode at the bottom of the falcon tube (*see Note 17*).
17. Transfer each protein microarray to a new 50 mL falcon tube and store at room temperature in darkness until scan (*see Note 18*).

3.4 Scanning and Data Analysis

A microarray scanner compatible with nitrocellulose surfaces and with the 532 nm (red channel) and 635 nm (green channel) solid-state lasers is needed, as the GenePix 4000B. The red channel is used for the detection of Cy5 and Alexa Fluor 647, while the green one is used for the detection of Cy3 and Alexa Fluor 555 (if it is also used). The laser power percentage should be adjusted during pre-view scanning, and probe at, at least, two different laser powers (i.e., 100% and 10%) for a better analysis of low- and high-reactive spots. Finally, all arrays have to be scanned at the same laser power for proper normalization.

The quantification of the signal intensity of each spot is performed using a microarray acquisition and analysis software compatible with the previously obtained images with the scanner (i.e., GenePix Pro 7.1 program) that allow the intra-array normalization using the background signal around each spot. For such a purpose, the array list (*.gal) containing the information associated to each

spot printed onto the array (protein ID, protein name, gene name ... together with the x and y coordinates of this location in the array) is required. This array list is provided by the manufacturers of commercial protein microarrays. Alternatively, for custom-made protein microarrays the array list can be generated with GenePix Pro 7.1 or Microsoft Office Excel.

Then, after the analysis of the microarrays, the signal intensity obtained will be recorded in the array list associated to the corresponding spots. Finally, data obtained for each spot in the microarray is inter-array normalized and processed using the median intensity of each spot. For data processing, free available bioinformatics tools, such as Babelomics, Pomelo II, or MultiExperiment Viewer [31–33], can be used to identify those proteins interacting with the protein of interest and ranked according to the signal intensity displayed in the microarrays. Alternatively, commercial protein array manufacturers provide bioinformatic tools for analysis and identification of protein interactors, such as Protoarray Prospector v5.2.

3.5 Validation

Validation of results should be made using orthogonal techniques in order to avoid false positives and to ensure the accuracy of the results. Lack of validation would increase the identification of erroneous protein-protein interactions and the incorrect definition of their interactome.

Validation of protein-protein interactions identified by high-throughput microarrays might be performed by means of qualitative and quantitative techniques, including dot-blot, WB, ELISA, surface plasmon resonance (Biacore), interferometry (Blitz), phage display, or yeast two-hybrid assays (Fig. 1c) [34, 35]. Surface plasmon resonance and interferometry are recommended to get the affinity constants of the interacting proteins [36–38]. Another accurate technique for validation of protein-protein interactors is immunoprecipitation followed by WB using HRP-labeled antibodies against the protein of interest and their partners (Fig. 1c). However, some of these techniques might not be available due to the absence of adequate and affordable antibodies that recognize the peptides or proteins of interest. In that case, it would be recommended to use fusion proteins with different tags and anti-tag antibodies for the validation of the interactions, or alternatively use mass spectrometry for validation of the interactor partners [28]. Finally, it would also be recommended to perform reciprocal assays to doubly verify protein-protein interactions.

Validation assays might take into consideration the objectives of the study, the nature of the methodology, and the biological variability. Collectively, validation assays should result in high-quality validated bioanalytical data available to the scientific community.

4 Notes

1. DTT is a reagent used to reduce protein disulfide bonds to sulfhydryl groups in peptides and proteins. DTT should not be added to the probe buffer until use, and after adding DTT, probe buffer should be stored at 4 °C in a container tightly sealed and protected from oxygen for less than 1 week to avoid its oxidation.
2. Blocking solution and probe buffer should be prepared the day before the assay, filtered, and stored at 4 °C until use.
3. Primary antibodies used for detecting protein-protein interactions by means of protein microarrays might be unlabeled, biotin-labeled, or fluorophore-labeled antibodies. For those unlabeled antibodies, a secondary fluorescently labeled antibody has to be used to detect specific signals.
4. When using biotinylated proteins or antibodies, to further amplify the signal with fluorescently labeled streptavidin do not block the membrane with skimmed milk (use BSA as blocking agent). The presence of biotin in milk could abrogate the specific signal of subsequent steps or increase the background signal of the microarray.
5. Quality of purified protein samples should be assessed by Coomassie blue staining and WB after 10% SDS-PAGE under reducing conditions. Alternatively, for protein extracts assessed as above, use α -tubulin, GAPDH, β -actin, or RhoGDi as loading controls by WB. Diffused protein bands or multiple bands observed by WB suggest protein sample degradation, and then, new protein samples should be obtained prior to labeling and hybridization with the protein microarray.
6. Sample volumes could change after dialysis. If the protein samples were equally concentrated before the dialysis, the sample volumes should be the same after the dialysis. However, it is always recommended to measure the collected volume and quantify the protein concentration for subsequent steps.
7. To check for a correct labeling, perform a WB analysis using HRP-streptavidin for detection of biotinylated proteins. If the labeling was correctly done, multiple bands should be detected for protein extracts and an only band for purified proteins.
8. Samples with any fluorophore (Cy3/Cy5 or, alternatively, Alexa Fluor 555/Alexa Fluor 647 pairs) should be kept away from light by using aluminum foil during any step of the experimental procedure involving them (labeling, screening, etc.).
9. Obtain a spectrum of the labeled protein from 750 to 220 nm using a NanoDrop system to determine the dye/protein ratio.

Absorbance at 280 nm will permit to obtain the protein concentration and absorbance at 555 and 635 nm for dye concentration. Usual optimal ratios range from 2–3 to 6–8 for Cy5 and Cy3, respectively. Additionally, the protein separated after SDS-PAGE can be recorded onto a LICOR Odyssey CLx equipment to confirm the labeling of the protein.

10. Avoid dryness of nitrocellulose slides after any incubation or during washes to avoid unspecific signal or an increase in the background signal of the microarrays.
11. For hybridization with unpurified protein samples, 250 µg of protein from cell or tissue lysate is recommended. For purified protein samples, test different amounts of protein between 0.1 and 10 µg. Those spots appearing in all or multiple concentrations of protein are more prone to be actual interacting partners. Therefore, if possible, the titration of the optimal protein concentration is recommended to get the best results in the microarrays.
12. Optimal sample concentrations for hybridization depend on the nature of the sample. Conditioned media from cell culture usually does not require any dilution. When using serum samples, a 1:300 dilution of serum is the standard dilution in most experiments. However, it is recommended to test the optimal serum dilution to be used in the protein microarrays between 1:50 and 1:600 prior to incubation with a large set of samples. Plasma instead of serum could also be used.
13. Although it is recommended to incubate each array with one labeled sample, if each array will be incubated with two differently labeled samples it is recommended to hybridize protein microarrays with a primary antibody against the tag protein fused to the human proteins printed onto the microarrays (positive control primary antibody), followed by incubation with the appropriate secondary antibody, as printing quality control. Furthermore, it is also recommended to probe a protein microarray with only the secondary labeled antibodies to identify potential unspecific reactive proteins recognized by the secondary antibodies (negative control).
14. If protein microarrays are incubated with labeled protein samples or serum samples at **step 3 (Subheading 3.3)**, they could be incubated with the positive control primary antibody in **step 8**, as described in **Note 13**. On the contrary, if microarrays are incubated with unlabeled protein samples, **steps 11–13** should be repeated twice, firstly for hybridization with the primary antibody against the protein of interest and its corresponding secondary antibody, and secondly for hybridization only with the secondary antibody.

15. If protein samples or primary antibodies are fluorescently labeled, **steps 11–13** (Subheading 3.3) are not required and the protocol should be further continued at **step 14** (Fig. 1a). If protein samples or primary antibodies are unlabeled, protein microarrays should be incubated with either Cy5 or Alexa Fluor 647 secondary antibody (Fig. 1b). When protein samples or primary antibodies are biotinylated, protein microarrays should be subsequently incubated with Cy5- or Alexa Fluor 647-conjugated streptavidin in probe buffer (1:1000, 30 min at 4 °C and 50 rpm), and washed with probe buffer (**steps 5–7**) prior to **step 14**. Be careful when choosing primary antibodies and protein labels and tags if using fusion proteins (i.e., most proteins printed in the arrays are fused to GST).
16. Salt traces present at the end of the procedure produce high background during laser excitation and recording of the images.
17. Put the microarray with the barcode at the bottom of the falcon tube; otherwise during the centrifugations the barcode glue can potentially affect the signal and significantly increase the background on the microarray.
18. Prior to microarray scanning, protein microarrays might be further dried by applying filtered air and to remove at the same time any particle from the surface of the slides. It is also recommended to scan the nitrocellulose slides the day after hybridization to allow the slides to completely dry and to avoid the presence of any traces of moisture that can interfere during scanning.

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References

1. Berezovsky IN, Guarnera E, Zheng Z, Eisenhaber B, Eisenhaber F (2017) Protein function machinery: from basic structural units to modulation of activity. *Curr Opin Struct Biol* 42:67–74. <https://doi.org/10.1016/j.sbi.2016.10.021>
2. Vazquez A (2010) Protein interaction networks. In: Alzate O (ed) *Neuroproteomics*, Frontiers of Neuroscience series. CRC Press, Boca Raton, FL
3. Pawson T, Nash P (2000) Protein-protein interactions define specificity in signal transduction. *Genes Dev* 14:1027–47. <https://doi.org/10.1101/gad.14.9.1027>
4. Barderas R, Mendes M, Torres S, Bartolome RA, Lopez-Lucendo M, Villar-Vazquez R, Pelaez-Garcia A, Fuente E, Bonilla F, Casal JI (2013) In-depth characterization of the secretome of colorectal cancer metastatic cells identifies key proteins in cell adhesion, migration,

- and invasion. *Mol Cell Proteomics* 12 (6):1602–1620. <https://doi.org/10.1074/mcp.M112.022848>
5. Barderas R, Babel I, Casal JI (2010) Colorectal cancer proteomics, molecular characterization and biomarker discovery. *Proteomics Clin Appl* 4(2):159–178. <https://doi.org/10.1002/prca.200900131>
 6. Mendes M, Pelaez-Garcia A, Lopez-Lucendo M, Bartolome RA, Calvino E, Barderas R, Casal JI (2017) Mapping the spatial proteome of metastatic cells in colorectal cancer. *Proteomics* 17(19). <https://doi.org/10.1002/pmhc.201700094>
 7. Kim MS, Pinto SM, Getnet D, Nirujogi RS, Manda SS, Chaerkady R, Madugundu AK, Kelkar DS, Isserlin R, Jain S, Thomas JK, Muthusamy B, Leal-Rojas P, Kumar P, Sahasrabudhe NA, Balakrishnan L, Advani J, George B, Renuse S, Selvan LD, Patil AH, Nanjappa V, Radhakrishnan A, Prasad S, Subbannayya T, Raju R, Kumar M, Sreenivasamurthy SK, Marimuthu A, Sathe GJ, Chavan S, Datta KK, Subbannayya Y, Sahu A, Yelamanchi SD, Jayaram S, Rajagopalan P, Sharma J, Murthy KR, Syed N, Goel R, Khan AA, Ahmad S, Dey G, Mudgal K, Chatterjee A, Huang TC, Zhong J, Wu X, Shaw PG, Freed D, Zahari MS, Mukherjee KK, Shankar S, Mahadevan A, Lam H, Mitchell CJ, Shankar SK, Satishchandra P, Schroeder JT, Sirdeshmukh R, Maitra A, Leach SD, Drake CG, Halushka MK, Prasad TS, Hruban RH, Kerr CL, Bader GD, Iacobuzio-Donahue CA, Gowda H, Pandey A (2014) A draft map of the human proteome. *Nature* 509 (7502):575–581. <https://doi.org/10.1038/nature13302>
 8. Uhlen M, Fagerberg L, Hallstrom BM, Lindskog C, Oksvold P, Mardinoglu A, Sivertsson A, Kampf C, Sjostedt E, Asplund A, Olsson I, Edlund K, Lundberg E, Navani S, Szgyarto CA, Odeberg J, Djureinovic D, Takanen JO, Hober S, Alm T, Edqvist PH, Berling H, Tegel H, Mulder J, Rockberg J, Nilsson P, Schwenk JM, Hamsten M, von Feilitzen K, Forsberg M, Persson L, Johansson F, Zwahlen M, von Heijne G, Nielsen J, Ponten F (2015) Proteomics. Tissue-based map of the human proteome. *Science* 347(6220):1260419. <https://doi.org/10.1126/science.1260419>
 9. Legrain P, Aebersold R, Archakov A, Bairoch A, Bala K, Beretta L, Bergeron J, Borchers C, Corthals GL, Costello CE, Deutsch EW, Domon B, Hancock W, He F, Hochstrasser D, Marko-Varga G, Salekdeh GH, Sechi S, Snyder M, Srivastava S, Uhlen M, Hu CH, Yamamoto T, Paik YK, Omenn GS (2011) The human proteome project: current state and future direction. *Mol Cell Proteomics*. <https://doi.org/10.1074/mcp.O111.009993>
 10. Hu Z, Scott HS, Qin G, Zheng G, Chu X, Xie L, Adelson DL, Oftedal BE, Venugopal P, Babic M, Hahn CN, Zhang B, Wang X, Li N, Wei C (2015) Revealing missing human protein isoforms based on ab initio prediction, RNA-seq and proteomics. *Sci Rep* 5:10940. <https://doi.org/10.1038/srep10940>
 11. Blakeley P, Siepen JA, Lawless C, Hubbard SJ (2010) Investigating protein isoforms via proteomics: a feasibility study. *Proteomics* 10 (6):1127–1140. <https://doi.org/10.1002/pmhc.200900445>
 12. Regnier FE, Kim J (2018) Proteins and proteoforms: new separation challenges. *Anal Chem* 90(1):361–373. <https://doi.org/10.1021/acs.analchem.7b05007>
 13. Sutandy FX, Qian J, Chen CS, Zhu H (2013) Overview of protein microarrays. *Curr Protoc Protein Sci Chapter 27:Unit 27 21*. <https://doi.org/10.1002/0471140864.ps2701s72>
 14. Babel I, Barderas R, Diaz-Uriarte R, Martinez-Torrecuadrada JL, Sanchez-Carbayo M, Casal JI (2009) Identification of tumor-associated autoantigens for the diagnosis of colorectal cancer in serum using high density protein microarrays. *Mol Cell Proteomics* 8 (10):2382–2395. <https://doi.org/10.1074/mcp.M800596-MCP200>
 15. Babel I, Barderas R, Diaz-Uriarte R, Moreno V, Suarez A, Fernandez-Acenero MJ, Salazar R, Capella G, Casal JI (2011) Identification of MST1/STK4 and SULF1 proteins as autoantibody targets for the diagnosis of colorectal cancer by using phage microarrays. *Mol Cell Proteomics* 10(3):M110 001784. <https://doi.org/10.1074/mcp.M110.001784>
 16. Uhlen M, Oksvold P, Fagerberg L, Lundberg E, Jonasson K, Forsberg M, Zwahlen M, Kampf C, Wester K, Hober S, Wernerus H, Bjorling L, Ponten F (2010) Towards a knowledge-based human protein atlas. *Nat Biotechnol* 28(12):1248–1250. <https://doi.org/10.1038/nbt1210-1248>
 17. Manzano-Roman R, Fuentes M (2019) A decade of nucleic acid programmable protein arrays (NAPPA) availability: news, actors, progress, prospects and access. *J Proteome* 198:27–35. <https://doi.org/10.1016/j.jprot.2018.12.007>
 18. Yu X, Song L, Petritis B, Bian X, Wang H, Vilorio J, Park J, Bui H, Li H, Wang J, Liu L, Yang L, Duan H, McMurray DN, Achkar JM,

- Magee M, Qiu J, LaBaer J (2017) Multiplexed nucleic acid programmable protein arrays. *Theranostics* 7(16):4057–4070. <https://doi.org/10.7150/thno.20151>
19. Ramachandran N, Hainsworth E, Bhullar B, Eisenstein S, Rosen B, Lau AY, Walter JC, LaBaer J (2004) Self-assembling protein microarrays. *Science* 305(5680):86–90. <https://doi.org/10.1126/science.1097639>
20. Ramachandran N, Raphael JV, Hainsworth E, Demirkan G, Fuentes MG, Rolf A, Hu Y, LaBaer J (2008) Next-generation high-density self-assembling functional protein arrays. *Nat Methods* 5(6):535–538. <https://doi.org/10.1038/nmeth.1210>
21. Garranzo-Asensio M, San Segundo-Acosta P, Martinez-Useros J, Montero-Calle A, Fernandez-Acenero MJ, Haggmark-Manberg A, Pelaez-Garcia A, Villalba M, Rabano A, Nilsson P, Barderas R (2018) Identification of prefrontal cortex protein alterations in Alzheimer's disease. *Oncotarget* 9 (13):10847–10867. <https://doi.org/10.18632/oncotarget.24303>
22. Hospach I, Joseph Y, Mai MK, Krasteva N, Nelles G (2014) Fabrication of homogeneous high-density antibody microarrays for cytokine detection. *Microarrays (Basel)* 3(4):282–301. <https://doi.org/10.3390/microarrays3040282>
23. Barderas R, Bartolome RA, Fernandez-Acenero MJ, Torres S, Casal JI (2012) High expression of IL-13 receptor alpha2 in colorectal cancer is associated with invasion, liver metastasis, and poor prognosis. *Cancer Res* 72(11):2780–2790. <https://doi.org/10.1158/0008-5472.CAN-11-4090>
24. Huang RP (2001) Detection of multiple proteins in an antibody-based protein microarray system. *J Immunol Methods* 255(1–2):1–13. [https://doi.org/10.1016/s0022-1759\(01\)00394-5](https://doi.org/10.1016/s0022-1759(01)00394-5)
25. San Segundo-Acosta P, Montero-Calle A, Fuentes M, Rabano A, Villalba M, Barderas R (2019) Identification of Alzheimer's disease autoantibodies and their target biomarkers by phage display and protein microarrays. *J Proteome Res* 18 (7):2940–2953. <https://doi.org/10.1021/acs.jproteome.9b00258>
26. San Segundo-Acosta P, Garranzo-Asensio M, Oeo-Santos C, Montero-Calle A, Quiralte J, Cuesta-Herranz J, Villalba M, Barderas R (2018) High-throughput screening of T7 phage display and protein microarrays as a methodological approach for the identification of IgE-reactive components. *J Immunol Methods* 456:44–53. <https://doi.org/10.1016/j.jim.2018.02.011>
27. Chatterjee M, Mohapatra S, Ionan A, Bawa G, Ali-Fehmi R, Wang X, Nowak J, Ye B, Nahhas FA, Lu K, Witkin SS, Fishman D, Munkarah A, Morris R, Levin NK, Shirley NN, Tromp G, Abrams J, Draghici S, Tainsky MA (2006) Diagnostic markers of ovarian cancer by high-throughput antigen cloning and detection on arrays. *Cancer Res* 66(2):1181–1190. <https://doi.org/10.1158/0008-5472.CAN-04-2962>
28. Garranzo-Asensio M, San Segundo-Acosta P, Poves C, Fernandez-Acenero MJ, Martinez-Useros J, Montero-Calle A, Solis-Fernandez G, Sanchez-Martinez M, Rodriguez N, Ceron MA, Fernandez-Diez S, Dominguez G, de Los Rios V, Pelaez-Garcia A, Guzman-Aranguez A, Barderas R (2020) Identification of tumor-associated antigens with diagnostic ability of colorectal cancer by in-depth immunomic and seroproteomic analysis. *J Proteomics* 214:103635. <https://doi.org/10.1016/j.jpro.2020.103635>
29. Wisniewski JR, Gaugaz FZ (2015) Fast and sensitive total protein and peptide assays for proteomic analysis. *Anal Chem* 87 (8):4110–4116. <https://doi.org/10.1021/ac504689z>
30. Pablo San Segundo-Acosta MG-A, Montero-Calle A, Oeo-Santos C, Villalba M, Guzmán-Aranguéz A, Barderas R (2017) Chapter 4: Protein microarrays in neurodegenerative diseases. In: Fernández-Irigoyen ESJ (ed) Current proteomic approaches applied to brain function, Springer series in Neuromethods, vol 124, Springer, New York, pp 43–62. https://doi.org/10.1007/978-1-4939-7119-0_4
31. Morrissey ER, Diaz-Uriarte R (2009) Pomelo II: finding differentially expressed genes. *Nucleic Acids Res* 37(Web Server issue):W581–W586. <https://doi.org/10.1093/nar/gkp366>
32. Alonso R, Salavert F, Garcia-Garcia F, Carbonell-Caballero J, Bleda M, Garcia-Alonso L, Sanchis-Juan A, Perez-Gil D, Marin-Garcia P, Sanchez R, Cubuk C, Hidalgo MR, Amadoz A, Hernansaiz-Ballesteros RD, Aleman A, Tarraga J, Montaner D, Medina I, Dopazo J (2015) Babelomics 5.0: functional interpretation for new generations of genomic data. *Nucleic Acids Res* 43(W1):W117–W121. <https://doi.org/10.1093/nar/gkv384>
33. Howe EA, Sinha R, Schlauch D, Quackenbush J (2011) RNA-Seq analysis in MeV. *Bioinformatics* 27(22):3209–3210. <https://doi.org/10.1093/bioinformatics/btr490>
34. Fivash M, Towler EM, Fisher RJ (1998) BIAcore for macromolecular interaction. *Curr Opin Biotechnol* 9(1):97–101. [https://doi.org/10.1016/s0958-1669\(98\)80091-8](https://doi.org/10.1016/s0958-1669(98)80091-8)

35. Roden LD, Myszka DG (1996) Global analysis of a macromolecular interaction measured on BIAcore. *Biochem Biophys Res Commun* 225 (3):1073–1077. <https://doi.org/10.1006/bbrc.1996.1297>
36. Oeo-Santos C, Lopez-Rodriguez JC, Garcia-Mouton C, San Segundo-Acosta P, Jurado A, Moreno-Aguilar C, Garcia-Alvarez B, Perez-Gil J, Villalba M, Barderas R, Cruz A (1862) Biophysical and biological impact on the structure and IgE-binding of the interaction of the olive pollen allergen Ole e 7 with lipids. *Biochim Biophys Acta Biomembr* 2020 (6):183258. <https://doi.org/10.1016/j.bbamem.2020.183258>
37. Barderas R, Shochat S, Timmerman P, Hollestelle MJ, Martinez-Torrecuadrada JL, Hoppener JW, Altschuh D, Meloen R, Casal JI (2008) Designing antibodies for the inhibition of gastrin activity in tumoral cell lines. *Int J Cancer* 122(10):2351–2359. <https://doi.org/10.1002/ijc.23395>
38. Barderas R, Desmet J, Timmerman P, Meloen R, Casal JI (2008) Affinity maturation of antibodies assisted by in silico modeling. *Proc Natl Acad Sci U S A* 105 (26):9029–9034. <https://doi.org/10.1073/pnas.0801221105>



Chapter 7

Detection of Posttranslational Modification Autoantibodies Using Peptide Microarray

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Abstract

Autoantibodies are humoral antibodies against self-proteins and play vital roles in maintaining the homeostasis. Autoantibodies can also target posttranslational modifications (PTMs) of proteins and the identification of new PTM autoantibodies is important to identify biomarkers for the early diagnosis of cancer and autoimmune diseases. In this chapter, we describe a method to detect PTM autoantibodies using citrullinated peptide microarray as an example. This method can be used to screen serum autoantibodies for different human diseases.

Key words Posttranslational modification, Citrullination, Autoantibody, Peptide microarray, Biomarker

1 Introduction

Autoantibodies are antibodies that bind to the body's self-proteins, and their production is affected by factors such as genetic predisposition, environment, and chemical substances [1–3]. The accumulated evidences have indicated the utility of autoantibodies as biomarkers in clinical diagnosis [1, 3, 4]. In addition, autoantibodies can also target posttranslational modification (PTM) processes, including phosphorylation, citrullination, and acetylation [1, 5–7]. The study of PTM autoantibodies is helpful in the understanding of autoimmunity, diagnosis, and treatment of human diseases [5, 8].

Protein microarray technology is a high-throughput technology for proteomics research, which can analyze the interactions of serum antibodies and simultaneously display proteins on

Meng Li, Hongye Wang, Xianjiang Kang and Xiaobo Yu contributed equally with all other contributors.

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microarray [9, 10]. However, the identification of PTM autoantibodies is difficult because of the hard manipulation of PTM number and location on proteins. As an alternative solution, the PTM can be attached to peptide during synthesis. For an example, the anti-citrullinated peptide (anti-CCP) test has been widely used in the clinical diagnosis of rheumatoid arthritis (RA) [11–13]. Compared to proteins, the location and number of PTMs on peptides are more controllable and the synthesis of PTM peptides is much easier, cost effective, and reproducible.

Taking citrullination as an example, here we describe a method to detect PTM autoantibodies, in which the citrullinated peptides and correspondent unmodified peptides are synthesized and printed in duplicate on the slide. Using the citrullinated peptide microarray, we demonstrate the detection of PTM autoantibodies in the serum of patients with different diseases (Figs. 1 and 2).

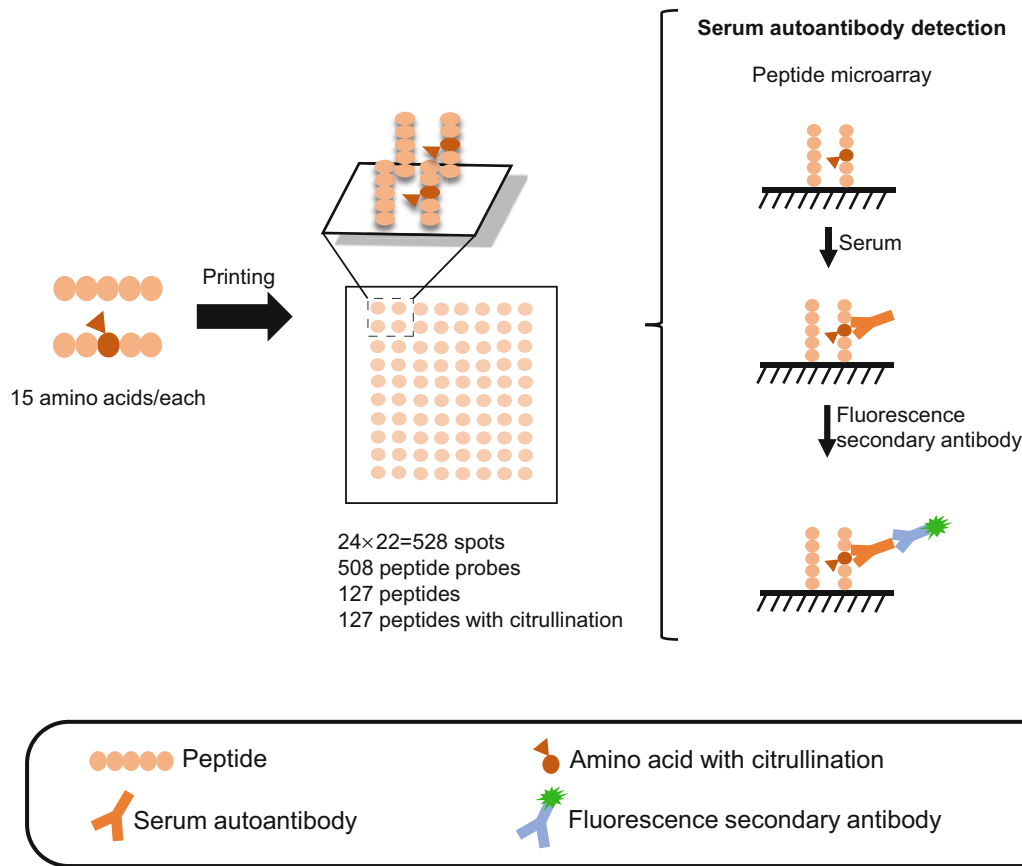


Fig. 1 Schematic illustration of citrullinated peptide microarray fabrication and serum autoantibody detection

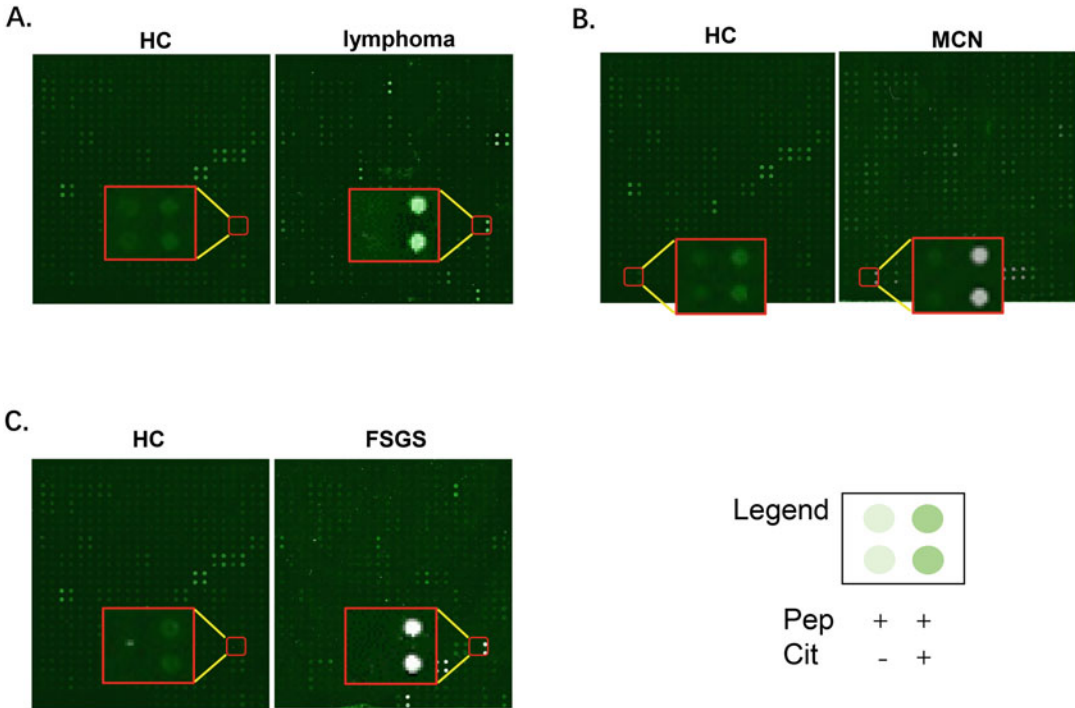


Fig. 2 Representative images of serum autoantibody detection using citrullinated peptide microarray. (a–c) are the serum from the healthy control (HC), minimal change nephropathy (MCN), and focal segmental glomerulosclerosis (FSGS), respectively. *Pep* peptide, *Cit* citrullinated peptide

2 Materials

1. Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.4.
2. Wash buffer (PBST): 0.05% (v/v) Tween 20 in PBS.
3. Blocking buffer: 5% (w/v) skim milk in PBST.
4. Secondary antibody: Cy3-Affinipure Donkey anti-human IgG (H + L) (Jackson ImmunoResearch Inc., West Grove, PA, USA).
5. Acrodisc[®] 32 mm Syringe Filters with 0.45 µm Supor[®] Membrane.
6. MS Rocking Shaker.
7. GenePix[®] 4300A microarray scanner (Molecular Devices, Sunnyvale, CA, USA).

3 Methods

3.1 Detection of Serum Autoantibody Using Peptide Microarray

1. The slide should be equilibrated to room temperature before use (*see Note 1*).
2. Place the slide in a designated chamber (*see Note 2*).
3. Add 400 μL blocking buffer to array chamber at room temperature for 1 h, gently rocking (*see Notes 3–6*).
4. The serum samples are thawed at 4 °C and centrifuged at $14,000 \times g$ for 10 min. Add 4 μL serum to 400 μL blocking buffer at a ratio of 1:100 (*see Notes 7 and 8*).
5. Remove the blocking buffer, add 400 μL diluted serum into the array chamber, and then incubate at room temperature for 2.5 h, gently rocking.
6. Wash the slide three times with 400 μL PBST, 10 min per wash.
7. Dilute secondary antibody (working concentration of 4 $\mu\text{g}/\text{mL}$) in 400 μL blocking buffer. Add 400 μL of diluted secondary antibody into the array chamber, and incubate for 1 h at room temperature, gently rocking (*see Note 9*).
8. Wash the slide three times with 400 μL PBST, 10 min per wash.
9. Wash the slide twice with 400 μL dH₂O, 2 min per wash.
10. Remove the remaining liquid, drain the array chamber with vacuum pump or pipette, and dry the slide.
11. All arrays are scanned using a GenePix 4300A microarray scanner, under the wavelength of 532 nm. The median of fluorescent signal intensity is extracted using GenePix Pro7 software (*see Note 10*).

3.2 Data Analysis

The average of fluorescent signal is calculated from duplicate spots. 10% of microarray signals is served as the background, and all microarray data is normalized using the fluorescence signal intensity divided by the background [14].

3.3 Results

According to our previous research and literature reports, we designed a citrullinated peptide microarray with 254 peptides related to citrullinated autoantibodies, including 127 peptides with citrullination and 127 their correspondent unmodified peptides, in which each peptide has 15 amino acids [15, 16]. All peptides were printed on a chemically modified glass slide in duplicate (Fig. 1).

Using citrullinated peptide microarray, we detected the autoantibodies in serum from the patients with lymphoma, minimal change nephropathy (MCN), and focal segmental glomerulosclerosis (FSGS) and healthy control (HC). The representative images are shown in Fig. 2. No significant difference was observed

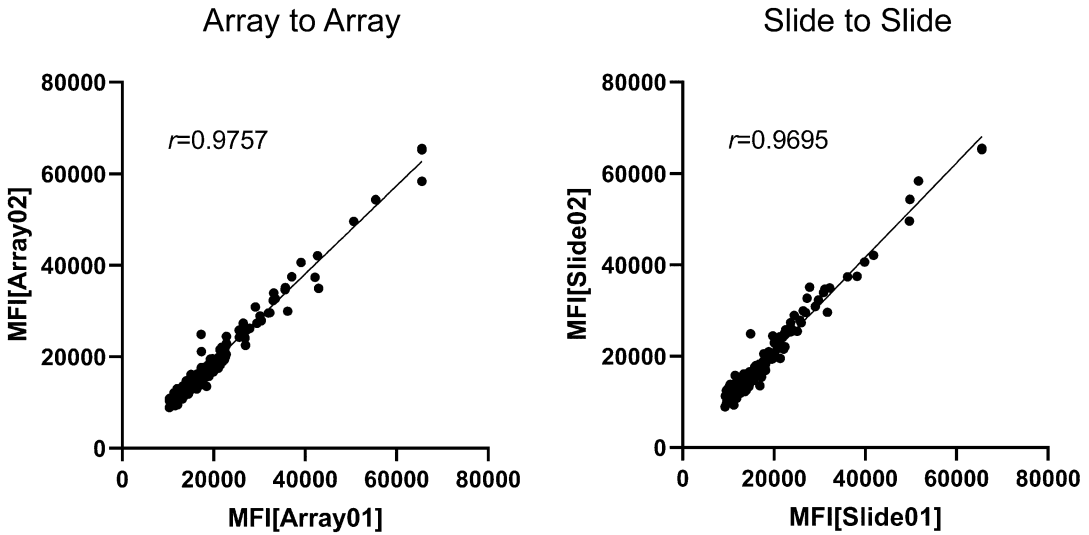


Fig. 3 Reproducibility of serum autoantibody detection using citrullinated peptide microarray. *MFI* median of fluorescence intensity

on the peptides with and without citrullination for healthy control. However, the fluorescence signals of citrullinated peptides were significantly higher than the peptides without citrullination. The r correlations of array to array and slide to slide were 0.9757 and 0.9695, respectively, demonstrating the high reproducibility of the citrullinated peptide microarray in the detection of serum autoantibodies (Fig. 3).

Taking lymphoma as an example, the profile of citrullinated autoantibodies is shown in Fig. 4. It can be observed that the autoantibodies were produced to the three unmodified peptides (sequence: P9: ELELRPTGEIEQYSV, P41: GSGGSYGRGSRGG SG, P101: IPEIPKRGELKTEL) using the criteria in which the normalized signal was higher than 3 (Fig. 4a). Furthermore, the autoantibodies of four different citrullinated peptides were produced (sequence: P27: ASTSTTI[Cit]SHSSRR, P35: LVLKLKE [Cit]PSPGPAA, P82: GSGRSSS[Cit]GPYESGS, P99: LLLSIKM [Cit]LEKEIET) (Fig. 4b). In order to know the specificity of autoantibodies to the citrullination, we further calculated the ratio of citrullinated peptides to the correspondent unmodified peptides. Using the ratio of 2 as the cutoff, four peptides were selected (Fig. 4c). However, the translational utility of these citrullinated autoantibodies needs to be validated in different clinical cohorts in the future.

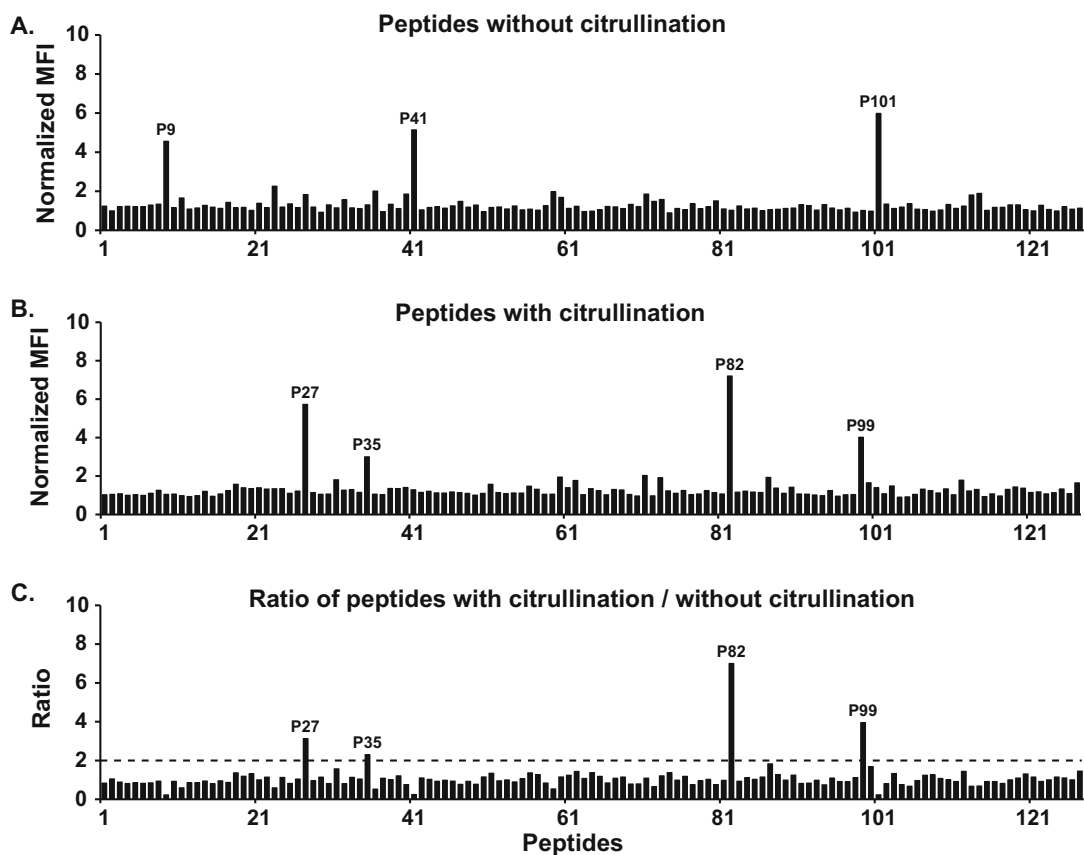


Fig. 4 The profile of citrullinated autoantibodies in a lymphoma patient. (a–c) are the normalized signals from the peptides without and with citrullination and the ratio of peptides with citrullination/without citrullination, respectively

4 Notes

1. The citrullinated peptide microarrays are usually stored at $-20\text{ }^{\circ}\text{C}$; if the experiments are completed within 1 week, it can be stored at $-4\text{ }^{\circ}\text{C}$.
2. Please be careful to avoid scraping spots on the array.
3. Before use, the PBST buffer should be filtered with $0.45\text{ }\mu\text{m}$ filter.
4. The blocking buffer should be centrifuged at $9800 \times g$ for 5 min, and then collect the supernatant to new tubes.
5. Each time the liquid is removed or added, it should be operated slowly in a fixed position.
6. The incubation on a shaker is helpful for the interactions of autoantibodies to their correspondent peptides on microarray.

7. Serum samples stored at -20°C should be thawed slowly at -4°C followed by centrifugation for 10 min at $14,000 \times g$ to remove precipitation.
8. The optimal serum dilution should be determined prior to the screening.
9. The secondary antibody is labeled with the fluorescein and the reaction should be performed in the dark.
10. The scanning wavelength should be determined by the fluorescein on secondary antibody.

Acknowledgments

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Contributions: J.L. and X.Q. provided the clinical samples. M.L., H.W., J.D., and J.R. prepared the microarrays. M.L., H.W., executed microarray experiments. M.L., H.W. and X.Y. executed the statistical and structural analysis. M.L., H.W., X.K. and X.Y. conceived the idea, designed experiments, analyzed the data and wrote the manuscript.

References

1. Zaenker P, Ziman MR (2013) Serologic autoantibodies as diagnostic cancer biomarkers—a review. *Cancer Epidemiol Biomark Prev* 22 (12):2161–2181
2. Elkon K, Casali P (2008) Nature and functions of autoantibodies. *Nat Clin Pract Rheumatol* 4 (9):491–498
3. Tan Q et al (2020) Autoantibody profiling identifies predictive biomarkers of response to anti-PD1 therapy in cancer patients. *Theranostics* 10(14):6399–6410
4. Tan EM, Zhang J (2008) Autoantibodies to tumor-associated antigens: reporters from the immune system. *Immunol Rev* 222:328–340
5. Zavala-Cerna MG et al (2014) The clinical significance of posttranslational modification of autoantigens. *Clin Rev Allergy Immunol* 47(1):73–90
6. Karthikeyan K et al (2016) A contra capture protein array platform for studying post-translationally modified (PTM) autoantigenomes. *Mol Cell Proteomics* 15 (7):2324–2337
7. Valesini G et al (2015) Citrullination and autoimmunity. *Autoimmun Rev* 14(6):490–497
8. Carubbi F et al (2019) Post-translational modifications of proteins: novel insights in the autoimmune response in rheumatoid arthritis. *Cells* 8(7):657
9. Yu X, Petritis B, LaBaer J (2016) Advancing translational research with next-generation protein microarrays. *Proteomics* 16 (8):1238–1250
10. Atak A et al (2016) Protein microarray applications: autoantibody detection and posttranslational modification. *Proteomics* 16 (19):2557–2569
11. Schellekens GA et al (2015) Citrulline is an essential constituent of antigenic determinants

- recognized by rheumatoid arthritis-specific autoantibodies. *J Immunol* 195(1):8–16
12. Schellekens GA et al (2000) The diagnostic properties of rheumatoid arthritis antibodies recognizing a cyclic citrullinated peptide. *Arthritis Rheum* 43(1):155–163
13. van Gaalen FA et al (2004) Autoantibodies to cyclic citrullinated peptides predict progression to rheumatoid arthritis in patients with undifferentiated arthritis: a prospective cohort study. *Arthritis Rheum* 50(3):709–715
14. Yu X et al (2013) Quantifying antibody binding on protein microarrays using microarray nonlinear calibration. *Biotechniques* 54(5):257–264
15. Wang D et al (2017) AAgAtlas 1.0: a human autoantigen database. *Nucleic Acids Res* 45(D1):D769–D776
16. Yu X et al (2017) Multiplexed nucleic acid programmable protein arrays. *Theranostics* 7(16):4057–4070



Chapter 8

Epitope Mapping of Allergenic Lipid Transfer Proteins

Clara San Bartolomé, Carmen Oeo-Santos, Pablo San Segundo-Acosta, Rosa Muñoz-Cano, Javier Martínez-Botas, Joan Bartra, and Mariona Pascal

Abstract

Food allergy is becoming a great problem in industrialized countries. Thus, there is the need for a robust understanding of all aspects characterizing IgE response to allergens. The epitope mapping of B-cell epitopes has the potential to become a fundamental tool for food allergy diagnosis and prognosis and to lead to a better understanding of the pathogenesis. Using this approach, we have worked on epitope mapping of the most important plant food allergens identified in the Mediterranean area. The final aim of this study is to define the immune response regarding B epitopes and its clinical relevance in LTP allergy. This chapter describes the protocol to produce microarrays using a library of overlapping peptides corresponding to the primary sequences of allergenic lipid transfer proteins.

Key words IgE-epitope mapping, Plant food allergy, Lipid transfer protein, Peptide microarray

1 Introduction

Food allergy has been increasing in prevalence in recent decades documenting up to 10% affected in Westernized regions. Thus, it has become an important health problem especially in industrialized countries [1]. Plant food allergy is the main cause of food allergy in adult population and the fourth cause in pediatric population at this moment [2]. In particular, in the Mediterranean countries and less frequently in the northern regions, lipid transfer proteins (LTPs) have emerged as a relevant family of plant panallergens and its sensitization is principally responsible for plant food allergy and food-induced anaphylaxis in adults [3]. The LTP family is widely distributed throughout the plant kingdom; therefore, LTP syndrome is defined by a complex pattern of multiple sensitizations to plant foods (i.e., fruits: specially *Rosaceae* and *Prunoideae*

family, vegetables, nuts, and cereals), and pollens containing LTPs [3–7] and even latex and *Cannabis sativa* have also been involved [8, 9].

The current diagnosis of food allergies is based on a detailed clinical history and both in vivo (skin prick test) and in vitro measurements of allergen extract- or component-specific serum IgE (sIgE) levels are performed looking for sensitization. In this sense, molecular or component resolved diagnostics has largely improved the accuracy of allergy diagnosis as well as the optimization of the treatment strategy in patients. Nevertheless, oral food challenge is still the gold standard for culprit identification and clinical relevance identification [10]. Otherwise, some studies involving milk [11, 12], peanut [13, 14], egg [15], and fish [16] allergens have suggested that clinical sensitivity may correlate better with epitope-specific recognition. In this regard, epitope mapping of B-cell epitopes may become a fundamental tool to understand the pathogenesis and tolerance induction of food allergy, and contribute to the development of diagnostic tools and design of therapeutic approaches [17]. However, the characterization of specific epitopes of panallergens, such as plant food allergens belonging to the LTP family, remains inconclusive.

In the last years, new strategies have been tested to characterize regions with IgE-binding capacity. Most of them use mainly peach LTP, Pru p 3, as a suitable model to undertake the identification of IgE epitopes of allergenic LTPs of plant foods given that it has traditionally been considered the primary sensitizer in LTP allergy [18]. The identification of three sequential epitopes of Pru p 3 is highlighted analyzing synthetic decapeptides deduced from its amino acid sequence [19]. Likewise, two relevant conformational epitopes of Pru p 3 have been identified using a random peptide phage display library [20]. These mentioned epitopes have also been found preserved in other *Rosaceae* fruit and the identified N-terminal epitope seems to be less conserved and likely represents a *Rosaceae*-specific epitope, with lower homology to LTP from other families [21]. Later, a study about Api g 2 (celery LTP), an important plant food allergen source [22] associated with sensitization to *Artemisia vulgaris* and *Betula verrucosa* pollen in Central Europe [23], showed that the highest sequence identities of Pru p 3 to Api g 2 and Art v 3 are found in the C-terminal region which could constitute a general LTP cross-reactive epitope [24]. Additionally, several bioinformatic tools have been released for prediction allergenicity of new molecules based on their homology with known allergen, and guidelines to assess potential allergenicity of proteins. Regarding this, a study about in silico prediction of the allergenicity of a few LTP concludes the high IgE recognition in relation with the sequence homology to Pru p 3 [25]. Remarkably, another study highlights the inhomogeneity of LTP IgE

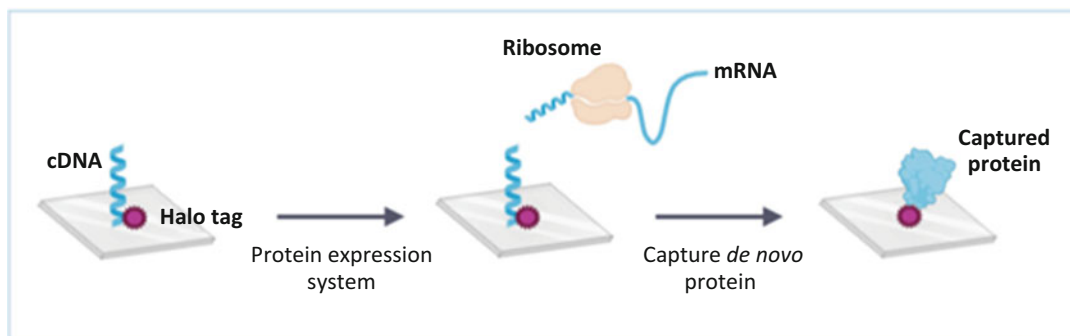


Fig. 1 Scheme of the approach to display the peptides fused to HaloTag in the surface of the microarrays using HeLa cell lysate protein expression system

recognition due to the observed sequence micro-heterogeneity and points out that a complete or partial epitope sharing can be found only when we compare closely related LTPs [26].

Besides these few studies about B-cell epitope mapping of LTP mentioned, epitope mapping of the most important plant food allergens identified in the Mediterranean basin from taxonomically unrelated allergenic sources, and its clinical relevance, in patients with LTP syndrome is still not available. For this reason, we propose and optimize a methodology for the performance of peptide microarray technology.

Peptide microarrays allow large-scale, simultaneous, and parallel analysis of hundreds to thousands of proteins, using small volumes of serum from the patients and allowing their analysis in a reasonable time. We used a nucleic acid programmable protein array (NAPPA) constructed by deposition of plasmid cDNA encoding the proteins of interest (fused to an affinity tag) onto a solid support. Proteins were expressed using the HeLa cell lysate system for in vitro transcription and translation reactions [27, 28] (Fig. 1).

In this chapter we describe the major steps for the epitope mapping of LTP using the amino acid sequence of the allergenic protein that has been divided into peptides with a length of 24 amino acids and an overlap of 12 amino acids. The subsequent steps consist of (a) cDNA sample preparation with buffer handling; (b) spotting process, which includes the transfer of peptides onto the solid slide; (c) incubation of the microarrays with human serum; and (d) data acquisition.

2 Materials

2.1 Microarray Printing

1. [sciFLEXARRAYER S3](#) with a PDC 70 Type 2 (Sciencion AG).
2. HaloLink™ Slides, coated with chloroalkanes (Promega) (*see Note 1*).

3. A library of overlapping peptides (24 amino acids overlapping 12 amino acids) corresponding to the primary sequences of the food allergen of interest was designed using SnapGene software (GSL Biotech). The subsequent obtaining of cDNA from the peptides was performed by polymerase chain reaction (PCR), the gateway cloning system (Thermo Fischer Scientific), and its purification. Each peptide includes the sequence of Halo protein in the carboxy-terminus to anchor the allergen to the surface of the array by a covalent bond.

The custom-made peptides are resuspended in Milli-Q water; the concentration is determined by absorbance using a spectrophotometer and stored at -20°C until use.

4. RNase-free water.
5. Ethanol 100%.
6. Ethanol 70%: For 1 L: 700 mL ethanol 100% containing 300 mL RNase-free water.
7. Glycogen (Thermo Fisher Scientific, Mol. Biology Grade).
8. Sodium acetate 3 M, pH 5.2.
9. 0.3125 mg/mL Suberic acid bis(3-sulfo-*N*-hydroxysuccinimide ester) sodium salt (BS^3) (Sigma).
10. 0.9 mg/mL Bovine serum albumin (BSA) containing 0.4% Tween-20 (Thermo Fischer Scientific).
11. Stop solution: Tris-HCl 1 M, pH 8.8 (Thermo Fischer Scientific).
12. V-bottom 384-well plates (Greiner Bio-One).
13. sciCLEAN8 wash solution (Scienion AG).

2.2 Microarray Hybridization

1. Multiwell cassette.
2. SuperBlock™ Blocking Buffer in PBS (Thermo Fischer Scientific).
3. Rocking shaker.
4. LifterSlip Microarray Coverslips (Erie Scientific Company).
5. Phosphate-buffered solution (PBS) pH 7.4: 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2PO_4 , and 1.5 mM KH_2PO_4 .
6. Milli-Q water.
7. Centrifuge.
8. 1-Step™ Human Coupled IVT Kit-DNA (Thermo Fischer Scientific).
9. Incubator.
10. Washing solution (0.1% PBST): For 1 L: 999 mL PBS containing 1 mL Tween-20.

11. Washing solution II (0.2% PBST): For 1 L: 998 mL PBS containing 2 mL Tween-20.
12. Blocking solution (5% skimmed milk): For 1 L: 50 g skimmed milk powder and 1 L 0.2% PBST.
13. BSA 3%: 3 g BSA and 100 mL 0.2% PBST.
14. Mouse anti-human IgE Fc-BIOT antibody (SouthernBiotech).
15. Alexa Fluor 555 monoclonal goat anti-mouse IgG (H + L) (Invitrogen).
16. Cy3-conjugated streptavidin (RayBiotech).
17. PicoGreen (Promega).
18. Anti-HaloTag Monoclonal Antibody (Promega).

2.3 Data Acquisition and Analysis

1. GenePix[®] 4100A Microarray Scanner (Axon Laboratories) with two lasers (532 and 635 nm).
2. GenePix Pro 7.1 Microarray Acquisition and Analysis Software.
3. Microsoft Excel (Microsoft Corporation) and TIGR MultiExperiment Viewer (MeV v4.8) software.

3 Methods

3.1 Ethanol Precipitation of DNA

1. Bring the sample to precipitate up to 100 μ L of RNase-free water.
2. Add 1/10 volumes of 3 M sodium acetate pH 5.2.
3. Add glycogen up to a final concentration of 1 μ g/ μ L.
4. Add 2.5 volumes of ethanol 100%.
5. Shake and incubate at -80°C at least for 30 min.
6. Centrifuge at 13,200 rpm ($16,000 \times g$) for 15 min.
7. Remove the ethanol 100% carefully and wash with 500 μ L of ethanol 70%.
8. Incubate at -80°C at least for 30 min.
9. Centrifuge at 13,200 rpm for 15 min (repeat **steps 7–9** twice).
10. Remove the ethanol 70%.
11. Air-dry the pellet in the laminar flow cabinet for 5–10 min until no alcohol is seen or detected.
12. Resuspend in 45 μ L of RNase-free water.
13. Quantification of DNA and agarose gel 0.7% expression.
14. A final concentration of 1 μ g/ μ L is required for printing (*see Note 2*); therefore we have to dilute or concentrate the DNA.
15. Prepare aliquots of 20 μ L of DNA.

3.2 Peptide Microarray Printing

1. To prepare the printing plate, dilute each aliquot of 20 μ L of DNA with 10 μ L of BSA containing 0.4% Tween-20, then add 10 μ L of BS³, and incubate for 4 min. Stop the reaction with 3 μ L of Tris-HCl 1 M pH 8.8 (*see Note 3*). Transfer 43 μ L/well to V-bottomed 384-well plates. pANT7-cHalo empty vector and printing buffer are also printed as negative controls. The final concentration of each cDNA is 0.5 μ g/ μ L.
2. For printing, a sci FLEXARRAYER S3 is used with PDC 70 Type 2. Each feature (cDNA and negative control) was printed in duplicate. The humidity for printing is adjusted to 60%. To minimize the cost of the slides and to increase the number of samples that can be processed simultaneously, 14 microarrays are printed on each slide (*see Note 4*). After the printing procedure is complete, the microarrays are left overnight in the deck of the printer, and the printer is kept closed, to allow the slow equilibration of the humidity with the ambient conditions and the spots to dry slowly. The slides produced are labeled with barcode tags, stored in a box, and sealed in a plastic bag with a desiccant bag (silica gel packet).

3.3 Microarray Hybridization

As a quality control approach, DNA deposited at each printed spot is quantified by staining with PicoGreen.

3.3.1 Detection of DNA Printed on NAPPA Slides

1. Obtain two slides from the printing run, and place in the incubation dish with 4 mL per slide of SuperBlock Blocking Buffer on a rocking shaker for 30–60 min.
2. Prepare PicoGreen stock solution according to the manufacturer's procedure. Then dilute this stock 1:100 in SuperBlock Blocking Buffer.
3. Remove slides from the incubation dish.
4. For a single slide apply 300 μ L of PicoGreen solution and gently cover with a lifter slip. Let it sit for 5 min protected from light at room temperature.
5. Remove the coverslip.
6. Wash with PBS (1 $\times$) 3 times, 5 min each in agitation. Rinse quickly with Milli-Q water.
7. To dry, place the slides into Falcon tube without lids and centrifuge at 1000 rpm for 10 min.
8. Scan the slide (*see Note 5*).

3.3.2 Express Protein on DNA-Printed NAPPA Slides

1. Obtain slides from the printing run, and place in the incubation dish with 4 mL per slide of SuperBlock Blocking Buffer on a rocking shaker for 30–60 min.
2. Gently rinse the slides with Milli-Q water 2 min and dry putting the slides into Falcon tube without lids and centrifuge at 1000 rpm for 10 min.

3. Apply a HybriWell gasket to the printed surface, carefully aligned. Ensure that the gasket is well adhered.
4. Preheat the incubator to be used for IVT at 30 °C.
5. Prepare IVT mixture as follows (each slide will require 150µL):
75µL of HeLa lysate.
15µL of Accessory proteins.
30µL of Reaction mix.
30µL of Nuclease-free water.
6. Add carefully 175µL of IVT mix to the inlet of one end of the gasket, ensuring uniform coverage and avoiding generation of bubbles. When done, apply inlet seals to both openings of the gasket.
7. Place the slides inside the incubator. Incubate for 1.5 h at 30 °C for protein expression, followed by 20 min at room temperature for the query protein to bind to the immobilized protein.
8. Remove the slides from the incubator, peel away the HybriWell, and rinse once with PBS (1×).
9. Immerse each slide in PBS containing 5% skim milk and 0.2% Tween-20 and wash two times, 5 min each, in an incubation dish. Use about 4 mL per slide/wash.
10. Block using 5% skim milk on a rocking shaker at room temperature for an additional 30–45 min.

*3.3.3 Serum, PicoGreen,
and Anti-HaloTag Antibody
Incubation*

1. Dilute thawed serum (1:2 in PBS containing 5% skim milk and 0.2% Tween-20; thus 60µL of serum and 60µL of skim milk are needed).
2. Quality control: Prepare the PicoGreen doing a 1:1000 dilution in BSA 3% and anti-HaloTag antibody doing a 1:1000 dilution in BSA 3% (*see Note 6*).
3. Gently rinse the slides with PBS (1×).
4. Insert the multiwell cassette on the slide.
5. Apply 120µL of serum, PicoGreen, and anti-HaloTag antibody in each well according to the designed template. Incubate overnight at 4 °C on a rocking shaker.

*3.3.4 IgE and IgG
Detection*

1. Pour and remove the multiwell cassette.
2. Wash the slides once with PBS (1×), 5–10 min in agitation.
3. Wash with PBS containing 0.1% Tween-20 three times, 5 min each in agitation.
4. Prepare the anti-human IgE antibody, and do a 1:100 dilution in 5% skim milk (prepare 600µL for each slide or 120µL per well).

5. Apply the anti-human IgE antibody on serums, and incubate for 1 h at room temperature.
6. Wash with PBS containing 0.1% Tween-20 three times, 5 min in agitation.
7. Apply according to template in the well with:
PicoGreen: 120 μ L of BSA 3%.
Anti-HaloTag antibody: 120 μ L of secondary antibody IgG, do a 1:100 dilution in BSA 3%.
Serum: 120 μ L of Cy3-conjugated streptavidin, do a 1:500 dilution in BSA 3%.
8. Incubate for 30–45 min at room temperature (in agitation if the multiwell cassette is on).
9. Wash with PBS containing 0.1% Tween-20 three times, 5 min in agitation.
10. Wash once with PBS (1 $\times$) and once with Milli-Q water, 5 min in agitation.
11. Place the slides into Falcon tube without lids and centrifuge at 1000 rpm for 10 min.

3.4 Scanning and Data Analysis

1. Before scanning, make sure that the slides are completely dry.
2. The slides are scanned with GenePix[®] 4100A Microarray with 532 and 635 nm solid-state lasers and compatible with all used surfaces. The 635 nm laser is used for the detection of Cy5, Alexa Fluor 647, or spectrally similar dye signal (red channel), while the 532 nm laser should be used for the Cy3, Alexa Fluor 555, or spectrally similar dye signal (green channel). The scanner produces 16-bit TIFF image files.
3. These images are quantified using GenePix Pro 7.1 microarray acquisition software that permits the intra-array normalization using the background signal around each spot. The data obtained by the software for each spot in the microarray is, then, inter-array normalized and processed using the desired statistical tests using specific bioinformatics tools, such as MultiExperiment Viewer, which permit performing normalization and processing of the microarray data.

4 Notes

1. Good performance is also achieved using HaloBind-Slides (7BioScience GmbH, Neuenburg am Rhein, Germany). The slides coated with chloroalkanes facilitate the binding of cDNA.

2. The preparation of the print mix required a DNA concentration of 1 µg/µL, to ensure deposition of an adequate amount for robust protein expression and display. To achieve this, it is necessary to precipitate at least 45 µg of DNA.
3. The mix of BSA and BS³ generates a structure around the DNA, trapping it, which prevents its diffusion in the array and promotes the printing spots with similar size. This reaction is favored by temperature and it is important to stop it just in time to avoid excessive viscosity that makes the printing step difficult.
4. An important step in the printing procedure is to maintain the sciCLEAN8 wash solution always full.
5. DNA spotted should result in the staining and imaging of uniformly well-rounded features, which exhibit a reasonably narrow range of signal intensities upon imaging and quantitation.
6. Immobilization of cDNA is confirmed by incubation with PicoGreen, while protein expression is detected by incubation with the antibody anti-HaloTag. We performed this step in one slide to confirm the adequate quality of the microarray to carry out the experiments with the sera of interest.

References

1. Sicherer SH, Sampson HA (2018) Food allergy: a review and update on epidemiology, pathogenesis, diagnosis, prevention, and management. *J Allergy Clin Immunol* 141(1):41–58. <https://doi.org/10.1016/j.jaci.2017.11.003>
2. Alergológica 2015 (2017) Sociedad Española de Alergia e Inmunología Clínica. Draft-grupo de comunicación healthcare, Madrid
3. Asero R, Piantanida M, Pinter E, Pravettoni V (2018) The clinical relevance of lipid transfer protein. *Clin Exp Allergy* 48(1):6–12. <https://doi.org/10.1111/cea.13053>
4. Pastorello EA, Robino AM (2004) Clinical role of lipid transfer proteins in food allergy. *Mol Nutr Food Res* 48(5):356–362. <https://doi.org/10.1002/mnfr.200400047>
5. Rial MJ, Sastre J (2018) Food allergies caused by allergenic lipid transfer proteins: what is behind the geographic restriction? *Curr Allergy Asthma Rep* 18(11):56. <https://doi.org/10.1007/s11882-018-0810-x>
6. Pascal M, Muñoz-Cano R, Reina Z, Palacín A, Vilella R, Picado C, Juan M, Sánchez-López J, Rueda M, Salcedo G, Valero A, Yagüe J, Bartra J (2012) Lipid transfer protein syndrome: clinical pattern, cofactor effect and profile of molecular sensitization to plant-foods and pollens. *Clin Exp Allergy* 42(10):1529–1539. <https://doi.org/10.1111/j.1365-2222.2012.04071.x>
7. Egger M, Hauser M, Mari A, Ferreira F, Gadermaier G (2010) The role of lipid transfer proteins in allergic diseases. *Curr Allergy Asthma Rep* 10(5):326–335. <https://doi.org/10.1007/s11882-010-0128-9>
8. Decuyper II, Rihs HP, Van Gasse AL, Elst J, De Puyseleir L, Faber MA, Mertens C, Hagedorens MM, Sabato V, Bridts C, De Clerck L, Ebo DG (2019) Cannabis allergy: what the clinician needs to know in 2019. *Expert Rev Clin Immunol* 15(6):599–606. <https://doi.org/10.1080/1744666X.2019.1600403>
9. Faber MA, Sabato V, Bridts CH, Nayak A, Beezhold DH, Ebo DG (2015) Clinical relevance of the Hevea brasiliensis lipid transfer protein Hev b 12. *J Allergy Clin Immunol* 135(6):1645–1648. <https://doi.org/10.1016/j.jaci.2014.12.1919>
10. Sato S, Yanagida N, Ebisawa M (2018) How to diagnose food allergy. *Curr Opin Allergy Clin Immunol* 18(3):214–221. <https://doi.org/10.1097/ACI.0000000000000441>

11. Järvinen KM, Beyer K, Vila L, Chatchatee P, Busse PJ, Sampson HA (2002) B-cell epitopes as a screening instrument for persistent cow's milk allergy. *J Allergy Clin Immunol* 110 (2):293–297. <https://doi.org/10.1067/mai.2002.126080>
12. Matsumoto N, Okochi M, Matsushima M, Kato R, Takase T, Yoshida Y, Kawase M, Isobe K, Kawabe T, Honda H (2009) Peptide array-based analysis of the specific IgE and IgG4 in cow's milk allergens and its use in allergy evaluation. *Peptides* 30 (10):1840–1847. <https://doi.org/10.1016/j.peptides.2009.07.005>
13. Shreffler WG, Beyer K, Chu TH, Burks AW, Sampson HA (2004) Microarray immunoassay: association of clinical history, in vitro IgE function, and heterogeneity of allergenic peanut epitopes. *J Allergy Clin Immunol* 113 (4):776–782. <https://doi.org/10.1016/j.jaci.2003.12.588>
14. Otsu K, Guo R, Dreskin SC (2015) Epitope analysis of Ara h 2 and Ara h 6: characteristic patterns of IgE-binding fingerprints among individuals with similar clinical histories. *Clin Exp Allergy* 45(2):471–484. <https://doi.org/10.1111/cea.12407>
15. Martínez-Botas J, Cerecedo I, Zamora J, Vlaicu C, Dieguez MC, Gómez-Coronado D, de Dios V, Terrados S, de la Hoz B (2013) Mapping of the IgE and IgG4 sequential epitopes of ovomucoid with a peptide microarray immunoassay. *Int Arch Allergy Immunol* 161 (1):11–20. <https://doi.org/10.1159/000343040>
16. Perez-Gordo M, Lin J, Bardina L, Pastor-Vargas C, Cases B, Vivanco F, Cuesta-Herranz J, Sampson HA (2012) Epitope mapping of Atlantic salmon major allergen by peptide microarray immunoassay. *Int Arch Allergy Immunol* 157(1):31–40. <https://doi.org/10.1159/000324677>
17. Lin J, Sampson HA (2017) IgE epitope mapping using peptide microarray immunoassay. *Methods Mol Biol* 1592:177–187. https://doi.org/10.1007/978-1-4939-6925-8_14
18. Salcedo G, Sánchez-Monge R, Barber D, Díaz-Perales A (2007) Plant non-specific lipid transfer proteins: an interface between plant defence and human allergy. *Biochim Biophys Acta* 1771(6):781–791. <https://doi.org/10.1016/j.bbali.2007.01.001>
19. García-Casado G, Pacios LF, Díaz-Perales A, Sánchez-Monge R, Lombardero M, García-Selles FJ, Polo F, Barber D, Salcedo G (2003) Identification of IgE-binding epitopes of the major peach allergen Pru p 3. *J Allergy Clin Immunol* 112(3):599–605. [https://doi.org/10.1016/s0091-6749\(03\)01605-1](https://doi.org/10.1016/s0091-6749(03)01605-1)
20. Pacios LF, Tordesillas L, Cuesta-Herranz J, Compes E, Sánchez-Monge R, Palacín A, Salcedo G, Díaz-Perales A (2008) Mimotope mapping as a complementary strategy to define allergen IgE-epitopes: peach Pru p 3 allergen as a model. *Mol Immunol* 45(8):2269–2276. <https://doi.org/10.1016/j.molimm.2007.11.022>
21. Borges JP, Barre A, Culerrier R, Granier C, Didier A, Rougé P (2008) Lipid transfer proteins from Rosaceae fruits share consensus epitopes responsible for their IgE-binding cross-reactivity. *Biochem Biophys Res Commun* 365 (4):685–690. <https://doi.org/10.1016/j.bbrc.2007.11.046>
22. Ballmer-Weber BK, Vieths S, Lüttkopf D, Heuschmann P, Wüthrich B (2000) Celery allergy confirmed by double-blind, placebo-controlled food challenge: a clinical study in 32 subjects with a history of adverse reactions to celery root. *J Allergy Clin Immunol* 106 (2):373–378. <https://doi.org/10.1067/mai.2000.107196>
23. Egger M, Mutschlechner S, Wopfner N, Gadermaier G, Briza P, Ferreira F (2006) Pollen-food syndromes associated with weed pollinosis: an update from the molecular point of view. *Allergy* 61(4):461–476. <https://doi.org/10.1111/j.1398-9995.2006.00994.x>
24. Gadermaier G, Hauser M, Egger M, Ferrara R, Briza P, Santos KS, Zennaro D, Girbl T, Zuidmeer-Jongejan L, Mari A, Ferreira F (2011) Sensitization prevalence, antibody cross-reactivity and immunogenic peptide profile of Api g 2, the non-specific lipid transfer protein 1 of celery. *PLoS One* 6(8):e24150. <https://doi.org/10.1371/journal.pone.0024150>
25. Garino C, Coïsson JD, Arlorio M (2016) In silico allergenicity prediction of several lipid transfer proteins. *Comput Biol Chem* 60:32–42. <https://doi.org/10.1016/j.compbiolchem.2015.11.006>
26. Bernardi ML, Giangrieco I, Camardella L, Ferrara R, Palazzo P, Panico MR, Crescenzo R, Carratore V, Zennaro D, Liso M, Santoro M, Zuzzi S, Tamburrini M, Ciardiello MA, Mari A (2011) Allergenic lipid transfer proteins from plant-derived foods do not immunologically and clinically behave homogeneously: the kiwifruit LTP as a model. *PLoS One* 6(11):e27856. <https://doi.org/10.1371/journal.pone.0027856>
27. Miersch S, LaBaer J (2011) Nucleic acid programmable protein arrays: versatile tools for array-based functional protein studies. *Curr*

- Protoc Protein Sci 64(1):27.22.21–27.22.26. <https://doi.org/10.1002/0471140864.ps2702s64>
28. Manzano-Román R, Fuentes M (2019) A decade of nucleic acid programmable protein arrays (NAPPA) availability: news, actors, progress, prospects and access. J Proteomics 198:27–35. <https://doi.org/10.1016/j.jprot.2018.12.007>



Epitope Mapping of Food Allergens Using Noncontact Piezoelectric Microarray Printer

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Abstract

Peptide microarrays have been used to study protein-protein interaction, enzyme-substrate profiling, epitope mapping, vaccine development, and immuno-profiling. Unlike proteins, peptides are cheap to produce, and can be produced in a high-throughput manner, in a reliable and consistent procedure that reduces batch-to-batch variability. All this provides the peptide microarrays a great potential in the development of new diagnostic tools. Noncontact printing, such as piezoelectric systems, results in a considerable advance in protein and peptide microarray production. In particular, they improve drop deposition, sample distribution, quality control, and flexibility in substrate deposition and eliminate cross-contamination and carryover. These features contribute to creating reproducible assays and generating more reliable data. Here we describe the methods and materials for epitope mapping of food allergens using peptide microarrays produced with a noncontact piezoelectric microarray printer.

Key words Epitope mapping, Food allergy, Peptide microarray, Piezoelectric microarray printer

1 Introduction

The diagnosis of allergy has experienced a significant advance with the appearance of purified and recombinant native allergens, as well as with the development of component-resolved diagnostics (CRD) and protein microarrays. The traditional diagnostic tools that used natural allergen extracts containing a mixture of allergenic and nonallergenic molecules have been replaced by new techniques using individual allergen molecules or components using purified native or recombinant allergens. The main advantage of CRD is the capability to identify various allergenic sources from patients who are poly-sensitized. Thus, it allows the generation of each patient's IgE sensitization profile against numerous sensitizing agents [1]. ImmunoCAP ISAC[®] was the first multiplex in vitro diagnostic tool for the allergy based on microarray technology and used

allergen components exclusively. At present, it contains a panel of 112 allergens (ISAC 112[®]). Numerous studies have focused on characterizing allergic patients' IgE profile and its correlation with current diagnostic techniques such as the prick test and ImmunoCAP [2–4]. In particular, the development of this technology has enabled large-scale epidemiological studies of the aeroallergen sensitization profile [5]. It has also been shown to be useful for accurate prescription of allergen-specific immunotherapy [6]. Although its primary application has been in aeroallergens, studies have also been performed to evaluate their diagnostic ability in food allergens such as milk, eggs, celery, peanuts, hazelnuts, or walnuts [7–9]. Therefore, testing for multiple specific IgE reactivities can clarify the nature and cause of allergic reactions and improve allergic patients' treatment, especially of the polysensitized ones. However, although this technique has generally achieved acceptable specificity and reproducibility values, some studies have indicated high variability detecting specific allergens, which has led to recommending their use along with quantitative tests such as specific IgE determination [9]. It has also been advised not to use it in cases where the results may significantly impact the prescribed therapy [10].

The foundations of protein microarray technology began in 1961 when Feinberg [11] published for the first time the proof of concept of the microspot technique, and 2 years later, using this same technique, the detection of autoimmune antibodies [12]. In 1983, Chang carried out the first work containing a microarray of immobilized antibodies on a solid glass surface used to detect human surface leukocyte antigens (HLA) [13]. Later, Ekins introduced in 1989 the concept of “multi-analyte immunoassay systems” and established the conceptual and methodological basis of protein microarrays [14].

In the past, epitope mapping was mainly performed using SPOTS membrane-based immunoassay, which requires the *in situ* synthesis of peptide arrays directly on a membrane support and incubation with the patient's sera [15–19]. But it was a time-consuming, labor-intensive, and expensive method that required a large volume of sera, and could only test a limited number of peptides [20, 21].

As a result of the rapid expansion of microarray technology and improved peptide synthesis processes, immunoassays based on the application of microarray peptide technology for epitope mapping began to be developed [22]. The main areas in which they have been developed are cancer [23], autoimmune diseases [24], bacterial [25, 26] and viral infections [27], and allergy [20].

The key advantage of the microarray-based immunoassay is the possibility to assay thousands of peptides simultaneously with a minimum amount of sample, which reduces the biological cost of the assays, increases the possibility of replicates, and therefore

strengthens the statistical analysis. Also, its capacity to analyze several immunoglobulins at the same time has particular relevance in studying the immune response, and particularly allergy.

In 2004, Shreffler et al. first applied this technology to determine the epitopes of binding to IgE of peanut allergens [28] and confirmed that the identified antigenic regions correlated with the areas obtained by SPOT membrane assays. Since then, several studies have used peptide microarrays for epitope mapping of food allergens such as anisakis, cow's milk, fish, hen's egg, lentil, peanut, shrimp, soybean, and walnut. To date, several studies have established that peptide microarray B-cell epitope mapping could improve food allergy diagnosis and prognosis [29]. In particular, sensitization to specific epitopes of milk, peanut, and shrimp has been proposed as biomarkers to determine clinical reactivity, persistence, severity of allergic reactions, and response to oral immunotherapy (OIT) [29].

We have been working for 15 years with a split-pin contact printer, first dedicated to DNA microarray production and later on to producing protein microarrays. More recently, in the last 2 years, we have been working with a noncontact piezoelectric system. This implies a significant improvement in the design and quality of protein microarrays. Contact printers are historically widely used for DNA microarrays. Although they have also been used for protein microarrays, the inherently complex nature of the proteins makes this deposition technology not the most suitable for these applications [30]. Reliable production of protein microarrays depends on many parameters, many of which cannot be achieved with contact printers: full control of drop deposition and missing spots, minimization of carryover and cross-contamination, and unaffected surface after the printing process. Noncontact printers are usually more flexible in terms of the substrates that can be used, as they can use both standard slides and more complex substrates such as membranes, plates, or electronic devices [31]. On the other hand, the main disadvantage of noncontact printers, such as piezoelectric systems, compared to contact printing is that they are very limited in the range of viscosity they can work with. On the other hand, optimizing the droplet dispensing parameters such as voltage or frequency can be adjusted individually to compensate for differences in fluid properties.

2 Materials

2.1 Microarray Printing

1. sciFLEXARRAYER S3 with PDC 70 Type 2 (Sciencion).
2. Dendron-modified slides, functionalized with *N*-hydroxyl succinimidyl (NSB27 NHS), were obtained from NanoSurface Biosciences POSTECH (*see Note 1*).

3. sciCHIP epoxy slides (Scienion) (*see* **Note 1**).
4. 96-Well microtiter plates for protein microarrays sci PLEX PLATE Type 2 (Scienion).
5. Hybond ECL Nitrocellulose Membrane (GE Healthcare Bio-Sciences Corp.).
6. Substrate plates V-bottomed 384-well plates (781280; Greiner Bio-One).
7. A library of overlapping peptides corresponding to the primary sequences of the food allergens of interest are synthesized commercially, with a purity >70%, and analyzed with high-performance liquid chromatography and mass spectrometry (GenScript) (*see* **Note 2**). The custom-made peptides are usually supplied as lyophilized powder, resuspended in phosphate-buffered saline at a stock concentration of 20 mg/mL, and stored at -80°C until use.
8. Protein printing buffer (PPB; ArrayIt) (*see* **Note 3**).
9. sciSPOT Protein D1 and D11 (Scienion) (*see* **Note 3**).
10. Biotin-SP (long spacer) AffiniPure Goat Anti-Rat IgG (H + L) was spotted as reference control (112-065-062, Jackson ImmunoResearch).
11. Purified Mouse Anti-Human IgE (555894, BD Pharmingen).
12. Purified Mouse Anti-Human IgG4 (555881, BD Pharmingen).
13. Human IgE Myeloma (401152, Calbiochem).
14. Human IgG4 Myeloma (400126, Calbiochem).
15. Human Angiotensin II DRVYIHPF octa peptide (A9525, Sigma-Aldrich).
16. Poly-DL-alanine (P9003, Sigma-Aldrich).
17. Human serum albumin (HAS) solution 200 g/L (703586, CSL Behring).
18. Purified proteins from bovine milk: casein (C4032), α -casein (C6780), β -casein (C6905), κ -casein (C0406), α -lactoalbumin (L6010), β -lactoglobulin (L3908) from Sigma-Aldrich.
19. Aluminum seal tape (232699; Nalge Nunc International).

2.2 Microarray Hybridization

1. microBOX™ incubation chamber for glass slides (Quantifoil Instruments) with a slide holder for four glass slides.
2. MixMate orbital shaker (Eppendorf AG).
3. Multi-Array Slide System (Sigma Aldrich).
4. Protein Microarray Activation Buffer (ArrayIt).
5. SciBlock Protein DIM 2× (Scienion).
6. Protein Microarray Wash Buffer (ArrayIt).

7. Protein Microarray Wash Buffer 5× (ArrayIt).
8. Protein Microarray Rinse Buffer (ArrayIt).
9. ArrayIt Microarray Air Jet (ArrayIt).
10. Protein Microarray Reaction Buffer (ArrayIt).
11. SciBind Protein D1 2× (ArrayIt).
12. Biotin Mouse Anti-Human IgE Clone G7-26 (555858, BD Pharmingen).
13. Mouse Anti-Human IgG4 Fc-Alexa Fluor[®] 647 Clone HP6025 (9200-31, SouthernBiotech).
14. Cy3-Streptavidin 1 mg (PA43001, GE Healthcare Bio-Sciences).
15. HRP-Streptavidin (Item G) (EL-HRP, RayBiotech).
16. Substrate for colorimetric staining of microarrays sciColor T2 (CD-5600-50).

2.3 Data Acquisition and Analysis

1. ScanArray[®] Express microarray scanner (PerkinElmer) with two lasers (543 and 633 nm) and the associated software.
2. Microsoft Excel (Microsoft Corp.) and TIGR MultiExperiment Viewer (MeV v3.1) (<http://www.tm4.org/>) software.

3 Methods

3.1 Peptide Microarray Printing

1. To prepare the printing plate, stock solutions of peptides (20 mg/mL) are diluted 1:10 with PBS and transferred (10μL/well) to V-bottomed 384-well plates, according to the printing protocol. As positive reference control, an anti-rat IgG biotinylated antibody (0.1μg/μL) is printed at the beginning and at the end of the microarray in an asymmetric manner to avoid flipping or rotating the image in the acquisition process. Anti-human IgE (1, 0.5, and 0.25μg/μL), human IgE (0.2, 0.04, and 0.008μg/μL), and human IgG4 (0.2, 0.04, and 0.008μg/μL) are also printed as positive controls. PBS, angiotensin II octa peptide (1μg/μL), poly-DL-alanine (1μg/μL), and HSA (1, 0.2, and 0.04μg/μL) are printed as negative controls. Once the plate is complete, 10μL of Protein Printing Buffer (ArrayIt) (*see Note 3*) is added to each well. The final concentration of each peptide is 1 mg/mL. The printing plates are sealed with aluminum seal tape and stored at −80 °C.
2. For printing, a sciFLEXARRAYER S3 piezoelectric spotter is used with PDC 70 Type 2 (Fig. 1). Before starting to work, it is required to adjust the nozzle position offset and set the parameters of voltage, pulse, and frequency to optimize the drops (shape, speed, distance, volume, volume reproducibility—volume standard deviation—and alignment) (Fig. 2). To avoid

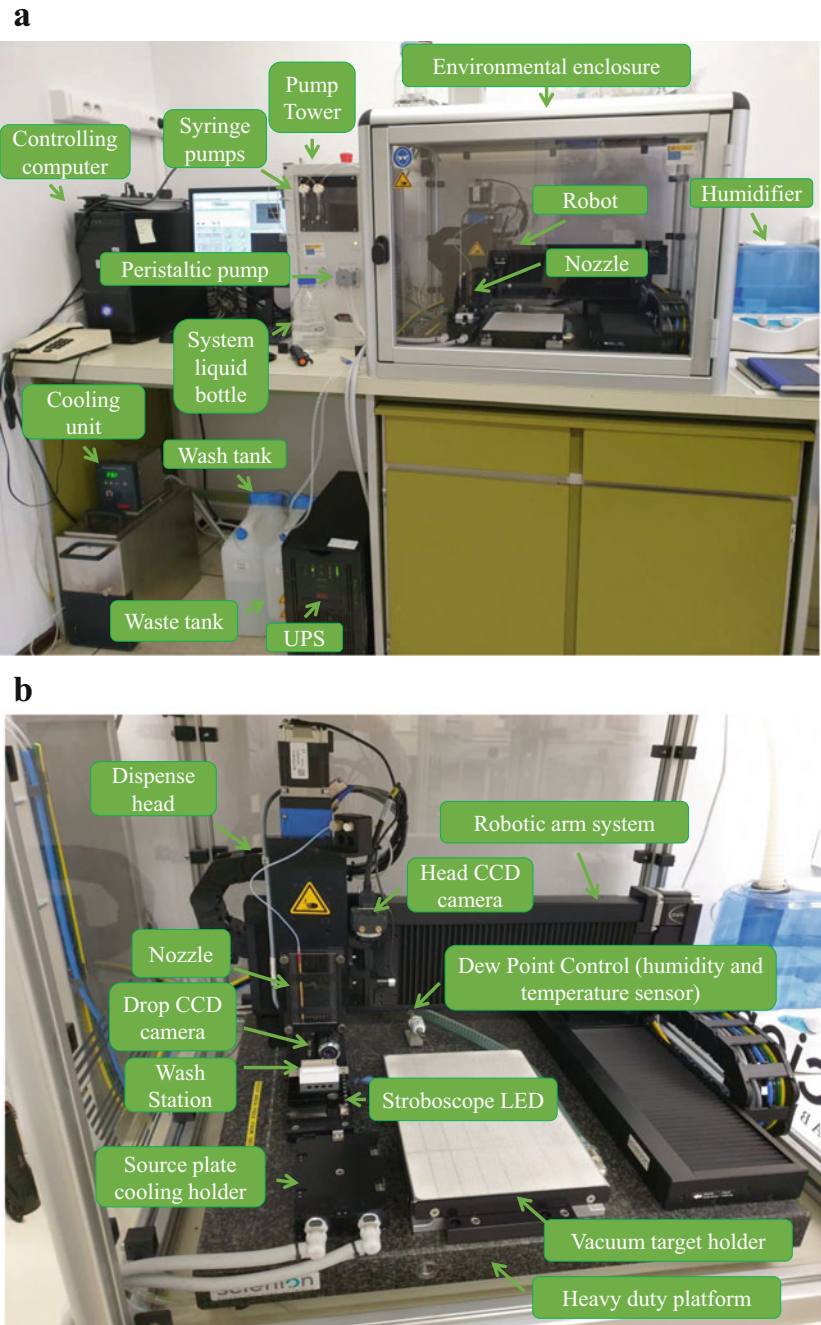
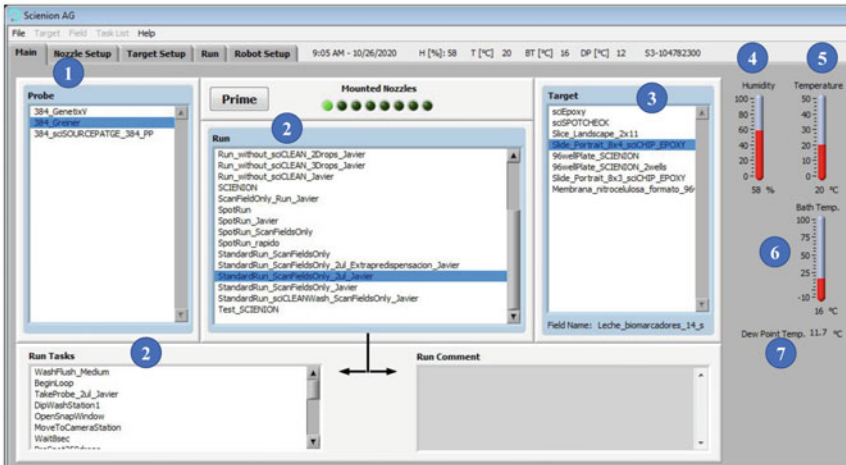


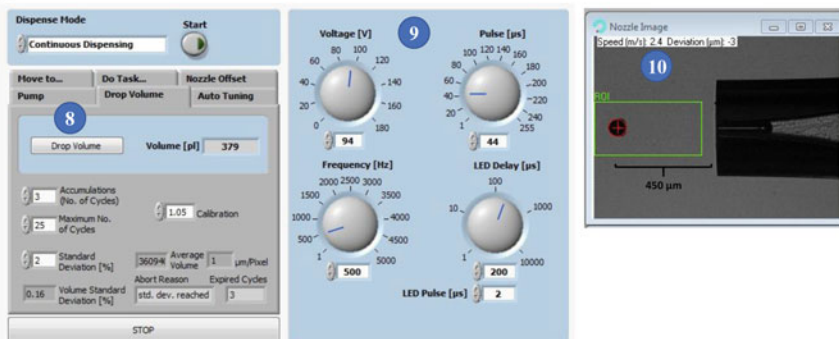
Fig. 1 (a) sciFLEXARRAYER S3 piezoelectric spotter components, (b) printing deck components

evaporation, the humidity for printing is adjusted to 60%, and the source plate's temperature is adjusted to 15 °C. These conditions correspond approximately to the dew point at which evaporation and condensation are minimal. Then, the substrates (slices, membranes, or plates) are placed on the

a



b



c

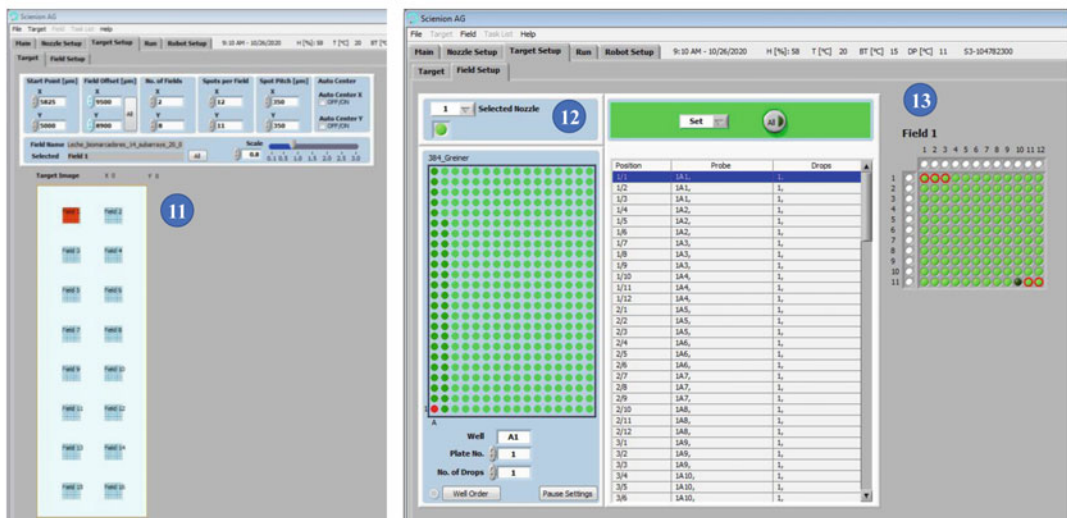


Fig. 2 (a) sciFLEXARRAYER S3 software controls main window, (b) drop optimization window, and (c) microarray design windows. (1) Probe source, (2) printing protocol, (3) target substrates, (4) humidity, (5) temperature, (6) source plate temperature, (7) dew-point temperature, (8) drop volume control window, (9) PDC control window, (10) head camera drop quality image, (11) microarray design window, (12) target setup printing routine, and (13) field layout

target holder and the vacuum pump is activated to secure the substrates to the print tray. The robot is programmed to take 2 μ L of each sample and deposit drops of approximately 370 pL and 200 μ m in diameter. Before and after each impression, the system performs automatic quality control of the drops and PDC washings both inside and outside the capillary, taking advantage of the piezoelectric system's ultrasonic vibration (*see Note 4*). To minimize the slides' cost and increase the number of samples that can be processed simultaneously, we print several microarrays on each slide (for instance, for a microarray of 10 $\times$ 10 spots, we print 16 microarrays per slide). We print also on 96-well microtiter plates and membranes (*see Note 5*). In 96-well microtiter plates an array of 12 $\times$ 12 spots in each well can be printed safely. Each feature (peptide, protein, or control) is usually printed in triplicate. After the printing procedure is complete, the microarrays are left overnight in the deck of the printer, and the printer is kept closed, to allow the slow equilibration of the humidity with the ambient conditions and the spots to dry slowly. The slides produced are labeled with barcode tags, stored in a box, and sealed in a plastic bag with a desiccant bag (silica gel packet).

3.2 Microarray Hybridization

3.2.1 Blocking

1. The slides are inserted into the 2 $\times$ 8 multi-well incubation chamber making sure that the printing surface is facing up, and each microarray is aligned to the silicone gasket.
2. To establish constant humidity in the microBOX™ incubation chamber, gauze moistened with deionized water is placed in a basin within the chamber.
3. Up to three slides are loaded into the slide holder and placed in the incubation chamber microBOX™ (up to six slides can be processed simultaneously in two holders) (Fig. 3).
4. 100 μ L of Protein Microarray Activation Buffer is added onto each microarray to block nonspecific binding. Plates and membranes are blocked with 200 μ L of sciBlock Protein DIM.
5. The microBOX™ is placed on a MixMate orbital shaker (Eppendorf AG) for gentle agitation (300 rpm).
6. The microarrays are incubated for 1 h at room temperature.
7. The blocking solution is removed by aspiration and the samples are washed twice with 100 μ L of Protein Microarray Wash Buffer. For plates or membranes the blocking solution is removed by aspiration and the protocol is continued with **step 1** of serum incubation.
8. The microarrays are rinsed with 100 μ L of Protein Microarray Rinse Buffer. The rinse solution is removed by aspiration and microarrays are dried under a particle-free air stream produced with the ArrayIt Microarray Air Jet.



Fig. 3 Microarray hybridization: (1) multi-well incubation chamber, (2) microBOX™ incubation chamber, (3) orbital shaker, (4) multi-well incubation holder, (5) vacuum system for aspiration

3.2.2 Serum Incubation

1. The patient's serum (50 μ L), diluted 1:1 with Protein Microarray Reaction Buffer, is placed onto each microarray and then into the microBOX™. For plates and membranes, the patient's serum is diluted with sciBind Protein D1.
2. The microarrays are incubated overnight on an orbital shaker at 4 °C with gentle agitation.
3. The microarrays are washed twice (3 min each) with 100 mL of Protein Microarray Wash Buffer at room temperature on an orbital shaker with gentle agitation. For plates and membranes, 200 μ L of Scienion Protein Microarray Wash Buffer is used.

3.2.3 IgE and IgG4 Detection

1. The microarrays are incubated for 2 h at room temperature with a mixture of antibodies diluted in Protein Microarray Reaction Buffer: anti-human IgE antibody covalently tagged with biotin and diluted 1:100 and anti-human IgG4 antibody covalently tagged with Alexa® 647 and diluted 1:1000. For plates and membranes, only anti-human IgE antibody covalently tagged with biotin diluted 1:100 with sciBind Protein D1 is used.
2. The microarrays are washed twice (3 min each) with 100 mL of Protein Microarray Wash Buffer at room temperature on an orbital shaker with gentle agitation. For plates and membranes, 200 μ L of Scienion Protein Microarray Wash Buffer is used.

3. The microarrays are incubated for 30 min at room temperature with 100 mL of Cy3-streptavidin diluted 1:500 with Protein Microarray Reaction Buffer. For plates and membranes incubation is done for 30 min at room temperature with 100 mL of HRP-streptavidin diluted 1:300 with sciBIND Protein D1.
4. The microarrays are washed twice (3 min each) with 100 mL of Protein Microarray Wash Buffer at room temperature on an orbital shaker with gentle agitation. Plates and membranes are washed twice (3 min each) with 200 μ L of Scienion Protein Microarray Wash Buffer. After these washes, 100 μ L of the sciColor T2 (Scienion) substrate is added per well or membrane and left to incubate at room temperature without agitation until the color is visualized. sciColor T2 solution is removed by aspiration and washed twice with PBS and a picture is taken.

3.3 Data Acquisition and Analysis

3.3.1 Microarray Scanning

1. Before scanning, the slides are washed quickly with deionized water and dried under a particle-free airstream produced with the ArrayIt Microarray Air Jet (ArrayIt). The slides are scanned with ScanArray Express (PerkinElmer). The Cy-3 signal is scanned with a green laser (543 nm) and the Alexa[®] 647 signal with a red laser (633 nm). The scanner produces green Cy-3 and red Alexa[®] 647 16-bit TIFF image files.
2. Gridding, spot finding, and quantitation: The Cy-3 and Alexa[®] 647 Tiff images are loaded into the ScanArray Express software. The grid information from the .gal file generated by the sci FLEXARRAYER S3 software is loaded, and the positive reference control spots at the beginning and end of the microarray (anti-rat IgG) are used for grid alignment. The adaptive circle algorithm is used for spot finding and quantitation. Before the quantitative data are saved, visual inspection of the spots is required to confirm that all the spots are correctly detected and have the appropriate shape and size. After visual inspection, any unreliable spots are flagged and removed from further analysis. The quantitation results are saved in the comma-separated value (CSV) file format.

3.3.2 Data Analysis and Visualization

The microarray analysis is performed according to the method of Lin et al. [20, 32]. This method is based on the calculation of a Z -score, which is a measure of the standardized fluorescence intensity for IgE and IgG4 recognition. The Z -score is calculated for each peptide spot (Z_i) according to the formula $Z_i = [S_i - S_{NC}] / \text{MAD}(S_{NC})$, where S for each peptide spot (S_i) or negative control (S_{NC}) is the median fluorescent signal of the spot divided by the local background and log2 transformed. $\text{MAD}(S_{NC})$ is the median absolute deviation of all the readouts for negative control spots (PBS, human angiotensin II, poly-DL-alanine, and HSA). The total Z -

value for each peptide is the median of the Z -scores for the replicate spots within the same microarray. Finally, to accommodate overlapping peptides, the weighted average of Z -values is calculated according to the formula $Z = (0.25 \times Z_{-1}) + (0.5 \times Z_0) + (0.25 \times Z_{+1})$. An individual peptide sample is considered positive if the standardized fluorescence intensity (represented as the weighted average Z -score) exceeds 3. A Microsoft Excel Visual Basic Application script created by our laboratory was used to calculate the Z -score for each dataset and combine the different datasets.

Data visualization and clustering are performed with the TIGR MultiExperiment Viewer (MeV v4.1) software (<http://www.tm4.org/>). MeV is an open-source software tool used to analyze and visualize processed data from microarray experiments. Although MeV has been developed for gene expression microarrays, many of its features are useful in epitope-mapping peptide microarray analyses. To visualize the IgE and IgG4 Z -score, the data are usually log2 transformed. Log2 (ratio) measures allow data representation, stabilize the variance, compress the range of the data, and increase the normal distribution of the data, which allow the data to be analyzed statistically. The MeV software includes different methods of clustering. A hierarchical clustering analysis can be used to identify differential recognition patterns between allergic patients. It can also be used in several statistical analyses, such as the t -test or one-way ANOVA, to identify peptides that are differentially recognized in groups of patients. More complex analyses can be performed using the R programming language, including linear and nonlinear modeling, time-series analysis, classification, clustering, data mining, and machine learning analysis (<http://www.r-project.org>).

4 Notes

1. We have tested a dendron-modified slide functionalized with N -hydroxyl succinimidyl (NSB27 NHS; NanoSurface Biosciences POSTECH) and several slides coated with epoxide groups. In our experience, NSB27 NHS and sciCHIP epoxy slides give the best results (Fig. 4). NSB27 NHS slides are coated uniformly with a cone-shaped organic compound, called NanoCone, functionalized with N -hydroxyl succinimidyl (NHS). NHS ester surface forms avid covalent bonds with primary amine groups. This coating allows controlled regular spacing between the proteins or peptides, minimizes the steric hindrance, and enhances the binding kinetics [33].
2. Several peptide lengths have been reported in the literature and the signal intensity generally increases for both IgE and IgG4 as the peptide length increases [34]. In our experience, the best

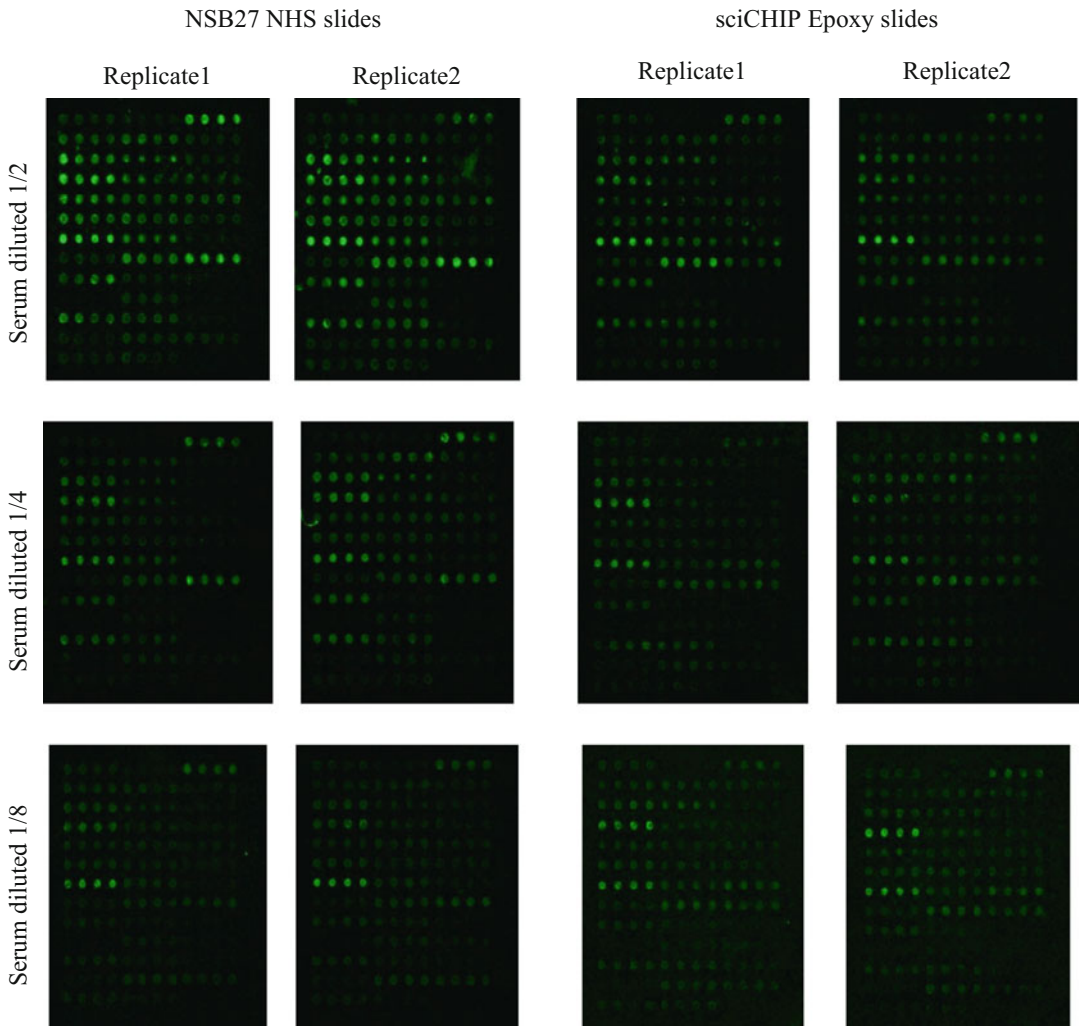


Fig. 4 Performance test for different microarray slides. Different dilutions of serum from a milk-allergic patient were incubated with two different types of slides and labeled with anti-human IgE secondary antibody

performance is achieved with 20-amino-acid peptides [35]. To design a library of overlapping peptides, it is important to exclude the sequences corresponding to the signal peptide because it is not present in the mature protein and is therefore not antigenic.

3. We have tested several printing buffers: sciSPOT Protein D1 and D11 from Scienion and protein printing buffer (PPB) from ArrayIt. As shown in Fig. 5a, D1 and D11 buffers gave very homogeneous spots with small diameter variations between proteins, with a diameter ranging from 167.91 to 182.08μm for D1 buffer and 188.33 to 205.83μm for D11 buffer. In contrast, PPB had more considerable diameter variations,

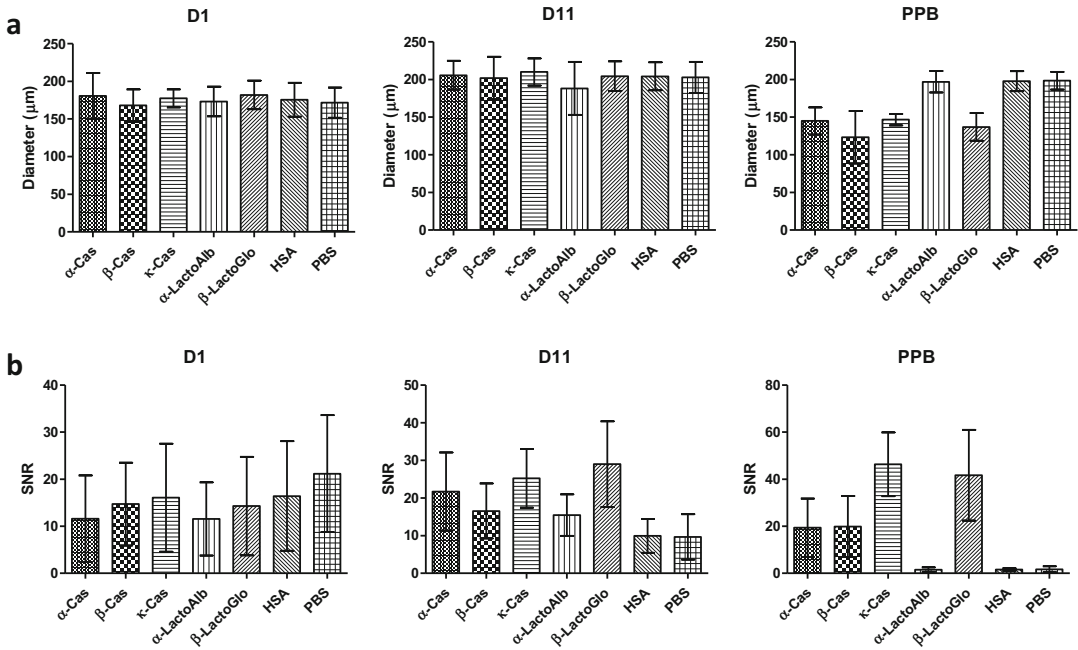


Fig. 5 Optimization of printing buffer (sciSPOT Protein D1 and D11) from Scienion and protein printing buffer (PPB) from Arraylt. **(a)** Average diameter of printing spot. **(b)** IgE fluorescence measures as signal-to-noise ratio (SNR). Six arrays and four spot replicates ($n = 24$) hybridized with the same serum of a milk-allergic patient on NSB27 NHS slides

ranging from 123.33 to 198.61μm. On the other hand, we analyze the fluorescence signal corresponding to the binding of IgE calculated as the signal-to-noise ratio (SNR). As shown in Fig. 5b, buffers D1 and D11 show a high fluorescence signal in the negative controls (HSA and PBS), in some cases even higher than that observed for milk proteins. This suggests that some components of these buffers interfere with the patients' serum giving a nonspecific signal. On the contrary, PPB did not present nonspecific signal in any of the negative controls (Fig. 5b). These results suggested that, although the morphology of the spots obtained with PPB was not the best, this buffer was suitable for printing microarrays to analyze cow's milk-allergic patients.

4. In our experience, the main advantage of piezoelectric noncontact printers, compared to contact printing, is the improvement of the washing system. Piezoelectric noncontact printers use ultrasonic vibration of the piezoelectric capillary, which eliminates carryover and cross-contamination. This point is especially crucial due to the complex nature of proteins, for example, in comparison to nucleic acids. This has a high impact on the design of microarrays; when we used a contact printer,

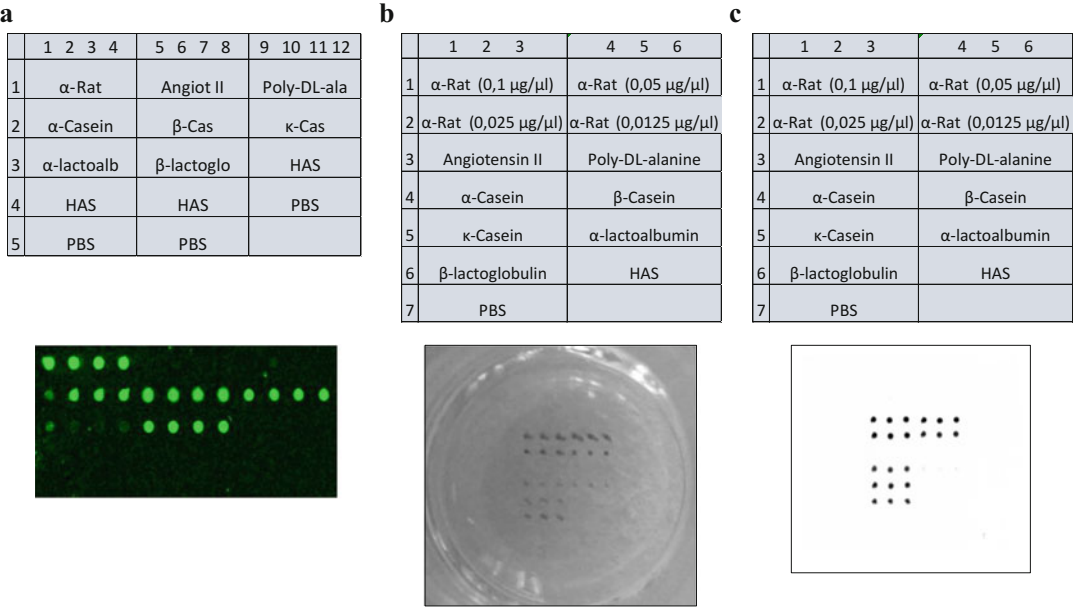


Fig. 6 Spotting scheme and microarray images obtained on **(a)** NSB27 NHS slides, **(b)** sciPLEXPLATE Type 2, and **(c)** Hybond ECL Nitrocellulose. Slides were developed using an anti-human IgE secondary antibody [biotin] and Cy3-streptavidin. Plates and membranes were visualized using an anti-human IgE secondary antibody [biotin] and HRP-streptavidin using colorimetric staining sciColor T2

we were forced to include at least three spots of PBS for each probe, which increased three times the microarrays' size [36]. Since we use a piezoelectric noncontact printer, we have substantially reduced the microarrays' size and, therefore, the number of arrays that we print on each slide.

- 5. We have tested printing on slides, 96-well microtiter plates, and membranes. The pattern of recognition obtained on the different substrates was similar, both in protein (Fig. 6) and in peptide microarrays (Fig. 7).

Acknowledgments

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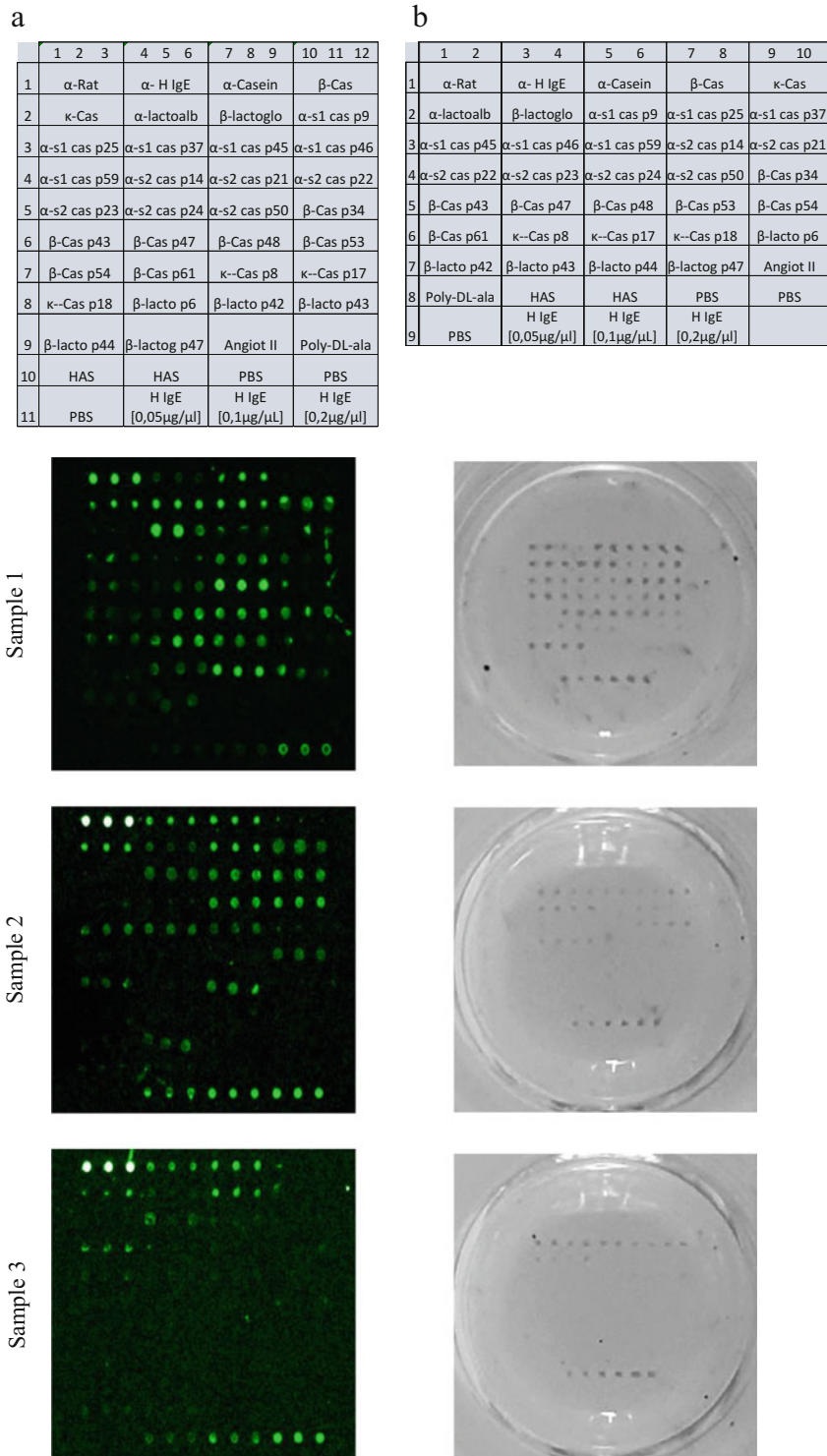


Fig. 7 Spotting scheme and microarray images obtained on **(a)** NSB27 NHS slides and **(b)** sciPLEXPLATE Type 2. Slides were developed using an anti-human IgE secondary antibody [biotin] and Cy3-streptavidin. Plates were developed using an anti-human IgE secondary antibody [biotin] and HRP-streptavidin using colorimetric staining sciColor T2

References

1. Incorvaia C, Mauro M, Ridolo E, Makri E, Montagni M, Ciprandi G (2015) A pitfall to avoid when using an allergen microarray: the incidental detection of IgE to unexpected allergens. *J Allergy Clin Immunol Pract* 3 (6):879–882
2. Williams P, Onell A, Baldracchini F, Hui V, Jolles S, El-Shanawany T (2016) Evaluation of a novel automated allergy microarray platform compared with three other allergy test methods. *Clin Exp Immunol* 184(1):1–10
3. Mothes-Luksch N, Jordakieva G, Hinterholz L, Jensen AN, Hallmann PK, Kundi M, Jensen-Jarolim E (2018) Allergy diagnosis from symptoms to molecules, or from molecules to symptoms: a comparative clinical study. *World Allergy Organization J* 11(1):22
4. Griffiths RLM, El-Shanawany T, Jolles SRA, Selwood C, Heaps AG, Carne EM, Williams PE (2017) Comparison of the performance of skin prick, ImmunoCAP, and ISAC tests in the diagnosis of patients with allergy. *Int Arch Allergy Immunol* 172(4):215–223
5. Scala E, Alessandri C, Bernardi ML, Ferrara R, Palazzo P, Pomponi D, Quarantino D, Rasi C, Zaffiro A, Zennaro D, Mari A (2010) Cross-sectional survey on immunoglobulin E reactivity in 23,077 subjects using an allergenic molecule-based microarray detection system. *Clin Exp Allergy* 40(6):911–921
6. Sastre J, Landivar ME, Ruiz-Garcia M, Andregnette-Rosigno MV, Mahillo I (2012) How molecular diagnosis can change allergen-specific immunotherapy prescription in a complex pollen area. *Allergy* 67 (5):709–711
7. Martínez-Aranguren R, Martínez-Botas J, Díaz-Perales A, Villalba M, de la Hoz B, Gomez F, Bartra J, Blanca-Lopez N, Alvarado MI, Moya C, Feo Brito F, Alonso MD, Gonzalez-Mancebo E, Terrados S, Garcia BE, Gamboa P, Parra A, Amelio CD, Goikoetxea MJ, Sanz ML (2016) Clinical performance of commercial ISAC 112 allergen microarray versus noncommercial RIRAAF platform for the diagnosis of plant food and olive pollen allergies. *J Investig Allergol Clin Immunol* 26 (3):185–187
8. Goikoetxea MJ, D'Amelio CM, Martínez-Aranguren R, Gamboa P, Garcia BE, Gomez F, Fernandez J, Bartra J, Parra A, Alvarado MI, Alonso MI, Gonzalez E, Terrados S, Moya C, Blanca N, Feo-Brito F, Villalba M, Díaz-Perales A, Sanz ML (2016) Is microarray analysis really useful and sufficient to diagnose nut allergy in the Mediterranean area? *J Investig Allergol Clin Immunol* 26(1):31–39
9. Cabrera-Freitag P, Goikoetxea MJ, Gamboa PM, Martínez-Aranguren R, Beorlegui C, Fernandez J, Sanz ML (2011) A study of the variability of the in vitro component-based microarray ISAC CDR 103 technique. *J Investig Allergol Clin Immunol* 21(5):414–415
10. Martínez-Aranguren R, Lizaso MT, Goikoetxea MJ, Garcia BE, Cabrera-Freitag P, Trellez O, Sanz ML (2014) Is the determination of specific IgE against components using ISAC 112 a reproducible technique? *PLoS One* 9(2):e88394
11. Feinberg JG (1961) A 'microspot' test for antigens and antibodies. *Nature* 192:985–986
12. Feinberg JG, Wheeler AW (1963) Detection of auto-immune antibody and tissue antigens by the 'microspot' technique. *J Clin Pathol* 16:282–284
13. Chang TW (1983) Binding of cells to matrixes of distinct antibodies coated on solid surface. *J Immunol Methods* 65(1–2):217–223
14. Ekins RP (1989) Multi-analyte immunoassay. *J Pharm Biomed Anal* 7(2):155–168
15. Frank R (2002) The SPOT-synthesis technique. Synthetic peptide arrays on membrane supports—principles and applications. *J Immunol Methods* 267(1):13–26
16. Chatchatee P, Jarvinen KM, Bardina L, Beyer K, Sampson HA (2001) Identification of IgE- and IgG-binding epitopes on alpha (s1)-casein: differences in patients with persistent and transient cow's milk allergy. *J Allergy Clin Immunol* 107(2):379–383
17. Chatchatee P, Jarvinen KM, Bardina L, Vila L, Beyer K, Sampson HA (2001) Identification of IgE and IgG binding epitopes on beta- and kappa-casein in cow's milk allergic patients. *Clin Exp Allergy* 31(8):1256–1262
18. Jarvinen KM, Beyer K, Vila L, Bardina L, Mishoe M, Sampson HA (2007) Specificity of IgE antibodies to sequential epitopes of hen's egg ovomucoid as a marker for persistence of egg allergy. *Allergy* 62(7):758–765
19. Jarvinen KM, Beyer K, Vila L, Chatchatee P, Busse PJ, Sampson HA (2002) B-cell epitopes as a screening instrument for persistent cow's milk allergy. *J Allergy Clin Immunol* 110 (2):293–297
20. Lin J, Bardina L, Shreffler WG, Andreae DA, Ge Y, Wang J, Bruni FM, Fu Z, Han Y, Sampson HA (2009) Development of a novel peptide microarray for large-scale epitope mapping

- of food allergens. *J Allergy Clin Immunol* 124 (2):315–322, 322 e311–313
21. Lin J, Sampson HA (2009) The role of immunoglobulin E-binding epitopes in the characterization of food allergy. *Curr Opin Allergy Clin Immunol* 9(4):357–363
 22. Sykes KF, Legutki JB, Stafford P (2013) Immunosignaturing: a critical review. *Trends Biotechnol* 31(1):45–51
 23. Linnebacher M, Lorenz P, Koy C, Jahnke A, Born N, Steinbeck F, Wollbold J, Latzkow T, Thiesen HJ, Glocker MO (2012) Clonality characterization of natural epitope-specific antibodies against the tumor-related antigen topoisomerase IIa by peptide chip and proteome analysis: a pilot study with colorectal carcinoma patient samples. *Anal Bioanal Chem* 403(1):227–238
 24. Andresen H, Zarse K, Grotzinger C, Hollidt JM, Ehrentreich-Forster E, Bier FF, Kreuzer OJ (2006) Development of peptide microarrays for epitope mapping of antibodies against the human TSH receptor. *J Immunol Methods* 315(1–2):11–18
 25. Gaseitsiwe S, Valentini D, Mahdavi S, Magalhaes I, Hoft DF, Zerweck J, Schutkowski M, Andersson J, Reilly M, Maeurer MJ (2008) Pattern recognition in pulmonary tuberculosis defined by high content peptide microarray chip analysis representing 61 proteins from *M. tuberculosis*. *PLoS One* 3(12):e3840
 26. Weber LK, Isse A, Rentschler S, Kneusel RE, Palermo A, Hubbuch J, Nesterov-Mueller A, Breitling F, Loeffler FF (2017) Antibody fingerprints in Lyme disease deciphered with high density peptide arrays. *Eng Life Sci* 17 (10):1078–1087
 27. Legutki JB, Johnston SA (2013) Immunosignatures can predict vaccine efficacy. *Proc Natl Acad Sci U S A* 110(46):18614–18619
 28. Shreffler WG, Beyer K, Chu TH, Burks AW, Sampson HA (2004) Microarray immunoassay: association of clinical history, in vitro IgE function, and heterogeneity of allergenic peanut epitopes. *J Allergy Clin Immunol* 113 (4):776–782
 29. Sanchez-Ruano L, de la Hoz B, Martinez-Botas J (2020) Clinical utility of microarray B-cell epitope mapping in food allergies: a systematic review. *Pediatr Allergy Immunol* 31 (2):175–185
 30. Austin J, Holway AH (2011) Contact printing of protein microarrays. *Methods Mol Biol* 785:379–394
 31. Barbulovic-Nad I, Lucente M, Sun Y, Zhang M, Wheeler AR, Bussmann M (2006) Bio-microarray fabrication techniques—a review. *Crit Rev Biotechnol* 26(4):237–259
 32. Lin J, Bardina L, Shreffler WG (2009) Microarrayed allergen molecules for diagnostics of allergy. *Methods Mol Biol* 524:259–272
 33. Kwak JW, Jeong H, Han SH, Kim Y, Son SM, Mook-Jung I, Hwang D, Park JW (2014) Phosphokinase antibody arrays on dendron-coated surface. *PLoS One* 9(5):e96456
 34. Shreffler WG, Lencer DA, Bardina L, Sampson HA (2005) IgE and IgG4 epitope mapping by microarray immunoassay reveals the diversity of immune response to the peanut allergen, Ara h 2. *J Allergy Clin Immunol* 116(4):893–899
 35. Martinez-Botas J, Cerecedo I, Zamora J, Vlaicu C, Dieguez MC, Gomez-Coronado D, de Dios V, Terrados S, de la Hoz B (2013) Mapping of the IgE and IgG4 sequential epitopes of ovomucoid with a peptide microarray immunoassay. *Int Arch Allergy Immunol* 161 (1):11–20
 36. Martinez-Botas J, de la Hoz B (2016) IgE and IgG4 epitope mapping of food allergens with a peptide microarray immunoassay. *Methods Mol Biol* 1352:235–249

Part IV

Deciphering Immune Responses in Diseases



Protein Arrays for the Identification of Seroreactive Protein Markers for Infectious Diseases

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Abstract

The protein array is a powerful platform to study humoral responses to infectious agents using small sample volumes [$<3 \mu\text{L}$]. Its success can be largely attributed to the development of new strategies for high-throughput cloning and expression, and improved manufacturing techniques for the construction of arrays. Here, we describe a method to hybridize protein arrays with malaria patients' sera in order to identify seroreactive antigens, some of which may have a high potential of conferring protection from severe forms of malaria.

Key words Protein arrays, Seroreactive antigens, *Plasmodium vivax*, *Plasmodium falciparum*, Malaria exposure, Protective immunity

1 Introduction

Antibodies are key players in the body's adaptive response to infectious agents. They destroy or neutralize pathogens by binding specifically to molecules called antigens. The response is highly regulated and includes a strong set of defenses. A unique feature is the generation of a long-lasting memory that prevents or reduces the chances of secondary infection from previously encountered pathogens. Immunological memory is, therefore, a crucial part of the body's adaptive defense system. Vaccines trigger the development of memory cells in the body by mimicking antigens. Subsequently, antibodies are produced and we develop immunity even without being previously infected by the disease-causing pathogen [1]. Insights into what kind of antigens can trigger a protective and effective immune response are central to developing and evaluating vaccines. Equally critical is an understanding of how the pathogen interacts with the host immune system. Protein arrays provide a convenient platform to characterize humoral immune responses to

infectious pathogens [2–5]. They can be used to identify, quantify, and compare antibody responses to thousands of antigens, across hundreds of different clinical samples, simultaneously [6, 7]. This high-throughput technology is regularly employed in clinical and translational research to identify vaccine candidates [8, 9], to measure disease prevalence and exposure [10, 11], and to identify serodiagnostic markers of infection [12–15].

The success of protein arrays over other proteomics technologies stems from the development of high-throughput cloning methods and improved array manufacturing capabilities [6]. For example, advances in cell-free expression systems have made it possible to efficiently synthesize thousands of proteins in a short time, including large proteins and those that are difficult to purify [16]. This approach is cost effective because it does not involve a protein purification step. It uses PCR-generated DNA fragments or plasmid cDNAs to express proteins in an in vitro system that includes the entire machinery for transcription and translation [6, 16, 17]. Reactions containing unpurified proteins are then printed directly onto nitrocellulose slides to construct protein arrays [17]. Arrays for more than 30 infectious microorganisms have been developed by the Protein Microarray Laboratory at UC Irvine [18]. Using arrays designed for malaria studies [Pf/Pv500], we analyzed antibody responses to parasite antigens (*Plasmodium vivax* and *Plasmodium falciparum*) in a low-malaria-transmission setting [19]. Our findings point to specific leads for possible protective immunity against severe malaria. Here, we describe a method to determine infectious disease seroreactive antigens from protein arrays, using Pf/Pv500 arrays (Antigen Discovery, Inc., Irvine, CA) as an example.

Each array contains eight pads, allowing eight different clinical samples to be probed and analyzed simultaneously. Each pad is printed with 515 *P. vivax* protein spots, 500 *P. falciparum* protein spots, and several control spots. Proteins are expressed as polypeptide fragments and printed on a nitrocellulose membrane as cell-free in vitro transcription and translation (IVTT) reactions. Larger proteins are printed as overlapping peptides or exon fragments. The expressed proteins have an N-terminal poly-histidine tag and a C-terminal HA tag. The quality of each array batch is determined using appropriate antibodies to test the efficiency of protein expression.

For the assay, the arrays are rehydrated with blocking buffer to prevent nonspecific binding on the active surface. Meanwhile, diluted sera from malaria-positive patients and controls are incubated with *E. coli* lysate to block anti-*E. coli* antibodies, which are usually present in large concentrations in human sera. Arrays are probed with diluted sera overnight and visualized using an affinity-purified anti-human IgG secondary antibody labeled with biotin. To detect the antibody, arrays are probed with a streptavidin

conjugate. They are then washed, dried, and scanned at a wavelength of 635 nm. The output files are exported as grayscale .tiff images and quantified using a microarray software as a unit-less relative median intensity, also called the median signal intensity (MSI). Prior to data analysis, the data is normalized using IVTT control spots to reduce background signals. Heat maps are generated and seroreactive antigens are defined.

In order to identify markers of protection from severe diseases, two groups of patients were studied: severe and non-severe malaria patient cohorts. All severe patients were hospitalized and showed at least one severe symptom for malaria, while non-severe patients were recruited from the outpatient department. We compared the antibody binding across both groups to identify the most differentially reactive antigens. A similar procedure can be used to identify clinically relevant antigens for other infectious diseases and for broader applications, using sera from different patient cohorts and controls [20]. Profiling before transmission season and correlating outcomes at the end of transmission season are also ways to identify protective antigens.

2 Materials

Prepare all solutions using double-distilled water (ddH₂O) and analytical grade reagents. Store the reagents at their respective temperatures mentioned in Subheading 2.1. Human sera are potential sources of aerosol-transmitted and blood-borne pathogens. Handle all human serum samples according to locally approved institutional biosafety protocols. Diligently follow all waste disposal regulations when disposing waste materials.

2.1 Reagents

Buffer (TBS): To prepare 1× TBS working solution, add 20 mL of 1 M Tris-HCl (20 mM), pH 7.5, and 30 mL of 5 M NaCl (150 mM) to a glass beaker containing 800 mL ddH₂O. Mix and make up the volume to 1 L with ddH₂O. Sterilize by filtering and store at room temperature (RT). Alternatively, you can prepare a 10× TBS stock solution. Dissolve 24 g Tris and 87.6 g NaCl in 800 mL ddH₂O. Mix and adjust pH to 7.5 with HCl (*see Note 1*). Make up the volume to 1 L with ddH₂O water. Sterilize by filtering and store at 4 °C till further use.

Buffer (TBST): To prepare 1× TBST working solution, add 20 mL of 1 M Tris-HCl (20 mM), pH 7.5 to a glass beaker containing 800 mL ddH₂O (filter if you see precipitates). Add 30 mL 5 M NaCl (150 mM) and 500 µL Tween 20 (0.05%). Mix and make up the volume to 1 L with ddH₂O.

E. coli lysate: *E. coli* lysates for microarray experiments are commercially available as lyophilized powders (*see Note 2*). Upon arrival, store at RT till required. When you take out a vial, reconstitute as per manufacturer's instructions and store the leftover at -20°C .

Array blocking buffer (BB): Blocking solutions are commercially available. Recipes vary by protocol (*see Note 3*).

Primary sera: Always store sera at -80°C in small aliquots of 10 μL (*see Note 4*). Do not use a frost-free freezer. To prepare sera for the microarray experiment, first prepare 10% *E. coli* lysate in blocking buffer (EBB) (e.g., 9 mL BB + 1 mL *E. coli* lysate). Aliquot the required volume of diluted EBB into Eppendorf tubes. Then in a class II biosafety cabinet, add sera into the EBB tubes to obtain a final dilution of 1:100 just before starting the experiment (*see Note 5*). Incubate for 30 min at RT on a slow platform rocker (4–6 rpm).

Secondary antibody (2° Ab): Use a biotin-conjugated anti-human IgG secondary Ab (e.g., biotin-conjugated affinity-purified goat α -human IgG Fc fragment). Upon delivery, label the tube with the date of arrival and store the lyophilized Ab at 4°C . Reconstitute as per manufacturer's instructions, but reconstitute with 50% glycerol, then aliquot, and store at -20°C (*see Note 6*). Aliquot in small volumes (25 μL). Discard all aliquots if not used within 1 year. For the experiment, dilute 2° Ab in BB (1:200), vortex, and leave on the platform rocker to mix for a couple of minutes at RT.

Tertiary antibody (3° Ab): Use a streptavidin Cy5 fluorophore. Upon delivery, label the tube with date of arrival and store lyophilized Ab at 4°C . Reconstitute as per manufacturer's instructions, but reconstitute with 50% glycerol, then aliquot, and store at -20°C . For the experiment, dilute the streptavidin conjugate in BB (1:100), vortex, and leave on platform rocker to mix for a couple of minutes at RT.

2.2 Equipment

Blocking: Pf-Pv500 arrays.

Lightproof box, desiccator cabinet.

Array chamber, frame slide plates, and clips.

Standard 96-well PCR plate.

Pipettes, micropipettes, autopipettes.

Sterile disposable micropipette tips.

Fine-nosed tweezers.

Magnetic stirrer.

Vortex.

Ultracentrifuge.
Class II biosafety cabinet.
Orbital shaker.
4 °C Refrigerator.
−20 °C Freezer.
Aluminum foil.
Paper towels.
Vacuum system (optional).
Microarray scanner.

2.3 Software

Software for array scanning and quantification.
Excel, SAM module.
R Studio, Perl, Python (optional).

3 Methods

3.1 Blocking

Carry out this step on day 1 (late afternoon):

1. Using an alcohol-resistant pen/diamond pen, label each array carefully on the non-active side (label array type, sera number, sera type, and array number) (*see* **Note 7**).
2. Position a chamber over the array and carefully place it into a frame slide plate (Fig. 1).
3. Rehydrate each pad with 1× array blocking buffer (BB) (*see* **Note 8**) and leave at RT for 30 min on a slow platform rocker (4–6 rpm).

3.2 Probing

1. Briefly vortex the sera (3 s), and then centrifuge the cryovials (in case there are particulates) in 15 mL falcon tubes for 1 min at 4000 rpm ($1252 \times g$ for a rotar radius of 7cm) or microfuge for 30 s at 14,000 rpm ($15,339 \times g$ for a rotar radius of 7cm) (Fig. 2). This step is optional (*see* **Note 9**).
2. Label sterile Eppendorf tubes with sera #.
3. Make up fresh 10% *E. coli* lysate in blocking buffer (EBB).
4. Aliquot the required volume of diluted EBB into Eppendorf tubes.
5. In a class II biosafety cabinet add the required volume of sera into the EBB mix (sera dilution: 1:100).
6. Immediately freeze aliquoted sera back into −80 °C. Place the diluted sera (1° Ab) onto the platform rocker at 50 rpm to block for anti-*E. coli* antibodies at RT for 30 min.

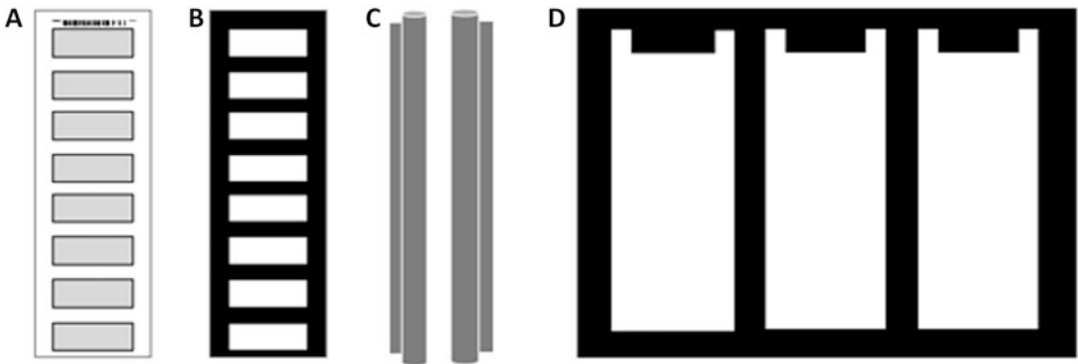


Fig. 1 Protein arrays and setup for experiment. (a) Active surface (barcode side) of a protein array having eight printed pads (gray boxes). Each pad contains a total of 500 *P. falciparum* and 515 *P. vivax* proteins (expressed as polypeptide fragments) printed as cell-free in vitro transcription-translation (IVTT) reactions (4 blocks). The pad also contains control spots; 24 IgG spots in 6 different concentrations (6 spots in each block), 24 anti-human IgG spots in 6 different concentrations, 32 TTBS (buffer) spots, 24 “no-DNA control” reaction spots lacking a DNA template in the plasmid vector, and 7 blank and 6 empty spots. Printed along with the control spots are 12 purified parasite proteins at two concentrations. White surface represents the glass surface of the protein array. (b) Chamber which is carefully positioned over the array before starting the experiment. (c) Clamps to fix the chamber over the array. (d) Frame slide plate to hold the arrays during the experiment. It can hold up to 3 arrays

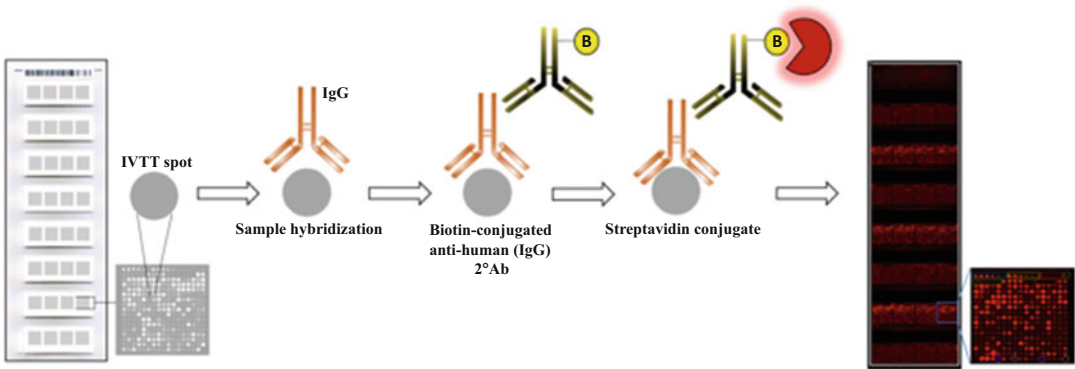


Fig. 2 Schematic representation of the microarray experiment design and workflow for the identification of seroreactive protein markers for infectious diseases

7. In a class II biosafety cabinet, aspirate BB from chambers with fine tips.
8. In a class II biosafety cabinet, add the required volume of diluted sera (e.g., 8-pad = 200 μ L).
9. Place a foiled lid on the chamber plate (to prevent evaporation) and a moist towel on the bottom.
10. Incubate overnight at 4 $^{\circ}$ C on a slow platform rocker (4–6 rpm).

3.3 Assay

Carry out this step on day 2 in the morning:

1. In a class II biosafety cabinet, aspirate sera (1° Ab) and wash the pads thrice (3×) with 100 µL TBST. After adding TBST, place on a fast rocker at 50 rpm for 5 min, aspirate, and repeat. Remember to change tips for each pad.
2. Dilute biotin-conjugated 2° Ab in BB (1:200), vortex the tubes, and leave them on a platform rocker at 50 rpm to mix for a few minutes at RT.
3. Aspirate TBST after three washes and add the required volume of diluted 2° Ab to the pads. Incubate for 1 h at 50 rpm on a RT platform rocker.
4. Aspirate 2° Ab and wash the pads thrice (3×) with 100 µL TBST.
5. During the washes, dilute 3° Ab–streptavidin conjugate in BB (1:100). Cover the 15 mL conical tube with aluminum foil to prevent exposure to light. Vortex and place the tube on a platform rocker.
6. After the third wash, aspirate TBST and add the required volume of 3° Ab. Place on a platform rocker at 50 rpm for 45 min at RT. Cover with a foiled lid (*see Note 10*).
7. Aspirate 3° Ab and wash the pads thrice (3×) with 100 µL TBST.
8. Then wash twice with 100 µL TBS. Place on a fast rocker at 50 rpm for 5 min, aspirate, and repeat.

3.4 Washing and Drying

1. Remove array from the frame and tap the edge lightly on a paper towel to remove excess fluid. Do not touch the active surface. For the washing step, carefully slide the array lengthwise into a 50 mL tube or slot the array into a slide box containing ddH₂O for a few seconds (*see Note 11*).
2. To remove excess fluid, spin the slide box or the 50 mL tubes containing the array in a centrifuge for 5 min at 2000 rpm ($\sim 300 \times g$ for a rotar radius of 7cm) (*see Note 12*).
3. Store the slides in a lightproof box in a desiccator until scanning.
4. Wash the plates and chambers in hot water with a few mL of TBST. Cover and soak for a little while, then tip out, and air-dry on tissue.

3.5 Scanning

1. To scan the array (Fig. 3), insert it carefully into the array holder space in the microarray scanner, with its active side down (*see Note 13*).
2. Click on the software icon and select the slide configuration. Then go to laser settings and select the appropriate wavelength

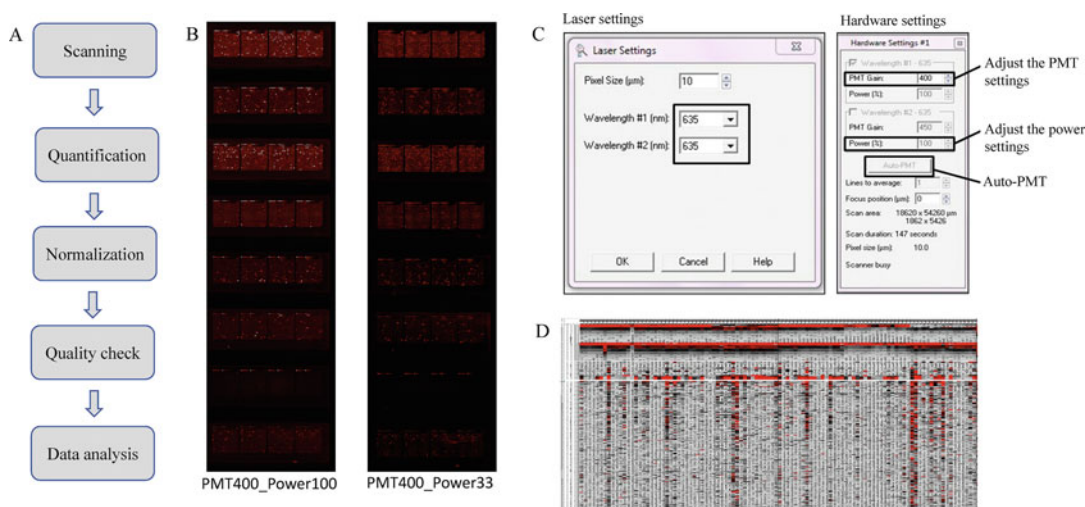


Fig. 3 (a) Flowchart describing the steps involved in a microarray experiment from scanning to data analysis. (b) Two arrays scanned at different power settings. A higher power increases the number of saturated spots (white) on the array. On lowering the power, a minimum number of spots are saturated while most of the low-intensity interactions are also captured. (c) Laser and hardware settings in the microarray software. (d) Heat maps with signal intensities for all protein spots across patients

(635 nm in this case). The wavelength depends on the conjugated fluorophore used for detection, in this case streptavidin-Cy5 conjugate.

3. Check preview scan images at different power settings and select the optimum PMT.
4. Scan the slide at optimum PMT value.
5. Once scanning is complete, save the image in the .tiff format.

3.6 Data Analysis

1. Open the saved .tiff image to be quantified and upload the .GAL file. It represents a file that contains the name and location of all protein spots on the array.
2. Lay the grid carefully over the image to quantify spot intensities and specify the folder in which you want the analysis output file to be saved. Adjust and align the grid manually over the array if required. This step is important in order to obtain only true positive signal intensities.
3. Once all the features are aligned, save the settings. Data is acquired as median signal intensities (MSI) for all spots on the array.
4. Save the file in the .gpr format.
5. Export the file in a format that is compatible with the software of your choice for data analysis. Normalize the data using control spots on the array. For sample-specific median

normalization, subtract the sample-specific median of the IVTT control spots from the raw intensity of all IVTT protein spots for that sample.

6. Generate a heap map using normalized signal intensities. The human IgG and anti-human IgG gradients are used as positive controls. Compare the signal intensities across different batches and rescan using different power settings, if necessary.
7. Identify seroreactive antigens. Antigens are considered seroreactive if the average spot intensity for a group of individuals is greater than a cutoff defined as the average plus two standard deviations of the reactivity to all 24 IVTT control spots.
8. For statistical data analysis, normalize the original data by dividing the raw IVTT signal intensities for each spot (after setting a floor of 100) by the sample-specific median of the IVTT control spots (fold-over control, FOC). Then log transform (log base 2) the values to make it easier to analyze (Log2 FOC).
9. Then analyze the raw data to compare the antibody binding across antigens and across serum specimens.
10. Identify differentially reactive proteins between any two groups using R, SAM (significance analysis of microarrays) or any other software of your choice. Various software and programming tools like R, Perl, and Python can be used for processing the data generated from microarray experiment.
11. Data can be graphically represented using R studio, GraphPad Prism (Prism v6.0, GraphPad Software Inc., La Jolla, CA) or any other software.

4 Notes

1. To avoid the need to adjust the pH of the buffer manually, commercial stock solutions may be purchased. Nevertheless, it is good to check the pH of TBS before starting the assay. Filtering, if you see any precipitates, can greatly reduce the chances of background signals.
2. Serum samples are incubated with diluted *E. coli* lysates to mask the effect of anti-*E. coli* antibodies that are present in high titers. They are available as lyophilized powders (10 mg total protein/tube), which have to be reconstituted carefully in 1 mL of blocking buffer. Follow the manufacturer's instructions and mix well by pipetting slowly. Freeze leftovers at -20°C .
3. The choice of a blocking buffer depends on the type of array being used. For instance, some buffers are specially formulated to suit nitrocellulose membrane surfaces. Stock solutions may

be purchased from many commercial vendors depending on the array. While rehydrating arrays with blocking buffer, dispense the solution from the corners to prevent damage to the printed spots. Ensure that the solution covers the entire surface of each array. After aspiration, immediately proceed to the next step to avoid drying of the array surface.

4. Always wear gloves and laboratory coats while handling serum samples. If you have cuts or abrasions on your palms or hands, cover them with adhesive dressing. Handle the serum samples in a class II biosafety cabinet according to institutional biosafety guidelines.
5. Serum dilution is determined based on the antibody concentration in the sample (e.g., IgG). Perform a test experiment before moving on to a larger hybridization assay, to determine serum and antibody dilutions for your assay and to test the antibody performance.
6. With glycerol, tubes can be stored at -20°C for a longer time and can be taken in and out of the freezer without the worry of freeze/thaw damage.
7. Wear gloves at all times during the assay. Ensure that you do not touch the active surface of the array (the surface with the bar code label) with hands or pipette tips. Keep the active surface up at all times. Never let the array dry out during the assay and do not let condensate form on the surface. It is best to store the arrays in a desiccator cabinet before use.
8. The math is for an 8-pad array. Calculate the volume of BB to be dispensed according to your array design (2-pad and 3-pad = $500\ \mu\text{L}$, 16-pad = $100\ \mu\text{L}$, 8-pad = $200\ \mu\text{L}$).
9. Thaw on ice before starting the assay. Mix slowly by inverting the tube several times and centrifuge to remove any floating debris. Wash your hands thoroughly after probing the arrays.
10. To prevent damage to the fluorescent label of the secondary Ab, protect the arrays from light during the incubation step. If the lights in the room cannot be switched off, cover the trays with aluminum foil or use a lightproof box. Remember that after this stage all other steps have to be performed in the dark.
11. Carefully remove the wash buffer from a corner by aspiration without scratching the active surface of the array. Do not let the arrays sit for long periods in wash buffer. Proceed to the drying stage immediately. Submerge the array in water at RT with the active surface facing up, with gentle shaking for 5 min.
12. This step is performed to remove excess water that would otherwise dry on the array surface leaving areas of high background. The slide boxes should be lined with filter paper or

Kimwipes to absorb excess moisture. Do not increase the centrifugation speed as higher speed may cause the arrays to break.

13. After the washes are completed, scan the microarrays immediately (highly preferred) or store the array, but it should not touch any other surfaces, including other arrays. The arrays may be placed at 4 °C after scanning (for storage). Scanner should be properly calibrated prior to scanning. Make sure that all scanner settings remain the same while scanning the arrays.

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References

1. Vetter V, Denizer G, Friedland LR, Krishnan J, Shapiro M (2018) Understanding modern-day vaccines: what you need to know. *Ann Med* 50 (2):110–120
2. Vigil A, Ortega R, Jain A, Nakajima-Sasaki R, Tan X, Chomel BB et al (2010) Identification of the feline humoral immune response to *Bartonella henselae* infection by protein microarray. *PLoS One* 5(7):e11447
3. Felgner PL, Roestenberg M, Liang L, Hung C, Jain A, Pablo J et al (2013) Pre-erythrocytic antibody profiles induced by controlled human malaria infections in healthy volunteers under chloroquine prophylaxis. *Sci Rep* 3:3549
4. Arévalo-Herrera M, Lopez-Perez M, Dotsey E, Jain A, Rubiano K, Felgner PL et al (2016) Antibody profiling in naïve and semi-immune individuals experimentally challenged with *Plasmodium vivax* sporozoites. *PLoS Negl Trop Dis* 10(3):e0004563
5. Uplekar S, Rao PN, Ramanathapuram L, Awasthi V, Verma K, Sutton P et al (2017) Characterizing antibody responses to *Plasmodium vivax* and *Plasmodium falciparum* antigens in India using genome-scale protein microarrays. *PLoS Negl Trop Dis* 11(1):e0005323
6. Yu X, Petritis B, LaBaer J (2016) Advancing translational research with next-generation protein microarrays. *Proteomics* 16 (8):1238–1250
7. Natesan M, Ulrich RG (2010) Protein microarrays and biomarkers of infectious disease. *Int J Mol Sci* 11(12):5165–5183
8. Kamuyu G, Tuju J, Kimathi R, Mwai K, Mburu J, Kibinge N et al (2018) KILchip v1.0: a novel *Plasmodium falciparum* Merozoite protein microarray to facilitate malaria vaccine candidate prioritization. *Front Immunol* 9:2866

9. Davies DH, Duffy P, Bodmer J-L, Felgner PL, Doolan DL (2015) Large screen approaches to identify novel malaria vaccine candidates. *Vaccine* 33(52):7496–7505
10. Baum E, Sattabongkot J, Sirichaisinthop J, Kiattibutr K, Davies DH, Jain A et al (2015) Submicroscopic and asymptomatic *Plasmodium falciparum* and *Plasmodium vivax* infections are common in western Thailand - molecular and serological evidence. *Malar J* 14:95
11. King CL, Davies DH, Felgner P, Baum E, Jain A, Randall A et al (2015) Biosignatures of exposure/transmission and immunity. *Am J Trop Med Hyg* 93(3 Suppl):16–27
12. Vigil A, Chen C, Jain A, Nakajima-Sasaki R, Jasinskas A, Pablo J et al (2011) Profiling the humoral immune response of acute and chronic Q fever by protein microarray. *Mol Cell Proteomics* 10(10):M110.006304
13. Huw Davies D, Jain A, Nakajima R, Liang L, Jasinskis A, Supnet M et al (2016) Serodiagnosis of acute typhoid fever in Nigerian pediatric cases by detection of serum IgA and IgG against hemolysin E and lipopolysaccharide. *Am J Trop Med Hyg* 95(2):431–439
14. Olaya-Abril A, Jiménez-Munguía I, Gómez-Gascón L, Obando I, Rodríguez-Ortega MJ (2015) A pneumococcal protein array as a platform to discover serodiagnostic antigens against infection. *Mol Cell Proteomics* 4(10):2591–2608
15. Felgner J, Juarez S, Hung C, Liang L, Jain A, Döşkaya M et al (2015) Identification of *Toxoplasma gondii* antigens associated with different types of infection by serum antibody profiling. *Parasitology* 142(6):827–838
16. Cook EA, He M (2014) Preparation of protein arrays using cell-free protein expression. *Methods Mol Biol (Clifton, NJ)* 1118:245–255
17. Davies DH, Liang X, Hernandez JE, Randall A, Hirst S, Mu Y et al (2005) Profiling the humoral immune response to infection by using proteome microarrays: high-throughput vaccine and diagnostic antigen discovery. *Proc Natl Acad Sci U S A* 102(3):547–552
18. Liang L, Felgner PL (2015) A systems biology approach for diagnostic and vaccine antigen discovery in tropical infectious diseases. *Curr Opin Infect Dis* 28(5):438–445
19. Venkatesh A, Jain A, Davies H, Periera L, Maki JN, Gomes E et al (2019) Hospital-derived antibody profiles of malaria patients in South-west India. *Malar J* 18(1):138
20. Sharon D, Snyder M (2014) Serum profiling using protein microarrays to identify disease related antigens. *Methods Mol Biol* 1176:169–178



Chapter 11

Glass Slide-Printed Protein Arrays as a Platform to Discover Serodiagnostic Antigens Against Bacterial Infections

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Abstract

Infectious diseases represent a major cause of morbidity and mortality worldwide. Early detection of infections is capital for managing life-threatening cases. So far, traditional diagnostic methods such as microbiological cultures are slow and, sometimes, inaccurate. In the molecular era, high-throughput techniques are essential for providing tools that are able to diagnose in a fast and reliable way, as well as they can be used for monitoring the humoral response of groups of people in a program of epidemiological surveillance when an outbreak occurs, or when a vaccine is being evaluated. Antigen-based protein microarrays are an ideal means for these purposes, as they can carry up to thousands of protein antigens from pathogenic sources and be probed with sera from different human groups (acute or chronic infected people, convalescent, controls). For the diagnosis of bacterial infections, the best antigens are in principle the surface proteins, as they have the highest chances to raise an effective immune response. Here we describe a general protocol for fabricating a glass slide-based protein microarray using recombinant bacterial surface antigens, according to our own expertise in the study of pneumococcal disease. The probing with human sera aims to evaluate differences between diseased and healthy people, in order to discover discriminating antigens that can be used, after appropriate validation, in further easy-to-use formats such as immunostrips.

Key words Protein array, Diagnostics, Infectious diseases, Humoral response, Antibodies, Serological assay

1 Introduction

Traditional methods for the diagnosis of infectious diseases have relied on microbiological cultures, when the suspected etiological agent is a bacterial or fungal pathogen, or the clinical examination of well-known symptoms for viral infections. Obviously, in the molecular era, these methods are not sufficient for many diseases because of their inaccuracy and slowness, in the case of microbiological diagnostics [1]. The promptness in having early detection of infections is often critical to make decisions about the treatment to

be applied to a patient, and even for saving lives. Thus, molecular diagnostics have represented an important advance in medicine for treating infectious diseases.

Within this group of molecular techniques, protein microarrays are excellent platforms for screening interactions of biomolecules [2]. Thus, they allow to test simultaneously tens to thousands of antigen-antibody interactions in a single assay, increasing the amount and speed of biological information that can be obtained, in contrast to other single antigen-based serological assays, such as Western blots, immunoprecipitation, or ELISA. This includes the study of protein-protein or protein-ligand interactions, discovery of disease biomarkers, analysis of posttranslational modifications, or identification of drug targets [3]. Particularly, in the field of infectious diseases protein microarrays are used extensively as high-throughput tools for diagnostics, as well as a means to discover/validate vaccine candidate antigens, and in programs of epidemiological surveillance [4, 5]. Different protein formats are available according to the surface to which the antigens are immobilized. Those employing solid surfaces as metal or glass slides are still the most widely used, because those based on multiplex bead-based assays like the Luminex[®] technology require costly platforms and are limited up to 500 antigens. Nevertheless, these latter platforms allow a very effective differentiation of patient and control groups at a quantitative level [6].

Typically, protein arrays for infectious diseases are printed with highly purified proteins from the pathogen(s) whose infection(s) are intended to detect, and they are probed with sera from patients as the source of antibodies, either IgM (to measure acute humoral response to infection) or IgG (to detect adaptive, secondary humoral response). The best candidate antigens being used in protein microarrays, either for diagnostic purposes, for vaccine discovery/evaluation, or for epidemiological surveillance, are surface proteins. These proteins, which include also those secreted to the extracellular milieu, have the highest chances to raise an effective host immune response because of their high exposure [7, 8], which makes them ideal targets for drug or vaccine development [9, 10]. After probing with a cohort of sera (which normally include at least two groups, i.e., that of the patients and a control with people not suffering the infection to be tested), secondary antibodies coupled to a fluorescent dye are added and a signal is recorded. Then, a complex statistical process is performed to normalize the data and to compare the groups under study, which is conducted to identify proteins that discriminate the groups. The proteins identified as discriminating antigens are normally validated with any immunohistochemical technique using another independent sera cohort. Once the discriminating antigens have been defined, these can be scaled out for use in a more suitable, user-friendly format like an immunostrip.

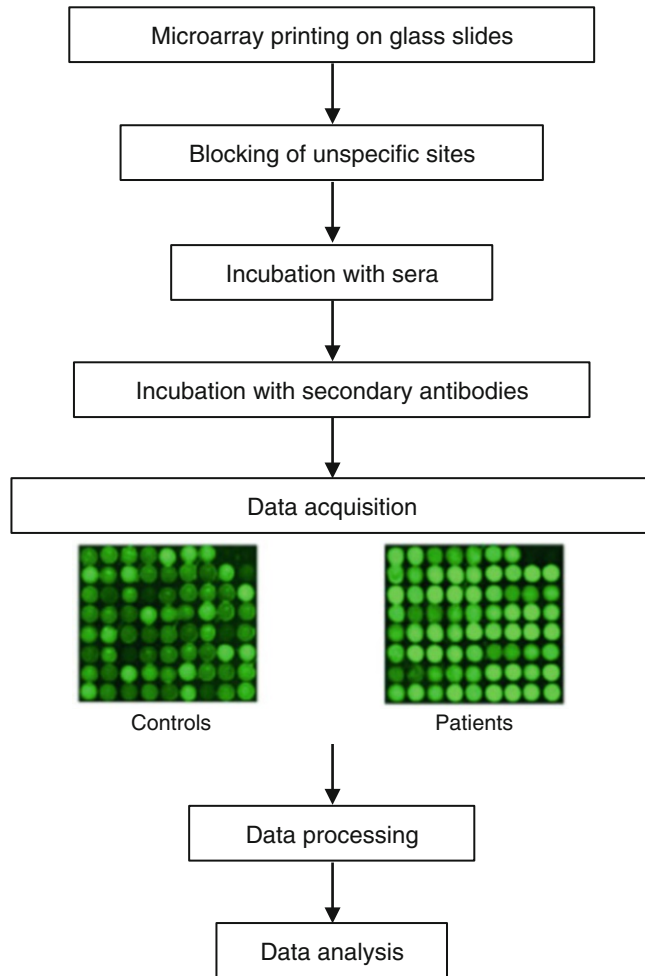


Fig. 1 Schematic workflow of bacterial protein microarray printing, hybridization with human sera, and further data acquisition, processing, and analysis

In this chapter, we describe the technical aspects to make a glass slide-based protein microarray of a collection of recombinant bacterial proteins, based on our own experience with pneumococcal proteins being used either as a platform to discover serological diagnostic markers of infection [5] or as an epidemiological surveillance tool (Fig. 1). Although of great importance, we do not annotate extensively other considerations because they have been already described in a highly comprehensive way, including the production of recombinant proteins [11], as well as the study design and the statistical treatment of the data [12, 13].

2 Materials

All buffers are prepared, using molecular biology-grade water, in different batches. It is recommended to prepare the solutions in excess (at least 20% more than the calculated amount to be used). Before the start of the procedure, buffers must be cooled at 4 °C. Depending on the slide and the number of proteins to be assayed, a different number of complete arrays may be printed. In the same way, the blocking, washing, and printing solutions will have to be adjusted to the number of slides to be used, being advisable to follow the manufacturer's instructions. Here we show how to prepare the materials according to the use of the Nexterion Slide H 3-D glass slides, as described in our previous work [5].

1. Recombinant proteins for printing in glass slide arrays: Proteins can be produced with a terminal tag to improve the printing procedure. For example, a double-fusion fragment containing an N-terminal GST fusion and a C-terminal 6 × His-tag could be produced (*see Note 1*). Once expressed, proteins should be purified using the appropriate procedure (as by using a Ni²⁺-agarose affinity chromatography for His-tags) and dialyzed against a specific buffer (such as phosphate-buffered saline, PBS) (*see Note 2*). Negative controls should be used (i.e., proteins not related to the variable or pathogen under study). Each protein should be printed on the glass slides, at least, per triplicate (although it is recommended to do it at least in quintuplicate), at 6 ng/spot (*see Note 3*). Printed slides are placed in a slide humidity chamber overnight at 75% relative humidity and stored at −20 °C until use.
2. Printing buffer: Using a graduated cylinder, prepare 1 L of a 150 mM phosphate and 0.01% sarkosyl buffer, using 3 M NaOH to adjust the pH to 8.5. Recombinant proteins are prepared in this buffer at a certain concentration (i.e., 250 µg/mL) (*see Note 4*).
3. Blocking buffer: For Nexterion Slide H 3-D glass slides, 25 mM ethanolamine in 200 mM sodium borate buffer, adjusted at pH 8.5, is recommended for blocking. Prepare 1 L using a graduated cylinder.
4. PBS-T: Prepare, using a graduated cylinder, 1 L of phosphate-buffered saline 1× (0.8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na₂HPO₄, and 0.24 g/L KH₂PO₄) and 0.1% Tween-20 solution adjusted to pH 7.4 (*see Note 5*). Sterilize it by filtering or autoclaving. This is the washing buffer.
5. Sera samples: Human serum has to be obtained according to the principles expressed in the Declaration of Helsinki and according to previously designed experimental criteria. Sera

are stored at -80°C in 25 μL aliquots and prepared on ice with washing solution (PBS-T) at the required dilution 2 h before probing (*see Note 6*).

6. Secondary antibodies and detection reagents: Cy3-coupled anti-human IgG and Cy5-coupled anti-human IgM should be purchased commercially (*see Note 7*).

3 Methods

Manipulation of human sera samples must be carried out by trained personnel in safety conditions, both methodologically and in terms of using protective equipment. This includes wearing appropriate lab material such as lab coat, goggles, gloves, and a face mask, if necessary. Studies involving human samples have to be performed according to the rules for Good Clinical Practice, as described by the Committee for Proprietary Medical Products (CPMP) of the European Union and the International Committee on Harmonisation (ICH) in “Note for Guidance on Good Clinical Practice, document CPMP/ICH/135/95,” effective since January 17th 1997, Clinical Trial Directive 2001/20/EC, the World Medical Declaration of Helsinki and its amendments effective since 1964 and in accordance with the Medical Research Involving Human Subjects Act (WMO). Once the procedure has started, drying of the slide should be avoided at some points, so the change from one step to the next one must be done quickly. In order to control the batch effect, the same array should be processed for all the days of the experiment.

1. Print the affinity-purified recombinant proteins on glass slides in quintuplicate (6 ng/spot) with split pins (4×4 pin tool) using a robotic array spotter (Genomic Solutions, BioRobotics MicroGrid II 610, Huntingdon, UK) (*see Note 8*). Distribute the proteins into 384-well plates at 2 wells per sample and 30 μL per well. Each component is prepared at 250 $\mu\text{g}/\text{mL}$ in printing buffer onto Nexterion Slide H 3-D glass slides. As negative controls, we used 12 commercially available irrelevant (i.e., nonrelated to pneumococcus) proteins from different biological sources. The pins are dwelled into the sample wells and blotted 15 times before printing. The humidity level in the arraying chamber is maintained at 55–60% during printing. Each of the components is printed five times in a grid of 140 μm diameter spots with 175 μm pitch. Place the printed slides in a slide humidity chamber overnight at 75% relative humidity and stored at -20°C until use.

2. The previously printed arrays are taken from the store at -20°C and thawed at 4°C , allowing their equilibration for up to 30 min (*see Note 9*).
3. In order to block nonspecific interactions and to reduce the background, add 75 μL of blocking solution on the sides or corners of each well and shake gently (<100 rpm) on a circular shaker for 1 h to ensure that the entire surface of the array is blocked. After that time, discard the blocking solution.
4. Wash the samples by adding 75 μL of PBS-T to each well and incubate with shaking as in the previous step during 1 min (*see Note 10*). After that, remove the washing buffer and repeat the process twice with washing solution and one more with distilled H_2O . Then, the slides must be dried by centrifugation ($350 \times g$ for 15 min) and assembled on 16-well slide holders (Nexterion Slide H MPX 16).
5. Once serum samples are thawed on ice, dilute them appropriately in PBS-T in a total volume of 45 μL per well (*see Note 11*). As another negative control, arrays without serum, but incubated with PBS-T, should be included. Then, incubate the different samples (two hybridizations per serum are recommended) for 1 h protected from light at room temperature (*see Note 12*).
6. Add 100 μL of PBS-T, incubate for 2 min with shaking, and remove by decantation of the washing buffer. Repeat the wash three more times (*see Note 13*).
7. Add 45 μL of anti-human IgG-Cy3 or anti-human IgM-Cy5 conveniently diluted, cover tightly with a seal strip, and incubate for 1 h at room temperature protected from light (*see Note 14*).
8. Remove slides from holders, wash twice for 10 min in PBS-T and then once in PBS for 10 min, and finally centrifuge at $350 \times g$ for 15 min to dry the array surface (*see Note 15*).
9. Scan the slides within 24 h of probing by using a properly calibrated scanner to acquire the raw values of mean fluorescence intensity (MFI) for each array spot (*see Note 16*).
10. Process the acquired raw data. Initial data processing includes not only the local background subtraction but also a correction of the intensity obtained (i.e., by using the corrected median) (*see Note 17*).
11. Finally, analyze the data. Statistical analysis of the collected and processed data may be carried out using standard techniques for normalization, comparison, clustering, or diagnosis depending on the purpose of the study (*see Note 18*).

4 Notes

1. The protocol here described is based on our experience on printing recombinant pneumococcal proteins for probing with human sera. As a general advice for producing recombinant proteins from bacterial sources, it is more suitable to use a prokaryotic expression system such as *Escherichia coli*. Although production of recombinant proteins in a prokaryotic heterologous system does not allow to get the original post-translational modifications, there are other advantages over the use of eukaryotic expression systems: prokaryotic systems are cheaper, easier, and faster than the eukaryotic ones. In our case, we used the pSpark[®] I vector (Canvax Biotech, Córdoba, Spain), and expressed the cloned genes in *E. coli* BL21, as described [5, 14], according to manufacturers' instructions. One major drawback of these expression systems is the formation of inclusion bodies in which recombinant proteins need further steps for their purification. However, if the purpose of the study is to use surface bacterial antigens in serodiagnostics, we propose here some general tips to avoid this problem and to easily recover the recombinant proteins in their soluble form from the *E. coli* cytoplasm. These tips consist of removing from the cloning fragments all the regions coding for exporting signals (e.g., signal peptide I for secreted and cell wall-anchored proteins; signal peptide II for lipoproteins), as well as the transmembrane helix for membrane proteins with one single transmembrane domain (TMD). For multi-transmembrane proteins, it is recommended to select sufficiently large domains between two adjacent TMD that are exposed to the outer side of the cell. This can be either predicted using subcellular localization algorithms (e.g., TMHMM, PsortB) or experimentally aided: a useful strategy is the "shaving" approach followed by LC-MS/MS analysis to identify the most exposed and abundant surface proteins [15, 16]. Also, this information can be supported by the prediction of antigenicity of the produced recombinant polypeptides, by using algorithms such as VaxiJen.
2. At this point it is necessary to check whether the change of the buffer produces the precipitation of the protein or not. If this is the case, the buffer to be used must be optimized. Once the protein concentration is quantified, the purification efficiency for each protein should also be tested, e.g., by densitometry analysis of SDS-PAGE gel bands. Finally, protein concentration must be adjusted at the same value for all proteins in order to use the same for glass slide printing in 384-well plates.

3. It is recommended to print each protein in quintuplicate and in two different slides. Subsequently, it is usual to eliminate the outlier values, so having ten data for each protein will guarantee getting enough values to carry out a robust statistical analysis. For this, two hybridizations per serum should be carried out.
4. The protein concentration to be used is one of the parameters to be optimized, and it will depend on several factors. These must be empirically determined, such as the affinity of the serum antibodies for the protein, the titer of antibodies against that protein, or the type of antibodies to be tested (e.g., IgM, IgG).
5. To reduce variability, it is recommended to use a stock solution from which to make the working solution. Then, a 10× PBS and a 10% Tween-20 stock solution could be made to ensure the use of the same stock throughout the experiment.
6. The volume of the aliquots of sera should be that which allows the use of such sera with the minimum waste of biological material; therefore, it depends on the experimental design. Avoid multiple freezing/thawing of samples to prevent the loss of sera viability.
7. IgM (either as primary or as secondary antibodies) requires a lower dilution than IgG (i.e., 1/1000 for IgG vs. 1/200 for IgM). On the other hand, Cy dyes are sensitive to photodamage, so they should be prepared, stored, and used in the dark.
8. In our case, we used the robotic array spotter BioRobotics MicroGrid II 610 (Genomic Solutions, Huntingdon, UK).
9. Arrays should be kept sealed to prevent exposure to air or moisture until they reach the temperature at which they are defrosted. On the other hand, avoid touching the areas of the array with printed proteins, and transport them on ice.
10. This step is carried out in order to remove any remaining blocking solution.
11. In this step it is necessary to be especially careful with cross contamination, so it is necessary to change the pipette tip between each serum sample.
12. Serum is usually a very valuable resource, so it should be used as diluted as possible, despite ensuring that the signal obtained is within the optimal detection range of the device used. In optimizing this process, beyond the dilution of the serum, the density of the printed protein and the dilutions of the secondary detection antibodies must be considered. Usually, serum is diluted in a range comprising from 1:100 to 1:500, but previous tests are required to choose the most appropriate dilution taking the precaution that the dilution to be established will

not be optimal for all the sera, but it will be for the majority. Ideally, each array should be repeated with at least three different serum dilutions to check the linearity of the signal and choose one of the results that yield reliable values within the detection range, but this would increase the cost and time required for the whole analysis.

13. If this is done so carefully that the walls of the well are not touched, the same pipette tip can be used throughout this step. However, if the operator is not sure or confident enough, changing the pipette tip is recommended.
14. Because dyes are used to be photosensitive, this step must be done quickly. One way to do this faster is to be careful in not touching the walls of the wells to avoid cross contamination, which will allow the same pipette tip to be used throughout this step. If not, please read **Note 13**.
15. It is important to wash well any secondary detection antibody residues not bound to serum antibodies to decrease background noise from the assay. The indicated washes are usually enough, but if not, the number of washes can be increased, ensuring that the sample is protected from light.
16. Spot size should be defined for the whole experiment and non-saturated pixels should be obtained. In our pneumococcal protein array, we used a GenePix 4000B microarray scanner (Molecular Devices Corporation, Union City, CA) at photo-multiplier voltage settings so that no saturated pixels were obtained. Image analysis was carried out with GenePix Pro 4.1 analysis software (Molecular Devices Corporation). Spots were defined as circular features with maximum diameter of 140 μm .
17. Based on our own protein microarray, local background subtraction from ten surrounding spots is performed and corrected median fluorescence intensity is used for initial data processing. Then, the mean background signal of negative controls is subtracted from each raw spot value after sera hybridization. Negative controls represent hybridizations of negative control proteins and buffer spots with sera and secondary antibodies. After background subtraction, negative or zero values are assigned a net value of 0. Then, outlier values for each spot are removed. The two hybridizations for each serum are averaged to get the signal mean intensity (SMI) values, and the mean and standard deviation (SD) are obtained from the five printed spots per protein in each group (patient, control, etc.). Finally, data normalization by background was carried out using Microsoft Excel as described [17]. Absolute SMI values <500 must be discarded for further statistical analysis, to avoid measures close to the detection limit.

18. Normalization usually involves *Z*-score calculation. For comparison of two or more groups, the Mann–Whitney *U*-test and the Student's *t*-test are those most widely used to determine which antigens discriminate the studied groups. In our previous study, we applied the Benjamini-Hochberg (BH) correction to control the false discovery rate. Protein targets are considered as immunogenic candidates if antibody levels are significantly different between pneumococcal infected and control groups at a certain level (e.g., with at least a 1.5-fold difference in their SMI values, BH-adjusted *p* values <0.05). Hierarchical clustering is normally used to group sera samples and antigens into subsets, such that those within each cluster (subset) are more closely related to one another than samples assigned to other clusters. To use single antigens or combinations of them for diagnostic purposes, receiver operating characteristic (ROC) curve analysis is usually performed, which includes the calculation of sensitivity, specificity, and area under the curve (AUC).

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References

1. Vernet G, Saha S, Satzke C, Burgess DH, Alderson M, Maisonneuve JF, Beall BW, Steinhoff MC, Klugman KP (2011) Laboratory-based diagnosis of pneumococcal pneumonia: state of the art and unmet needs. *Clin Microbiol Infect* 17(Suppl 3):1–13. <https://doi.org/10.1111/j.1469-0691.2011.03496.x>
2. Sharon D, Snyder M (2014) Serum profiling using protein microarrays to identify disease related antigens. *Methods Mol Biol* 1176:169–178. https://doi.org/10.1007/978-1-4939-0992-6_14
3. Syu GD, Dunn J, Zhu H (2020) Developments and applications of functional protein microarrays. *Mol Cell Proteomics* 19(6):916–927. <https://doi.org/10.1074/mcp.R120.001936>
4. Jiménez-Munguía I, van Wamel WJ, Olaya-Abril A, García-Cabrera E, Rodríguez-Ortega MJ, Obando I (2015) Proteomics-driven design of a multiplex bead-based platform to assess natural IgG antibodies to pneumococcal protein antigens in children. *J Proteomics* 126:228–233. <https://doi.org/10.1016/j.jprot.2015.06.011>
5. Olaya-Abril A, Jiménez-Munguía I, Gómez-Gascón L, Obando I, Rodríguez-Ortega MJ (2015) A pneumococcal protein array as a platform to discover serodiagnostic antigens against infection. *Mol Cell Proteomics* 14(10):2591–2608. <https://doi.org/10.1074/mcp.M115.049544>
6. Jiménez-Munguía I, van Wamel WJB, Rodríguez-Ortega MJ, Obando I (2017) Detection of natural antibodies and serological diagnosis

- of pneumococcal pneumonia using a bead-based high-throughput assay. *Methods Mol Biol* 1643:169–177. https://doi.org/10.1007/978-1-4939-7180-0_13
7. Grandi G (2006) Genomics and proteomics in reverse vaccines. *Methods Biochem Anal* 49:379–393
 8. Navarre WW, Schneewind O (1999) Surface proteins of gram-positive bacteria and mechanisms of their targeting to the cell wall envelope. *Microbiol Mol Biol Rev* 63(1):174–229
 9. Olaya-Abril A, Jiménez-Munguía I, Gómez-Gascón L, Rodríguez-Ortega MJ (2014) Surfomics: shaving live organisms for a fast proteomic identification of surface proteins. *J Proteomics* 97:164–176. <https://doi.org/10.1016/j.jprot.2013.03.035>
 10. Zagursky RJ, Anderson AS (2008) Application of genomics in bacterial vaccine discovery: a decade in review. *Curr Opin Pharmacol* 8(5):632–638. <https://doi.org/10.1016/j.coph.2008.06.009>
 11. Wadia PP, Sahaf B, Miklos DB (2011) Recombinant antigen microarrays for serum/plasma antibody detection. *Methods Mol Biol* 723:81–104. https://doi.org/10.1007/978-1-61779-043-0_7
 12. DeLuca DS, Marina O, Ray S, Zhang GL, Wu CJ, Brusic V (2011) Data processing and analysis for protein microarrays. *Methods Mol Biol* 723:337–347. https://doi.org/10.1007/978-1-61779-043-0_21
 13. Sibani S, LaBaer J (2011) Immunoprofiling using NAPPA protein microarrays. *Methods Mol Biol* 723:149–161. https://doi.org/10.1007/978-1-61779-043-0_10
 14. Olaya-Abril A, Gómez-Gascón L, Jiménez-Munguía I, Obando I, Rodríguez-Ortega MJ (2012) Another turn of the screw in shaving gram-positive bacteria: optimization of proteomics surface protein identification in *Streptococcus pneumoniae*. *J Proteomics* 75(12):3733–3746. <https://doi.org/10.1016/j.jprot.2012.04.037>
 15. Rodríguez-Ortega MJ (2018) “Shaving” live bacterial cells with proteases for proteomic analysis of surface proteins. *Methods Mol Biol* 1722:21–29. https://doi.org/10.1007/978-1-4939-7553-2_2
 16. Rodríguez-Ortega MJ, Norais N, Bensi G, Liberatori S, Capo S, Mora M, Scarselli M, Doro F, Ferrari G, Garaguso I, Maggi T, Neumann A, Covre A, Telford JL, Grandi G (2006) Characterization and identification of vaccine candidate proteins through analysis of the group A *Streptococcus* surface proteome. *Nat Biotechnol* 24(2):191–197. <https://doi.org/10.1038/nbt1179>
 17. Huber W, von Heydebreck A, Sultmann H, Poustka A, Vingron M (2002) Variance stabilization applied to microarray data calibration and to the quantification of differential expression. *Bioinformatics* 18(Suppl 1):S96–S104



Affinity Proteomics Assays for Cardiovascular and Atherosclerotic Disease Biomarkers

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Abstract

Systematic exploration of the dynamic human plasma proteome enables the discovery of novel protein biomarkers. Using state-of-the-art technologies holds the promise to facilitate a better diagnosis and risk prediction of diseases. Cardiovascular disease (CVD) pathophysiology is characterized for unbalancing of processes such as vascular inflammation, endothelial dysfunction, or lipid profiles among others. Such processes have a direct impact on the dynamic and complex composition of blood and hence the plasma proteome. Therefore, the study of the plasma proteome comprises an excellent exploratory source of biomarker research particularly for CVD. We describe the protocol for performing the discovery of protein biomarker candidates using the suspension bead array technology. The process does not require depletion steps to remove abundant proteins and consumes only a few microliters of sample from the body fluid of interest. The approach is scalable to measure many analytes as well as large numbers of samples. Moreover, we describe a bead-assisted antibody-labeling process that helps to develop quantitative assays for validation purposes and facilitate the translation of the identified candidates into clinical studies.

Key words Affinity proteomics, Plasma proteome, Multiplexity, High-throughput, Antibody, Novel biomarkers

1 Introduction

Cardiovascular disease (CVD) remains one of the major causes of mortality and morbidity worldwide. CVD includes atherosclerotic diseases (e.g., myocardial infarction, stroke) and non-atherosclerotic diseases (e.g., venous thromboembolism, valvular disease) [1]. Despite all considerable progress over decades in the diagnosis, treatment, and prognosis of the disease, there is still a great interest in defining novel biomarkers for risk prediction and yet for diagnosis and prognosis of CVD.

Blood is a valuable body fluid for monitoring the physical conditions of an individual and analysis of blood markers can assist the physician in assessing the patient's status and enable personalized management [2]. In relation to cardiovascular

pathophysiology, blood has a direct contact with key disease locations such as the endothelium cell surface, and is the vehicle for, among others, complement system, coagulation factors, and immune cells which all play a role in CVD. Additionally, blood is the body fluid where cells and tissues actively or passively secrete proteins [2–4] and/or release them by ectodomain cell membrane shedding [5]. Blood samples are routinely separated into serum or plasma for standard analytical clinical applications. Serum is prepared from blood drawn without any anticoagulant and is depleted of blood cells and coagulation factors. Plasma is prepared from blood drawn with the presence of an anticoagulant (e.g., heparin, K2-EDTA, or NA-citrate) and depleted of blood cells while the remaining protein composition is basically unchanged [2, 6]. Therefore, plasma is the more relevant sample specimen for exploratory biomarker research particularly for CVD physiopathology where the hemostatic system plays an important part.

Affinity proteomics based on using antibodies as capture reagents has been successfully employed for the identification of novel biomarkers in body fluids in several conditions and diseases [7–10], including cardiovascular disease (CVD) [11, 12]. Suspension bead array (SBA) is one of the affinity-based proteomic technologies applied for large-scale sample profiling. This technology, based on color-coded magnetic microspheres coupled with antibodies (Fig. 1), allows for a high degree of multiplexity of target analytes and large sample throughput [13, 14]. Moreover, the method requires small volumes of the body fluid of interest and a minimal sample processing compared to other proteomic techniques, such as mass spectrometry (MS). No depletion of high-abundance proteins (i.e., albumin) is necessary.

The suspension bead array can be built based on two strategies. In an agnostic, or unbiased, discovery-driven strategy, the choice of affinity reagents to be included is based on predicted and/or verified specificity and functionality for detection of protein targets in plasma and without considering prior knowledge of protein function and possible involvement in the particular disease or condition studies. This strategy will necessarily involve a very large number of candidates, as exemplified in a study of patients with advanced atherosclerosis where plasma samples were initially screened with more than 10,000 antibodies from the Human Protein Atlas project [12]. Alternatively, in a target-driven strategy, an educated selection of candidates is made based on prior knowledge and hypotheses of roles in the disease or condition under study. This strategy allows for more defined yet comprehensive plasma protein profiling, exemplified in a case/control study including patients with venous thromboembolism (VTE), where plasma samples were screened with 768 antibodies directed against a list of candidates compiled from literature, previous proteomic and genetic studies, data of tissue-specific expression genes, and known or

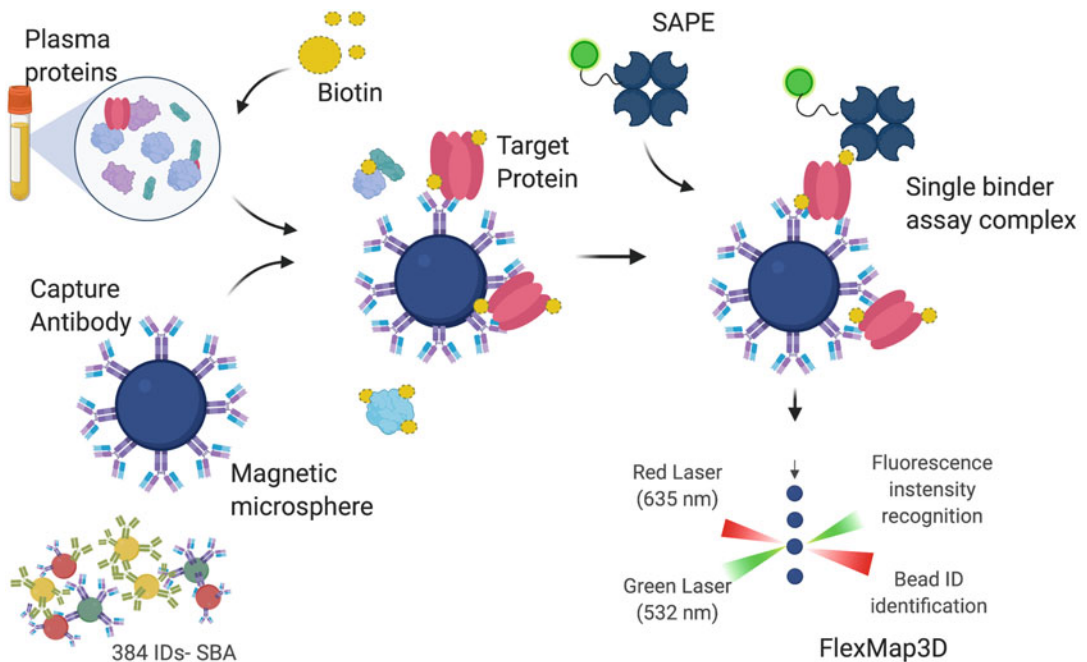


Fig. 1 Suspension bead array technology. Representation of the single-binder assay workflow for one ID bead. First, antibodies are covalently coupled onto the color-coded magnetic bead. In parallel, all plasma proteins are conjugated with biotin. Next, the biotin-labeled target protein is recognized by the antibody-bead complex. Finally, streptavidin-conjugated phycoerythrin specifically binds to the biotin. The beads are analyzed by a dual-laser system to identify (a) the internal color code of each bead (= antibody used) and (b) the fluorescence from the phycoerythrin as representation of the relative amount of protein captured by the antibody. (Created with [BioRender.com](https://www.biorender.com))

hypothesized involvement in pathways of relevance for VTE [11]. Both strategies were employed to successfully identify bio-marker candidates that could be validated in replication studies [11, 12]. However, the SBA technology platform has some limitations since antibodies have intrinsic characteristics in their affinity properties depending on the context they are used in. Hence, in a complex matrix such as plasma, antibodies may display a specificity different from that in other applications [15]. The categories for target binding are described in Table 1. Therefore, it can become necessary to confirm and/or identify the target specificity of antibodies not tested in the given sample and chosen technology. This will assist to deconvolute binding effects or factors influencing the binding, such as protein complex bound to the antibody target. Several approaches can be taken for validating antibody specificity [14, 16], and in our previous studies we have employed dual-binding assays that include using two different antibodies, acting as capture and detection for the intended target for validating our results [17] (*see* Chapter 5).

Table 1
Antibody selectivity classification in complex sample matrices

Category	Description
ON-target	The antibody binds to the intended protein target
CO-target	The antibody binds to the intended target and also enriches other proteins
OFF-target	The antibody does not enrich the intended target but binds to other proteins

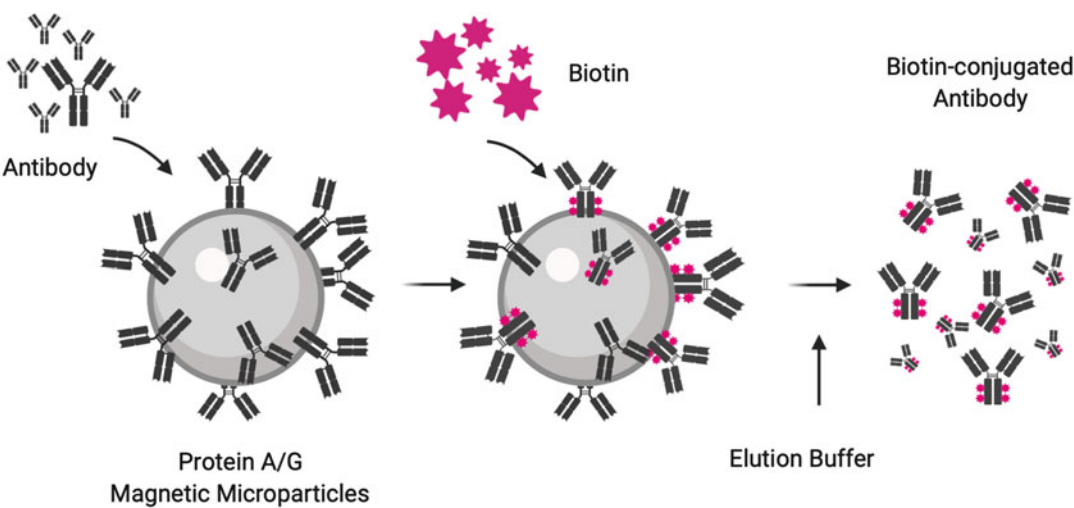


Fig. 2 Antibody biotinylation for the development of dual-binder sandwich assays. Sequential representation of the antibody biotinylation process from the starting step of antibody coupling to protein A beads, and subsequently addition of the biotin to the immobilized antibodies and the final elution of the biotinylated antibodies from the beads. (Adapted from Dezfouli M et al. [18]) [Image created with [BioRender.com](#)]

Eventually, there are no conjugated antibodies available that can be applied as detection antibodies, which was the case in one of our studies [12]. As an alternative, commercial kits for in-solution conjugating primary antibodies can be employed; however most of these require an initial large amount of antibody (e.g., >10µg). This can be solved by the application of the bead-assisted antibody labeling protocol described by Dezfouli et al. [18] (Fig. 2). The protocol allows the biotin conjugation of antibody reagents with low concentration of antibody, thereby enabling such reagents to be used as detection antibodies in dual-binding assays in the validation of results from the proteomic profiling.

In all, the protocol described in this chapter is divided into two sections. The first part of the protocol description is based on the profiling of CVD plasma samples as presented in our previous studies [11, 12] and adapted from Dobrin et al. [13]. As previously mentioned, the method is scalable in both dimensions, i.e., number of proteins and number of samples to be analyzed. To simplify the

protocol description, the following protocol part will describe the steps for a 96-well plate format as the minimum unit for both samples and antibody-bead suspension array. The protocol is subdivided into three main sections covering (a) plasma sample labeling with biotin, (b) covalent coupling of selected antibodies on magnetic microspheres in order to create the suspension bead array, and (c) affinity proteomics using the single-binder assay, by the combination of labeled samples and suspension bead array. The second part of the protocol section describes the antibody biotinylation process assisted by magnetic beads.

2 Materials

2.1 General Material and Equipment

1. PCR plates, 96-well non-skirted.
2. Flat-bottom clear polystyrene 96-well plate (alternatively 384-well plates).
3. Plate sealer [e.g., PlateLoc Thermal Microplate Sealer] (*see Note 1*).
4. Bench vortex.
5. Bench centrifuge with plate holders.
6. Bench plate shaker (e.g., PHMP-4 Thermoshaker) (*see Note 2*).
7. Standard manual or digital pipettes and disposable tips (1000 µL, 100 µL, 10 µL).
8. Standard polystyrene tubes (1.5 mL, 5 mL, 15 mL, 50 mL).
9. Liquid-handling robots (e.g., CyBio Selma; Freedom EVO[®] 150 platform).
10. Plate washer (e.g., EL406 Washer Dispenser).
11. Water bath.
12. Biosafety cabinet.
13. FlexMap3D (*see Note 3*).

2.2 Sample Labeling Reagents

1. Plasma samples (or other body fluid of interest).
2. Sample dilution buffer: Phosphate-buffered saline, pH 7.4 (1 × PBS), storage at room temperature (RT).
3. Labeling reagent: Lyophilized sulfo-*N*-hydroxysuccinimide polyethylene oxide biotin (EZ-Link[™] NHS-PEG4-Biotin) dissolved in sterile dimethyl sulfoxide (DMSO). Biotin is stored at −20 °C in the lyophilized form and stabilized at RT for 10 min prior to addition of DMSO to a final concentration of 10 mg/mL (*see Note 4*).
4. Labeling stop solution: 0.5 M Tris-HCl pH 8.0, prepared with Milli-Q-H₂O and stored at 4 °C.

2.3 Antibody-Bead Coupling Reagents

1. Selected antibodies: Preferably antibodies are formulated in BSA carrier-free and/or in amine-free buffers in order to avoid coupling competition (*see Note 5*).
2. MagPlex[®] microspheres: Magnetic carboxylated polystyrene microparticles containing internal dyes that generate specific and unique region identities (IDs). Stored at 4 °C and protected from light (*see Note 6*).
3. Activation buffer 1 (AB1): 0.1 M Monobasic sodium phosphate (NaH_2PO_4), pH 6.0–6.2, prepared in Milli-Q water, filtrated, and stored at 4 °C (*see Note 7*).
4. Activation buffer 2 (AB2): 50 mg/mL Sulfo-NHS (*N*-hydroxysulfosuccinimide) (NHS) and 50 mg/mL EDC (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride) (EDC) freshly prepared in activation buffer 1 prior to usage.
5. Antibody dilution buffer: 50 mM 2-(*N*-morpholino)-ethanesulfonic acid hydrate, pH 5.0 (MES), dissolved in Milli-Q water, filtrated, and stored at 4 °C (*see Note 7*).
6. Phosphate-buffered saline with 0.05% Tween[®] 20, pH 7.4 (PBS-T), stored at RT.
7. Bead-blocking reagent for ELISA (1 × BRE), stored at 4 °C (*see Notes 8 and 9*).

2.4 Labeling and Coupling Efficiency Testing Reagents

1. Assay buffer: 0.5% (w/v) Polyvinyl alcohol (PVA), 0.8% (w/v) polyvinylpyrrolidone (PVP) prepared in PBS, and 0.1% (v/v) casein, filtrated and stored at 4 °C (*see Notes 7, 8, and 10*).
2. PBS-T.
3. Labeling detection buffer: Streptavidin, R-phycoerythrin freshly diluted in PBS-T.
4. Coupling detection buffer: Anti-species (e.g., anti-rabbit, anti-mouse) antibody labeled with R-phycoerythrin diluted 1:2000 in PBS-T (*see Note 11*).

2.5 Single-Binder Assay Reagents

1. Biotinylated plasma samples (prepared as described in Subheading 3.1).
2. Suspension bead array (prepared as described in Subheading 3.2).
3. Assay buffer: 0.5% (w/v) Polyvinyl alcohol, 0.8% (w/v) polyvinylpyrrolidone prepared in PBS-T, and 0.1% (v/v) casein, filtrated and stored at 4 °C (*see Notes 7, 8, and 10*).
4. Washing buffer: PBS-T.
5. Cross-linking buffer: Paraformaldehyde 0.4% (v/v) dissolved in PBS, stored at 4 °C.
6. Detection buffer: Streptavidin-conjugated R-phycoerythrin (SAPE), diluted in PBS-T.

2.6 Antibody Labeling on Beads

1. Antibody: Preferably use antibodies formulated in a storage solution free of albumin and/or in buffers not containing amines.
2. Beads: Magnetic protein A or G. The selection of the beads will be guided by the commercial provider depending on the antibody species to be labeled.
3. Plates or tubes: The labeling process can be performed in 96-well flat-bottom surface plates or microcentrifuge 1.5 mL tubes.
4. Magnets: A magnetic separator suitable to the selected labeling support. A 96-well plate or standard microcentrifuge tube magnetic separator [e.g., DynaMag™-2 magnet].
5. Phosphate-buffered saline with 0.05% Tween® 20, pH 7.4 (PBS-T).
6. Labeling solution: Biotin (EZ-Link™ NHS-PEG4-Biotin) diluted in DMSO.
7. Elution buffer: Acetic acid 0.2 M, pH 3.2.
8. Neutralizing solution: Tris-HCl 0.5 M, pH 8.0.

3 Methods

3.1 Sample Labeling Protocol

Description: This protocol section describes an in-solution biotinylation process where biotin molecules are added to the primary amine residues and N-terminus present in all the plasma proteins. The protocol has the advantage of minimal processing of the biological sample with no requirement for depletion of the unbound biotin groups once the reaction has been quenched.

1. Thaw raw plasma samples on ice, vortex, and centrifuge at 4 °C ($2000 \times g$ for 5 min). Keep them on ice until transferred into PCR plates.
2. Distribute the plasma accordingly to the experimental layout in non-skirted PCR plates (volume per well: 50–100 µL). This step can be done manually by standard pipetting or by liquid-handling robots (e.g., Freedom EVO® or CyBio Selma). Each 96 well-plate layout shall contain a minimum of one blank well and three wells used for replication analysis. Therefore, there is up room for a maximum of 92 plasma samples randomly distributed in one 96-well plate (*see Note 12*).
3. Sample plates are sealed and centrifuged at $2000 \times g$ for 5 min at 4 °C.
4. In a new PCR plate, dispense 22 µL of PBS per well and transfer 3 µL of each plasma sample to the individual wells. Seal the sample plate, vortex, and spin down for 1 min at $1000 \times g$.

5. The labeling solution is prepared directly before its use by dissolving NHS-PEO4-Biotin in DMSO to 10 mg/mL and subsequently diluted to 3.5 mg/mL in a 15 mL tube with PBS. Add 5 μ L of the working labeling solution to each sample (see **Note 13**).

As described for plasma, working solution and buffers can be dispensed by standard pipette or by liquid-handling robot using 96-well head holders. Liquid-handling robots are preferred, having the advantage of liquid dispensing at one time point, preventing artifacts/errors from delayed periods of manual dispense.

6. Immediately after this, seal, vortex, and centrifuge the plate for 1 min at $1000 \times g$.
7. The labeling incubation step is carried out for 2 h at 4 °C, with one short break after 1-h incubation, where the sample plate is quickly vortexed, spun down, and placed again at 4 °C.
8. Quench the labeling process by adding 12.5 μ L of Tris-HCl (0.5 M, pH 8.0) to each well, followed by sealing, a quick vortex, and centrifugation for 1 min at $1000 \times g$.
9. Incubate the plate for 20 min at RT on a plate shaker and directly afterwards store the labeled samples at -20 °C until usage.

3.2 Antibody-Bead Coupling Protocol

Description: The protocol describes the steps for the generation of the suspension bead array, where antibodies are bound covalently onto magnetic microspheres. The suspension bead array offers large flexibility in composition due to the independent immobilization of antibodies on beads before pooled to generate the multiplex array. Within the composition of the SBA we include two negative controls. One corresponds to normal IgG couple to a specific bead identity. The IgG species will be defined by the origin species of the assay antibodies (e.g., rabbit, mouse, goat). The aim is to identify nonspecific binding of plasma human proteins to an antibody of nonhuman origin. A second negative control corresponds to non-coupled beads that undergo coupling process. The readout from this well will reflect the background level of the binding on the assay. Moreover, two positive control antibody reagents are included in the array composition, anti-human IgG and anti-human albumin. Both antibodies report high signal intensities as confirmation of the transfer of labeled plasma into the assay plate, the single binding assay.

All steps described in the protocol can be performed manually; however when generating larger antibody-bead arrays, it is highly recommended to facilitate the process by the use of automated or semiautomated liquid-handling robots (e.g., Freedom EVO[®] 150).

1. Dilute the antibodies in MES buffer by taking 1.76 μ g of each selected antibody into individual wells of a PCR plate to a final volume of 100 μ L.

2. Seal the antibody dilution plate, vortex, centrifuge for 1 min at $1000 \times g$, and store at 4°C (*see Note 14*).
3. Sequentially, aliquot each independent MagPlex bead region ($40\mu\text{L}$ containing approximately 5×10^5 beads) into individual wells of a half-area 96-well plate, following either a column or a row distribution manner (*see Note 15*).
4. Seal the plate with beads and centrifuge for 1 min at $1000 \times g$.
5. After removing the plate seal, place the plate on the magnet for 30 s to allow bead precipitation.
6. Wash the bead plate in the plate washer with the following protocol (*see Note 16*):
 - (a) Aspirate the bead storage buffer with the plate on the plate magnet.
 - (b) Dispense $80\mu\text{L}$ of AB1 buffer into the wells while on magnet.
 - (c) After 30 s, aspirate AB1 buffer.
7. Move the plate off the magnet and add $50\mu\text{L}$ of AB1 for bead resuspension.
8. Prepare fresh activation buffer (AB2) by dissolving EDC and NHS in AB1 buffer (for detailed description *see Note 17*). Add $50\mu\text{L}$ of AB2 buffer to each well, seal the plate, cover with an opaque lid, and vortex to bring the beads into a suspension.
9. Incubate the plate onto a bench plate shaker, at 650 rpm for 20 min at RT.
10. Immediately afterwards, centrifuge the plate for 1 min at $1000 \times g$ and wash the beads with two cycles of MES buffer as follows:
 - (a) Place the plate on the plate magnet for 30 s.
 - (b) Aspirate the AB2 buffer while on the magnet.
 - (c) Dispense $100\mu\text{L}$ of MES into each well while on the magnet.
 - (d) After 30 s on the magnet, aspirate the MES buffer.
 - (e) Repeat **steps 10c** and **10d** twice.
11. Take the plate off the magnet and add $100\mu\text{L}$ of each diluted antibody to the corresponding activated bead (ID). Resuspend the beads by pipetting up and down.
12. Seal the plate, cover with an opaque lid, vortex, and incubate on a shaker (650 rpm) at RT for 2 h.
13. Centrifuge the plate for 1 min at $1000 \times g$.
14. Remove unbound antibody by washing the beads with three cycles of PBS-T washes as follows:

- (a) Place the plate on the magnet for 30 s.
 - (b) Aspirate the antibody MES buffer while on the magnet.
 - (c) Dispense 100 μ L of PBS-T while on the magnet ($\times 3$).
 - (d) After 30 s on the magnet, aspirate the PBS-T buffer ($\times 3$).
15. Add 50 μ L of the blocking reagent to the beads. Seal the plate, vortex, and incubate for 20 min on a shaker at RT.
 16. Centrifugate the plate for 1 min at $1000 \times g$ and store the beads at 4 °C and light protected until usage (*see Note 18*).
 17. For the composition of the bead stock solution, an equal volume of the different beads is mixed together in a 5 mL tube and diluted with blocking buffer until a desired bead count is obtained. Our recommendation for an experiment is between 50 and 75 beads of each bead ID region per μ L of bead stock.

3.3 Labeling and Coupling Efficiency Assessment

Description: It is recommended to assess labeling and coupling efficiencies prior to the continuation with the experimental work. Coupling efficiency is defined by the binding of a secondary antibody that will recognize the capture antibodies immobilized onto beads. Labeling efficiency is assayed by the positive control beads included on the SBA (antibody-beads targeting human IgG and human albumin).

3.3.1 Antibody Coupling Efficiency

1. Distribute 5 μ L of SBA stock into 4 wells of a flat-bottom 96-well plate.
2. Dispense 50 μ L of secondary antibody (1:2000) diluted in PBS-T in three of the wells and 50 μ L PBS-T only into the last one.
3. Incubate for 30 min at RT at constant shaking.
4. Wash three times with PBS-T while on a magnet.
5. Add 80 μ L PBS-T and run the samples in the Luminex instrument. A readout signal of 8000–15,000 median of fluorescence intensity (MFI) is obtained for efficient couplings.

3.3.2 Sample Labeling Efficiency

1. Distribute 5 μ L of SBA stock into 4 wells of a flat-bottom 96-well plate (*see Note 19*).
2. In separate 1.5 mL tubes, prepare a 1:50 dilution in assay buffer of the selected labeled samples.
3. Add 45 μ L of diluted samples to the bead stock and incubate for 1 h with constant shaking at RT.
4. On a magnet, wash the beads three times with PBS-T.
5. Add 50 μ L of SAPE (1:750) diluted with PBS-T and incubate for 30 min, at RT and with continuous shaking.

6. Repeat **step 4**.
7. Add 80 μ L PBS-T and run the samples in the Luminex instrument. Indicative readout values for adequate labeling efficiency are as follows: anti-human albumin: 13,000–17,000 median of fluorescence intensity (MFI) and anti-human IgG: 18,000–23,000 MFI (*see Note 20*).

3.4 Single-Binder Assay Protocol

Description: The protocol presented below describes the steps for analyzing the plasma samples with the selected antibodies in the single-binder assay. This assay relies on the ability of the antibodies (immobilized onto the beads) to capture the intended protein within a complex context of a large number of proteins and dynamic protein concentration range. In brief, the labeled samples are mixed with the suspension bead array to allow the specific antibody bindings to occur, followed by removal of non-stable binding and unbound proteins with buffer exchange washes. Only proteins stably bound to the antibodies will be detected after the addition of the signal amplifier (SAPE). The resultant MFI represents the relative amount of a given protein in the analyzed samples.

1. Thaw the labeled plasma samples on ice.
2. Dispense 50 μ L of assay buffer supplemented with 10% (v/v) of rabbit IgG into a PCR plate (*see Note 21*).
3. Transfer 1 μ L of labeled sample into the assay plate, preferably using a liquid handler (e.g., Cytbio SELMA) (*see Note 22*).
4. Seal the plate, vortex, and centrifuge for 1 min at $1000 \times g$.
5. Diluted samples are heat treated for 30 min at 56 °C in order to enable epitope retrieval (*see Note 23*).
6. Prepare a 96-well half-area flat-bottom plate with 5 μ L of the beads in each well. For larger number of samples, a 384-well plate format can be used. Protect from light with an opaque cover lid until the transfer of the heat-treated samples is done.
7. Let the samples stabilize for 10 min at RT. Thereafter, vortex and centrifuge the plate for 1 min at $1000 \times g$.
8. Transfer 45 μ L of the heat-treated diluted samples into the bead plate. Seal the plate, cover with a lid, and vortex thoroughly to resuspend the beads. Incubate the mix overnight in a plate shaker (650 rpm) at RT while light protected.
9. Centrifugate the plate for 1 min at $1000 \times g$ and wash away unbound proteins with three cycles of PBS-T wash. Volume per wash will differ if using 96-well plates (100 μ L) or 384-well plate (60 μ L).
 - (a) Unseal and place the plate on the magnet for 30 s.
 - (b) Aspirate the buffer while on magnet.

- (c) Dispense PBS-T on magnet ($\times 3$).
- (d) After 30 s on the magnet, aspirate the PBS-T buffer ($\times 3$).
- 10. Add to each well 50 μ L of the cross-linking reagent 0.4% (v/v) PFA dissolved in PBS. Seal the plate and incubate for 10 min while gently shaking at RT and protected from light. PFA will help to stabilize the antibody-protein complex.
- 11. Immediate thereafter, wash the bead plate as described in **step 9**.
- 12. Dispense 50 μ L of freshly prepared solution of SAPE (streptavidin-R-phycoerythrin conjugated) diluted to 1:750 in PBS-T.
- 13. Seal, vortex, and incubate the plate for 30 min.
- 14. Thereafter, wash the plate three times with PBS-T as described in **step 9**.
- 15. Finally, while off the magnet, resuspend the beads in PBS-T prior to obtaining the plate readout in the selected Luminex instrument. The specific volume of PBS-T will be determined by the user preferences within the instrument recommendation, which varies from 80 to 100 μ L in a 96-well plate, or 50 to 60 μ L if using a 384-well plate format.

The readout resulting from the run will report a fluorescence signal per each of the bead ID. The signal, expressed as median of fluorescence intensity (MFI), represents the relative amount of each specific protein bound to the corresponding antibody.

3.5 Bead-Assisted Antibody Labeling (Detection Antibody Labeling)

Description: The protocol describes the steps for biotin labeling of antibodies with the assistance of magnetic bead that helps to perform the purification steps without a reduction of the starting amount of the antibody of interest.

1. Gently resuspend the bead stock and pipette 5 μ L of beads into a half-bottom 96-well plate (Dynabeads Protein A or G, 30 mg/mL) and add 25 μ L PBS-T to resuspend the beads.
2. Place the plate in the magnet separator and let the beads stand for 1 min.
3. Wash the beads two times with 100 μ L PBS-T. In the final step remove all the supernatant.
4. While off magnet, add the antibody to the bead pellet, previously diluted in PBS-T (1 μ g antibody + 30 μ L PBS-T) (*see Note 24*), and resuspend the beads by pipetting up and down.
5. Seal the plate, vortex, and incubate for 30 min at RT. Keep beads in suspension with gentle shaking (650 rpm) on a bench plate shaker.
6. Bring the biotin vial to RT for 10 min. Resuspend the biotin in DMSO to a final concentration of 0.5 mg/mL.
7. Spin down the plate and remove the sealer cover.

8. Place the plate on the magnet standing for 1 min, gently aspirate the supernatant, and keep it in a microcentrifuge tube for testing the efficiency of antibody coupling to the Dynabeads. Add 100μL of PBS-T to the beads.
9. Determine the concentration of antibody in the supernatant on the Nanodrop instrument and calculate the efficiency by the following formula:

$$\%ab \text{ binding on beads} = \left(\frac{\text{Initial ab concentration} - \text{supernatant ab concentration}}{\text{Initial ab concentration}} \right) \times 100$$

10. Repeat the bead wash two times with 100μL PBS-T, discarding the supernatant.
11. Add 2.2μL of the labeling reagent (0.5 mg/mL biotin) into the well on the opposite side of the well to the beads. Immediately add 60μL of PBS-T off magnet and pipette up and down in order to homogenize the biotin solution and beads.
12. Seal and vortex the plate and incubate for 30 min at RT. Keep beads in suspension with constant and gentle shaking (650 rpm) (alternatively, the incubation can be done for 2 h at 4 °C while shaking).
13. Spin down the plate, remove the sealed cover, and gently wash the beads three times with 100μL PBS-T. Remove any remaining wash buffer before adding the elution buffer.
14. Add 12μL elution buffer (acetic acid 0.2 M, pH 3.2).
15. Incubate for 2 min with gentle vortex (alternatively by gently pipetting up and down).
16. Place the plate on a magnet and collect the eluted labeled antibody by pipetting, avoiding any disruption or carryover of the bead pellet. Transfer the elute into a microcentrifuge tube.
17. Add 5μL of the neutralization solution (Tris-HCl 0.5 M, pH 8.0) to the antibody-labeled eluate.
18. Add 3μL PBS-T to the labeled antibody. The final concentration for the labeled antibody is about ≈0.45μg/μL.
19. Store the labeled antibody at 4 °C until usage.

We have observed different degrees of biotinylation using same conditions, indicating variability due to the intrinsic properties of each antibody. For this reason, we strongly recommend to assess the labeling efficiency prior to the experimental application of each antibody. The following steps are tailored to using the Luminex technology.

1. Dispense 50μL of the labeled antibody (diluted 1:50) into a well of a half-bottom plate.
2. Add 5μL of beads coupled with anti-species IgG (*see Note 25*).

3. Incubate for 30 min at RT with constant and gentle shaking.
4. Wash the beads on a magnet with three cycles of 100 μ L PBS-T.
5. Incubate the beads with R-phycoerythrin-labeled streptavidin (1:750) for 30 min.
6. Repeat the wash cycles with 100 μ L PBS-T.
7. Add 80 μ L PBS-T and run in the Luminex instrument (*see Note 26*).

4 Notes

1. The selection of a thermal sealer is preferable in order to avoid contamination of samples and/or buffers with the glue component present on plastic adhesive films.
2. A plate shaker having an opaque cover is recommended for all incubations due to light-sensitive properties of the Luminex beads. Alternatively a cover lid can be used to light protect the plate.
3. FlexMap3D instruments cover up to 500 ID bead identities. For suspension bead arrays containing low number of bead IDs, the process can be carried out in other available MAP instrument models (Lx200 (up to $n = 100$ compatible IDs) or MAGPIX (IDs up to $n = 50$)).
4. Biotin should be prepared fresh prior to usage and not be kept as frozen stock.
5. To increase the efficiency of the coupling process, antibodies containing carrier proteins (e.g., BSA) or dissolved in Tris or glycine buffer (amine buffers) should be avoided. These elements will interfere in the coupling process diminishing the amount of antibody coupled to beads.
6. MagPlex[®] beads are internally labelled with an organic solution containing two fluorescent dyes. Large exposure to light might cause photobleaching of the internal dyes. It is recommended to protect the beads from light during all incubations.
7. AB1, MES, and assay buffer can be stored as 1 $\times$ frozen stocks ($-20\text{ }^{\circ}\text{C}$) for up to 3 months.
8. Supplement the buffer with 0.1% (v/v) of a preserving biocide (e.g., ProClin) for a longer storage at $4\text{ }^{\circ}\text{C}$.
9. Stock aliquots of BRE buffer (10 $\times$) are recommended to be prepared and stored at $-20\text{ }^{\circ}\text{C}$ for up to 3 months.
10. PVA and PVP are difficult components to dissolve in room-temperature solution; for facilitating the process, autoclave the solution at $121\text{ }^{\circ}\text{C}$ for 20 min. When the solution is back at RT, add the casein to the buffer.

11. Alternative secondary antibody-labeled dyes can be applied (e.g., Alexa Fluor[®] 555, 546, 532); however the usage of RPE antibodies in our Luminex setup offers a better performance in signal intensities.
12. Replication samples can be prepared by mixing few microliters of plasma samples from the cohort/sample set to be analyzed. Alternatively, one selected sample for which a large volume is available can also be used for replication purpose.
13. NHS-ester moiety ligated to the biotin has a quick hydrolyzation rate; therefore the labeling solution should be used directly after reconstitution and the process shall not be delayed.
14. Diluted antibodies can be stored at 4 °C for a maximum period of 24 h.
15. Antibody and bead ID plate layout should be coordinated for a correct identification of the unique antibody-bead complex. This is especially relevant while generating large antibody-bead arrays.
16. Manual washes are optional by using a magnetic plate holder (e.g., LifeSep magnetic separator unit). Buffer removal is achieved by plate inversion while both plate and magnetic adaptor are held tightly together.
17. Allow EDC and NHS reagents to equilibrate at RT for at least 30 min. Dissolve EDC and NHS independently to 50 mg/mL in AB1 buffer. Quickly mix 1:1 parts EDC and NHS into 1.5 times (EDC + NHS) AB1 buffer (e.g., 1 mL EDC + 1 mL NHS + 3 mL AB1) and apply to the beads. EDC and NHS are very susceptible to hydrolysis after being reconstituted; therefore for an efficiency coupling the process should not be interrupted at this point.
18. Blocking reagent can be renovated after overnight incubation in order to obtain lower background assay levels.
19. The number of samples selected for this step depends on the user. A minimum of 4 repeated sample wells per each 96-well plate is recommended.
20. Indicative values are based on our experimental data. Anti-human IgG levels are usually high and close to saturation detection limit.
21. Normal rabbit IgG added to the assay buffer helps to reduce unspecific protein binding from human plasma protein to the coupled antibodies, originated from immunized rabbit. When the affinity reagents used in the SBA have a different origin, the assay buffer should be supplemented with IgG from the species of interest.

22. The sample final dilution from original raw to experimental plasma is 1:500. This dilution covers a dynamic range from albumin to cytokines (e.g., IL6). In order to study abundant proteins larger dilution should be applied and optimized.
23. Human protein atlas antibodies have been generated by rabbit immunization of protein linear fragment (recombinant human Protein Epitope Signature Tags, PrEST); therefore a heat treatment step has been introduced in the protocol in order to facilitate the unfolding of protein and therefore the antibody recognition. However, this step can be omitted while using antibodies targeting conformational epitopes.
24. The antibody amount per reaction can be scaled up to 3µg in 100µL. In case of larger amount needed, multiple wells for the same antibodies will be prepared.
25. Beads coupled with anti-rabbit IgG (or any other species) are prepared as described in Subheading 3.2 (antibody-bead coupling protocol).
26. Indicative signal for antibody biotinylation varies from 5000 to 8000 MFI.

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References

1. Lyngbakken MN, Myhre PL, Røsjø H, Omland T (2019) Novel biomarkers of cardiovascular disease: applications in clinical practice. *Crit Rev Clin Lab Sci* 56(1):33–60. <https://doi.org/10.1080/10408363.2018.1525335>
2. Issaq HJ, Xiao Z, Ventra TD (2007) Serum and plasma proteomics. *Chem Rev* 107(8):3601–3620. <https://doi.org/10.1021/cr068287r>
3. Uhlén M, Karlsson MJ, Hober A, Svensson AS, Scheffel J, Kotol D, Zhong W, Tebani A, Strandberg L, Edfors F, Sjöstedt E, Mulder J, Mardinoglu A, Berling A, Ekblad S, Dannemeyer M, Kanje S, Rockberg J, Lundqvist M, Malm M, Volk AL, Nilsson P, Månberg A, Dodig-Crnkovic T, Pin E, Zwahlen M, Oksvold P, von Feilitzen K, Häussler RS, Hong MG, Lindskog C, Ponten F, Katona B, Vuu J, Lindström E, Nielsen J, Robinson J, Ayoglu B, Mahdessian D, Sullivan D, Thul P, Danielsson F, Stadler C, Lundberg E, Bergström G, Gummesson A, Voldborg BG, Tegel H, Hober S, Forsström B, Schwenk JM, Fagerberg L, Sivertsson Å (2019) The human secretome. *Sci Signal* 12(609):eaaz0274. <https://doi.org/10.1126/scisignal.aaz0274>

4. Ignjatovic V, Geyer PE, Palaniappan KK, Chaaban JE, Omenn GS, Baker MS, Deutsch EW, Schwenk JM (2019) Mass spectrometry-based plasma proteomics: considerations from sample collection to achieving translational data. *J Proteome Res* 18(12):4085–4097. <https://doi.org/10.1021/acs.jproteome.9b00503>
5. Hayashida K, Bartlett AH, Chen Y, Park PW (2010) Molecular and cellular mechanisms of ectodomain shedding. *Anat Rec* 293(6):925–937. <https://doi.org/10.1002/ar.20757>
6. Bowen RA, Remaley AT (2014) Interferences from blood collection tube components on clinical chemistry assays. *Biochem Med* 24(1):31–44. <https://doi.org/10.11613/BM.2014.006>
7. Byström S, Eklund M, Hong MG, Fredolini C, Eriksson M, Czene K, Hall P, Schwenk JM, Gabrielson M (2018) Affinity proteomic profiling of plasma for proteins associated to area-based mammographic breast density. *Breast Cancer Res* 20(1):14. <https://doi.org/10.1186/s13058-018-0940-z>
8. Lourido L, Ayoglu B, Fernández-Tajes J, Oreiro N, Henjes F, Hellström C, Schwenk JM, Ruiz-Romero C, Nilsson P, Blanco FJ (2017) Discovery of circulating proteins associated to knee radiographic osteoarthritis. *Sci Rep* 7(1):137. <https://doi.org/10.1038/s41598-017-00195-8>
9. Drobin K, Marczyk M, Halle M, Danielsson D, Papiez A, Sangsuwan T, Bendes A, Hong MG, Qundos U, Harms-Ringdahl M, Wersäll P, Polanska J, Schwenk JM, Haghdoust S (2020) Molecular profiling for predictors of radiosensitivity in patients with breast or head-and-neck cancer. *Cancers* 12(3):753. <https://doi.org/10.3390/cancers12030753>
10. Häggmark A, Hamsten C, Wiklundh E, Lindskog C, Mattsson C, Andersson E, Lundberg IE, Grönlund H, Schwenk JM, Eklund A, Grunewald J, Nilsson P (2015) Proteomic profiling reveals autoimmune targets in sarcoidosis. *Am J Respir Crit Care Med* 191(5):574–583. <https://doi.org/10.1164/rccm.201407-1341OC>
11. Bruzelius M, Iglesias MJ, Hong MG, Sanchez-Rivera L, Gyorgy B, Souto JC, Frånberg M, Fredolini C, Strawbridge RJ, Holmström M, Hamsten A, Uhlén M, Silveira A, Soria JM, Smadja DM, Butler LM, Schwenk JM, Morange PE, Trégouët DA, Odeberg J (2016) PDGFB, a new candidate plasma biomarker for venous thromboembolism: results from the VEREMA affinity proteomics study. *Blood* 128(23):e59–e66. <https://doi.org/10.1182/blood-2016-05-711846>
12. Matic LP, Jesus Iglesias M, Vesterlund M, Lengquist M, Hong MG, Saiced S, Sanchez-Rivera L, Berg M, Razuvaev A, Kronqvist M, Lund K, Caidahl K, Gillgren P, Pontén F, Uhlén M, Schwenk JM, Hansson GK, Paulsson-Berne G, Fagman E, Roy J, Hultgren R, Bergström G, Lehtiö J, Odeberg J, Hedin U (2018) Novel multiomics profiling of human carotid atherosclerotic plaques and plasma reveals biliverdin reductase B as a marker of intraplaque hemorrhage. *JACC Basic Transl Sci* 3(4):464–480. <https://doi.org/10.1016/j.jacbts.2018.04.001>
13. Drobin K, Nilsson P, Schwenk JM (2013) Highly multiplexed antibody suspension bead arrays for plasma protein profiling. *Methods Mol Biol* 1023:137–145. https://doi.org/10.1007/978-1-4614-7209-4_8
14. Schwenk JM, Nilsson P (2011) Assessment of antibody specificity using suspension bead arrays. *Methods Mol Biol* 785:183–189. https://doi.org/10.1007/978-1-61779-286-1_12
15. Fredolini C, Byström S, Sanchez-Rivera L, Ioannou M, Tamburro D, Pontén F, Branca RM, Nilsson P, Lehtiö J, Schwenk JM (2019) Systematic assessment of antibody selectivity in plasma based on a resource of enrichment profiles. *Sci Rep* 9(1):8324. <https://doi.org/10.1038/s41598-019-43552-5>
16. Edfors F, Hober A, Linderbäck K, Maddalo G, Azimi A, Sivertsson Å, Tegel H, Hober S, Szigyarto CA, Fagerberg L, von Feilitzen K, Oksvold P, Lindskog C, Forsström B, Uhlen M (2018) Enhanced validation of antibodies for research applications. *Nat Commun* 9(1):4130. <https://doi.org/10.1038/s41467-018-06642-y>
17. Häussler RS, Bendes A, Iglesias M, Sanchez-Rivera L, Dodig-Crnković T, Byström S, Fredolini C, Birgersson E, Dale M, Edfors F, Fagerberg L, Rockberg J, Tegel H, Uhlén M, Qundos U, Schwenk JM (2019) Systematic development of sandwich immunoassays for the plasma secretome. *Proteomics* 19(15):e1900008. <https://doi.org/10.1002/pmic.201900008>
18. Dezfouli M, Vickovic S, Iglesias MJ, Nilsson P, Schwenk JM, Ahmadian A (2014) Magnetic bead assisted labeling of antibodies at nanogram scale. *Proteomics* 14(1):14–18. <https://doi.org/10.1002/pmic.201300283>



Nucleic Acid Programmable Protein Arrays (NAPPA) for the Discovery of Autoantibodies in Osteoarthritis

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Abstract

Since a decade, the nucleic acid protein programmable array (NAPPA) technology has provided researchers with a high-throughput proteomic technique for deciphering immune signatures and screening for biomarkers, among other applications. In osteoarthritis (OA), the activation of proinflammatory pathways of innate immunity due to cellular stress response and cartilage degradation is now considered to be one of the pathophysiological drivers of the disease, perpetuating the catabolic process and the inflammation of the joint. Therefore, the identification of immunosignatures in OA would allow to detect autoantibodies (AABs) as a new source of disease-specific biomarkers. Herein, we describe a proteomic strategy combining NAPPA and in situ protein expression coupled with enzyme-linked immunoassays (ELISA) to search for novel serum AABs in OA.

Key words NAPPA, Biomarker discovery, Autoantibodies, Serum, Osteoarthritis

1 Introduction

The nucleic acid protein programmable array (NAPPA) technology offers a high-throughput miniaturized small-volume detection platform in which more than 16,000 proteins are in situ displayed on a single microscope slide [1]. NAPPA is based on immobilizing expression vectors encoding the desired proteins, which are fused with a common epitope tag in the C-termini. This tag facilitates the in situ and just-in-time protein capture by an anti-tag antibody that is printed simultaneously with the expression vector. To date, this technology has proved successful in identifying autoantibodies (AABs) as novel candidate biomarkers in several diseases, using different body fluids and avoiding sample pre-fractionation [2–7].

Since the presence of immune cells in the synovial membrane was considered to be a common finding in osteoarthritis (OA) patients, there is an increasing belief that fragments released

1- High-throughput screening on NAPPA

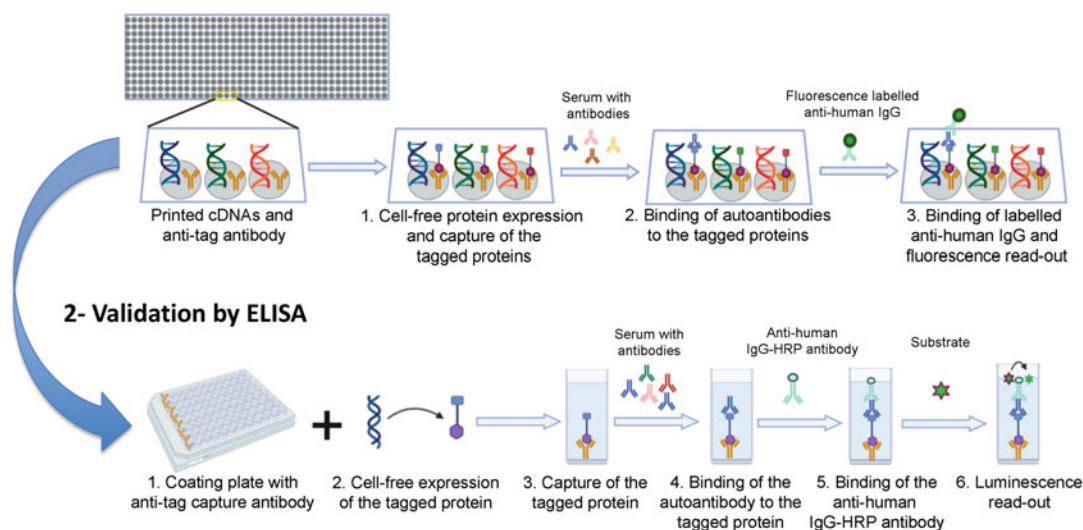


Fig. 1 Overview of the workflow for the discovery of serum autoantibodies in osteoarthritis by NAPPA and subsequent validation by ELISA

from cartilage breakdown could act as antigens and activate the humoral response, which usually involves the production of AAbs. These AAbs may be detected at asymptomatic stages of OA [8] and therefore be used to identify susceptible individuals and facilitate the disease diagnosis and management. With this aim, we describe a proteomic strategy that involves NAPPA combined with in situ protein expression coupled with enzyme-linked immunoassays (ELISA) as an approach to screen and validate serum AAbs in OA (Fig. 1) [8, 9]. In the ELISA procedure, the individual proteins coupled with the coated plates are synthesized using the same in situ expression system applied on the NAPPA screening.

2 Materials

2.1 Sample Preparation

1. Assay buffer: 5% (w/v) Fat-free milk powder prepared in 0.02% (v/v) Tween 20 in 1 × PBS pH 7.4 (PBST).

2.2 Serum Autoantibody Profiling on NAPPA

1. NAPPA arrays (*see Note 1*).
2. SuperBlock (1 ×): SuperBlock™ (TBS) Blocking Buffer (Thermo Fisher Scientific).
3. Milli-Q water.
4. Pipette tip box (*see Note 2*).
5. Plate shaker.

6. Nuclease-free tips.
7. Nuclease-free sterile tubes.
8. HybriWell gasket (Grace Bio-Labs, HBW2160).
9. Expression system: 1-Step Human Coupled IVT kit (Thermo Fisher Scientific) (*see* **Note 3**).
10. Bioassay dish with dividers (Genetix).
11. Programmable incubator (*see* **Note 4**).
12. Wash buffer: 0.02% (v/v) Tween 20 in 1× PBS pH 7.4 (PBST).
13. Blocking buffer (1×): 5% (w/v) Fat-free milk powder prepared in PBST.
14. Corning Hybridization Chamber (Sigma).
15. Rotator.
16. Conjugated detection antibody: Direct labeled Alexa Fluor 647 goat anti-human IgG (Thermo Fisher Scientific).
17. Deionized water.
18. Filtered compressed air (*see* **Note 5**).

2.3 Serum Autoantibody Profiling on ELISA

1. Clear flat-bottom 96-well plates.
2. Nuclease-free sterile tubes.
3. Nuclease-free tips.
4. Coating antibody: Polyclonal anti-GST antibody (GE Healthcare).
5. Coating buffer: 0.05 M Sodium carbonate, 0.05 M sodium bicarbonate, pH 9.6, 0.2 µm filtered.
6. cDNA expression plasmids (*see* **Note 6**).
7. Expression system: 1-Step Human Coupled IVT kit (Thermo Fisher Scientific).
8. Blocking and assay buffer (1×): 5% (w/v) Fat-free milk powder prepared in 0.02% (v/v) Tween 20 in 1× PBS pH 7.4 (PBST).
9. Wash buffer: 1× PBS pH 7.4.
10. Conjugated detection antibody: Horseradish peroxidase (HRP)-linked anti-human IgG (Jackson ImmunoResearch Laboratories).
11. Substrate for horseradish peroxidase (HRP): 3,3',5,5'-Tetramethylbenzidine (TMB Pierce™).
12. Stop solution: 2 M Sulfuric acid.
13. Plate shaker.
14. Thermoblock.

2.4 Data Analysis

1. Tecan Powerscanner™ (Tecan Group LTD) to scan NAPPAs arrays.
2. Absorbance plate reader for ELISA readout.

3 Methods

Herein, we describe a two-phase strategy for the massive screening of novel AAbs on serum sample pools using NAPPAs arrays and the subsequent validation of the potential autoantibody candidates in individual samples by ELISA. The number of sample pools to be analyzed on NAPPAs will correspond to each of the different sample groups under study. The potential hits distinguishing among sample groups resulting from NAPPAs screening will be then expressed in situ and immobilized on 96-well plates in order to analyze individual samples by the ELISA method. This strategy is optimized to detect immunoglobulin (Ig) G in OA serum samples, but other Ig classes could also be recognized by changing the detection antibody and probably adapting some experimental conditions such as serum sample dilutions to achieve a desired assay performance [10–12].

3.1 Protein Display and Serum Autoantibody Profiling on NAPPAs

1. Thaw serum samples on ice or overnight at 4 °C.
2. Vortex and quickly spin down the serum samples at 500 × *g*.
3. Prepare sample pools in a tube by mixing equal volumes of the corresponding sera (*see Note 7*).
4. Dilute each pool to 1:20 in assay buffer to a final volume of 2 mL and keep on ice until use (*see Note 8*).
5. Incubate the NAPPAs arrays with 30 mL of SuperBlock in a pipette tip box with divider for 1 h by gentle shaking (~150 rpm) at RT (*see Note 9*).
6. Preheat the incubator at 30 °C.
7. Thaw all provided reagents from the 1-Step Human Coupled IVT kit to prepare the human HeLa cell lysate-based IVT expression system on ice before its use (*see Note 10*).
8. Prepare the IVT by gently mixing 48 µL of HeLa lysate, 16 µL of accessory proteins, 32 µL of reaction mix, and 64 µL of nuclease-free water in the order listed and saved on ice until use (*see Note 11*).
9. After blocking, gently rinse the array for 2 min with Milli-Q water and dry by filtered compressed air.
10. Using sterile forceps, peel off the printed liner, and carefully place the HybriWell chamber to the printed side of the array, aligning the edges of the array with the edges of the HybriWell chamber, so that the printed features are centered (*see Note 12*).

11. Use a wooden stick to rub along the chamber to make sure that it is well sealed everywhere.
12. Gently pipet and inject 150 μ L of the IVT through one port on the HybriWell while allowing air to escape through the other port (*see Note 13*).
13. Remove all possible bubbles using a wooden stick to push out the air from the ports.
14. To seal the ports, first wipe away excess reagent on the surface of the chamber using a tissue. Use sterile forceps to remove a seal tab from the liner strip. Place a seal tab over each filling port. Press on both seals simultaneously, using finger pressure, to assure a secure seal.
15. Place the array on a bioassay dish or pipette tip box with divider and cover with Milli-Q water to preserve humidity.
16. Incubate at 30 °C for 1.5 h for protein expression followed by 30 min at 15 °C for protein capture in the programmable incubator.
17. Remove the HybriWell cover by grasping the tab end along the top edge of the HybriWell seal and peel it away from the array.
18. Rinse the array twice with wash buffer and once in blocking buffer, for 5 min each in a plate shaker (~150 rpm).
19. Block the array with blocking buffer in a plate shaker (~150 rpm) at RT for 30 min (*see Note 14*).
20. Vortex and quickly spin down the sample.
21. Remove the excess of blocking buffer by gently tapping the edge of the array onto a paper towel.
22. Place the array face up in a Corning hybridization chamber and add the 2 mL of serum sample pool.
23. Once the array is placed in the Corning chamber, place the top of the chamber directly over the gasket, and press and hold firmly while clipping the gasket together with the black clips.
24. Incubate the sample overnight at 4 °C.
25. Prepare 2 mL of direct labeled Alexa Fluor 647 goat anti-mouse IgG antibody diluted to 1:500 in blocking buffer and keep on ice until use.
26. Place out the array on a pipette tip box with divider and wash with blocking buffer, three times on a rocking shaker (~150 rpm), 5 min each.
27. Place the slides in the Corning chamber and add the 2 mL Alexa Fluor 647-labeled goat anti-mouse IgG antibody prepared before. Close the chamber and incubate for 1 h at RT on a rotator.

28. Place out the array on a pipette tip box with divider and wash three times with wash buffer, 5 min each in a plate shaker (~150 rpm).
29. Rinse thoroughly with deionized water and dry with compressed air.
30. Scan the array with the following scan settings: resolution 10, channel 2/647 nm, gain 50%, and intensity 50%.
31. Calculate the cutoff level by median intensity absolute deviation rule from all the spots through all the serum pools.

3.2 Coating Plates for ELISA

1. Prepare the coating solution by diluting anti-GST antibody in 0.05 M sodium carbonate/sodium bicarbonate buffer (pH 9.6) to a final concentration of 4 µg/mL into a volume enough for an extra 10% in terms of number of assay wells.
2. Add 100 µL of coating solution to each well and incubate at 4 °C overnight without shaking and protected from light.
3. Remove the excess of the antibody solution by turning the plate over a paper tower (*see Note 15*).
4. Block wells' surface with 200 µL of blocking buffer during overnight incubation at 4 °C by gentle shaking (~150 rpm).

3.3 Protein Expression for ELISA

1. Thaw the components of the expression system and the plasmid cDNA encoding human full length fused to GST recombinant protein on ice immediately before use.
2. Preheat the thermoblock to 30 °C.
3. Prepare the reaction for the desired protein expression according to the number of wells to be assayed including serum samples and negative controls. Each well will require 12.5 µL HeLa lysate, 2.5 µL accessory proteins, 5 µL reaction mix, 2 µL corresponding clone cDNA, and 3 µL nuclease-free water. All these reagents should be gently mixed in a nuclease-free 1.5 mL vial at RT in the order listed and saved on ice until use. A no-cDNA control reaction should be prepared to act as negative control of the assay. Here, clone cDNA volume will be replaced by nuclease-free water (*see Note 16*).
4. Incubate all the reactions for 3 h at 30 °C in a thermoblock. When the time is over put all the tubes on ice to stop the reaction (*see Note 17*).
5. Remove blocking buffer and wash each well 5× with 200 µL wash buffer.
6. Add 20 µL of the reaction by well and incubate at 4 °C overnight on a plate shaker (~150 rpm). In each plate, at least two wells should be incubated with 20 µL of the no-cDNA control reaction (*see Note 18*).

3.4 Serum Autoantibody Profiling on ELISA

1. Dilute each individual serum sample to 1:20 in blocking buffer to a final volume of 100 μ L.
2. Wash each well 5 $\times$ with 200 μ L blocking buffer.
3. Incubate the protein-coated wells with 100 μ L of individual sera diluted to 1:20 in blocking buffer and the no-cDNA control wells with 100 μ L blocking buffer at 4 °C overnight on a plate shaker (~150 rpm).
4. Dilute the horseradish peroxidase (HRP)-linked anti-human IgG to 1:1000 in blocking buffer.
5. Wash each well 3 $\times$ with 200 μ L blocking buffer.
6. Incubate the corresponding wells with 100 μ L of the diluted anti-human IgG for 1 h at RT on a rocking shaker (~150 rpm).
7. Wash 3 $\times$ with 200 μ L wash buffer.
8. Incubate each well with 100 μ L of substrate solution on a rocking shaker (~150 rpm) preserving from light until the reaction turns to blue color.
9. Stop the reaction by adding 50 μ L of stop solution.
10. Measure absorbance signals at 450 nm on a plate reader.
11. Identify sera with positive reactivity against the desired protein by subtracting the absorbance level to the negative control (blank) tested on the same run.

4 Notes

1. Among the different sets of arrays (HC1 to HC6) that are available at the NAPPA core CPD (Centre for Personalized Diagnostics) in the Biodesign Institute (Arizona State University, USA), we selected the one showing the greatest number of encoding protein genes previously related to OA. Each array contains 2352 total spots, including 2260 proteins (2125 unique) and 92 controls. The list of all protein-coding genes printed on each array is provided by the NAPPA core facility.
2. Pipette tip box or similar sized box with dividers that comfortably hold up to four slides.
3. The rabbit reticulocyte lysate expression system (Thermo Fisher Scientific) could also be used to express the proteins in their three-dimensional conformation and used according to the manufacturer's instructions. However, this in situ expression system shows lower efficiency for protein expression and more than ten times lower protein levels than the HeLa cell line [13].
4. In situ protein expression requires a first incubation step at 30 °C, followed by another step at 15 °C. Temperature precision during protein expression is critical; even a few degrees'

difference may reduce the yield. Therefore, it might be desired to have a programmable chilling incubator (Torrey Pines). However, two different incubators such as thermoblock could be used: one at 30 °C and the other at 15 °C.

5. If filtered compressed air is not available, a centrifuge with the proper glass slide adapter or a slide spinner (such as Labnet Slide Spinner Centrifuge) may also be used according to the manufacturer's instructions.
6. Purified DNA plasmids expressing the desired GST-tagged proteins-of-interest are stored at DNASU (www.dnasu.org) and provided by the NAPPA core CPD (Centre for Personalized Diagnostics) at the Biodesign Institute (Arizona State University, USA). For the in situ protein expression step, plasmids should be at 0.5 µg/µL.
7. The number of serum samples used to prepare the sample pool might differ depending on the research circumstances. We mixed ten different sera from each study group.
8. Serum dilution should be determined by the optimal sensitivity with minimum diffusion, through visual analysis to achieve a desired performance.
9. The printed site of the array should be faced up during all incubation and hybridization steps. It is also crucial not to let the arrays dry within the different steps and between them unless indicated.
10. Thirty minutes should be enough for the reagents to thaw completely.
11. Prepare the IVT using nuclease-free tubes and tips. Each slide requires 150 µL of IVT. However, we highly recommend preparing an extra volume of 10 µL per slide.
12. HybriWell chambers are RNase free and working surfaces are protected from RNase contamination by the removable liner. Take care to avoid contamination of exposed working surfaces.
13. We recommend applying a light pressure on the surface of the chamber during filling in order to dislodge air bubbles if they occur.
14. All steps for microarray processing are susceptible to automation by using Tecan's HS 400 Pro™ (1–4 slides per run) or HS 4800 Pro™ (1–12 or up to 48 slides per run) hybridization stations.
15. All the washing steps described in the ELISA protocol are carried out by turning the plate over a paper tower.
16. Each 1-Step Human Coupled IVT kits (Thermo Fisher Scientific) contains sufficient reagents to perform 40 reactions.

17. The reaction can be kept on ice for 90 min to 6 h or it might be stored at -20°C or more for long-term storage.
18. A shorter incubation for 2 h at RT is also effective at this step.

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References

1. Manzano-Román R, Fuentes M (2019) A decade of nucleic acid programmable protein arrays (NAPPA) availability: news, actors, progress, prospects and access. *J Proteome* 198:27–35. <https://doi.org/10.1016/j.jprot.2018.12.007>
2. Katchman BA, Chowell D, Wallstrom G et al (2017) Autoantibody biomarkers for the detection of serous ovarian cancer. *Gynecol Oncol* 146(1):129–136. <https://doi.org/10.1016/j.ygyno.2017.04.005>
3. Wang H, Demirkan G, Bian X et al (2017) Identification of antibody against SNRNPB, small nuclear ribonucleoprotein-associated proteins B and B', as an autoantibody marker in Crohn's disease using an immunoproteomics approach. *J Crohns Colitis* 11(7):848–856. <https://doi.org/10.1093/ecco-jcc/jjx019>
4. Bian X, Wasserfall C, Wallstrom G et al (2017) Tracking the antibody immunome in type 1 diabetes using protein arrays. *J Proteome Res* 16(1):195–203. <https://doi.org/10.1021/acs.jproteome.6b00354>
5. Wright C, Sibani S, Trudgian D et al (2012) Detection of multiple autoantibodies in patients with ankylosing spondylitis using nucleic acid programmable protein arrays. *Mol Cell Proteomics* 11(2):M9.00384. <https://doi.org/10.1074/mcp.M9.00384>
6. Gibson DS, Qiu J, Mendoza EA et al (2012) Circulating and synovial antibody profiling of juvenile arthritis patients by nucleic acid programmable protein arrays. *Arthritis Res Ther* 14(2):R77. <https://doi.org/10.1186/ar3800>
7. Anderson KS, Sibani S, Wallstrom G et al (2011) Protein microarray signature of autoantibody biomarkers for the early detection of breast cancer. *J Proteome Res* 10(1):85–96. <https://doi.org/10.1021/pr100686b>
8. Camacho-Encina M, Balboa-Barreiro V, Rego-Perez I et al (2019) Discovery of an autoantibody signature for the early diagnosis of knee osteoarthritis: data from the Osteoarthritis Initiative. *Ann Rheum Dis* 78(12):1699–1705. <https://doi.org/10.1136/annrheumdis-2019-215325>
9. Henjes F, Lourido L, Ruiz-Romero C et al (2014) Analysis of autoantibody profiles in osteoarthritis using comprehensive protein array concepts. *J Proteome Res* 13(11):5218–5229. <https://doi.org/10.1021/pr500775a>
10. Song L, Wallstrom G, Yu X et al (2017) Identification of antibody targets for tuberculosis serology using high-density nucleic acid

- programmable protein arrays. *Mol Cell Proteomics* 16(4 Suppl 1):S277–S289. <https://doi.org/10.1074/mcp.M116.065953>
11. Bian X, Wiktor P, Kahn P et al (2015) Antiviral antibody profiling by high-density protein arrays. *Proteomics* 15(12):2136–2145. <https://doi.org/10.1002/pmic.201400612>
12. Bian X, Wallstrom G, Davis A et al (2016) Immunoproteomic profiling of antiviral antibodies in new-onset type 1 diabetes using protein arrays. *Diabetes* 65(1):285–296. <https://doi.org/10.2337/db15-0179>
13. Festa F, Rollins SM, Vatter K et al (2013) Robust microarray production of freshly expressed proteins in a human milieu. *Proteomics Clin Appl* 7(5–6):372–377. <https://doi.org/10.1002/prca.201200063>



Chapter 14

Profiling Autoantibody Responses to Devise Novel Diagnostic and Prognostic Markers Using High-Density Protein Microarrays

Shabarni Gupta, Arghya Banerjee, Parvez Syed, and Sanjeeva Srivastava

Abstract

Protein microarrays are a diverse and high-throughput platform for screening biomolecular interactions, autoantigens, and protein expression profiles across tissues, etc. Autoantibodies produced against aberrant protein expression are often observed in malignancies which makes protein microarrays a powerful platform to elucidate biomarkers of translational interest. Early diagnosis of malignancies is an enduring clinical problem that has a direct impact on disease prognosis. Here, we provide an overview of a method employed to screen autoantibodies using patient sera in brain tumors. In case of brain malignancies, early diagnosis is particularly challenging and often requires highly invasive brain biopsies as a confirmatory test. This chapter summarizes the various considerations for applying a serum-based autoantibody biomarker discovery pipeline that could provide a minimally invasive initial diagnostic screen, potentiating classical diagnostic approaches.

Key words Protein microarrays, Brain tumors, Autoantibody, Diagnosis, Prognosis, Biomarker screening, Gliomas

1 Introduction

Effective early diagnosis of malignancies is one of the fundamental challenges in clinical oncology settings [1, 2]. Traditionally, malignancies are detected using histopathological examinations of biopsies, advanced imaging techniques or examining the expression of protein biomarkers in malignant tissues using immunoblots, enzyme-linked immunosorbent assay (ELISA), etc. While these techniques are robust, their ability to positively detect a malignancy is dependent on the tumor developing to a point, where it may not typically fit the definition of “early” diagnosis. For example, a tumor would be detected with efficacy on a primary screen like an

Shabarni Gupta and Arghya Banerjee are co-first authors.

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imaging technique only when it has grown to a size or density that would stand as a cause for caution to move on to a second, more invasive, validation technique like biopsies. Furthermore, a huge prerequisite for individuals to opt for such primary screens is the presentation of symptoms that contribute as compounding factors of late diagnosis of cancers such as colorectal cancer, ovarian cancer, and breast cancer. The lack of an initial, minimally invasive primary screen for malignancies is a translational void that has caught the attention of researchers all around the globe, over the last two decades. The emergence of next-generation sequencing (NGS) has helped overcome this void to a certain extent [3]. We observe not only clinicians routinely performing blood tests to probe for commonly mutated genes or circulating tumor DNA in patients, but also individuals opting for commercial NGS services that help flag mutations in genes associated with known tumors and other lifestyle diseases, especially where there may be a relevant family history weighing in. This is, however, plagued by the challenge of tumor heterogeneity and demographic variations [4]. Autoantibody screening for diagnosis and prognosis of malignancies using protein arrays fits well in this scenario to overcome several of these challenges in an unbiased manner.

A healthy immune system can differentiate between self and nonself entities. In an autoimmune disorder, the immune system fails to detect self-proteins and therefore attacks itself by generating antibodies against them, systemically deteriorating the subject's health [5, 6]. In malignancies, however, the underlying mutation causing the tumor may result in the formation of aberrant self-proteins [7]. The immune system can sense these aberrations and flag them as “nonself,” thereby leading to the production of antibodies against them. These aberrant self-proteins are termed as tumor-associated antigens (TAA) and antibodies produced against these self-antigens are termed autoantibodies [7]. As the immune system is activated on such an early-onset trigger (production of an aberrant tumor-causing self-protein), it serves as an ideal early diagnosis marker. Furthermore, a minimally invasive diagnostic blood test can be used to probe the production of such autoantibodies which is immensely useful in case of cancers of the brain as opposed to highly invasive biopsies, that may in some instances, pose as a life risk based on the location of the tumor [8].

Protein microarrays are a powerful proteomic platform for screening autoantibody production in individuals in a high-throughput manner [9]. Analogous, in principle to DNA microarrays, protein microarrays were developed to probe proteins and capture alterations in the proteome. Based on their application, protein microarrays can be designed to serve as functional arrays, analytical arrays, and reverse-phase protein arrays (RPA) [10]. Functional arrays consist of a microarray chip harboring a vast repertoire of proteins or peptides. These are probed against an enormous range of query molecules to identify protein-query molecule

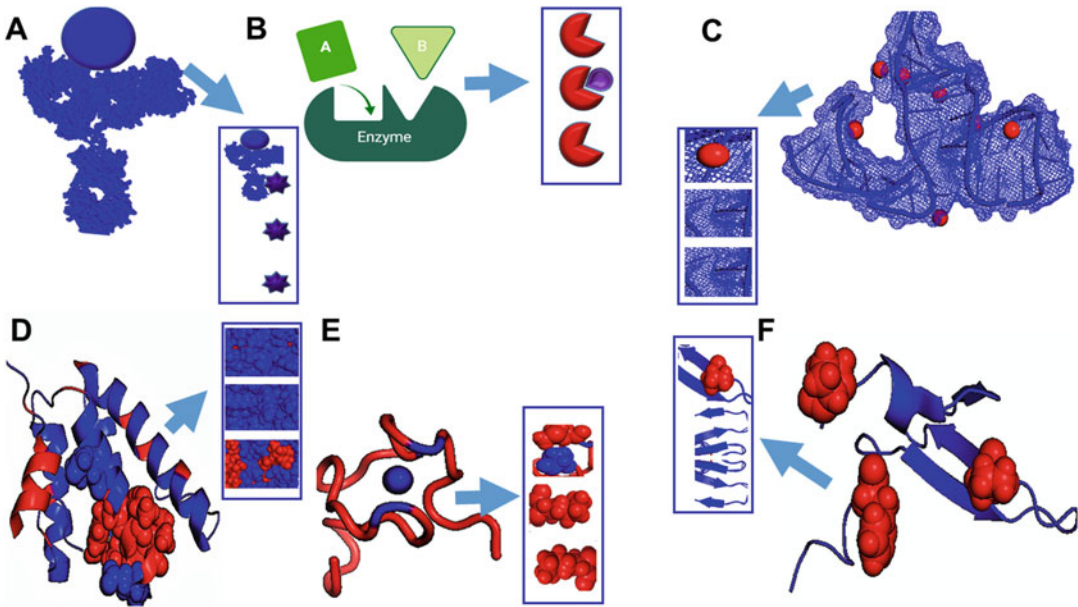


Fig. 1 Types of analytical microarrays: (a–f) represent antigen-antibody interaction, enzyme-substrate interaction, protein-DNA interaction, protein-protein interaction, protein-small molecule interaction, and protein-lipid interaction, respectively

interactions [11, 12]. Analytical microarrays, also termed capture microarrays, have some form of capture antibody, protein, lectin, and peptide printed on it (Fig. 1) [9, 13]. These are probed using a complex biofluid or sample like the serum, plasma, and tissue lysate to detect alterations in the sample proteome. Functional and analytical protein microarrays are also termed as forward-phase arrays. RPAs form the other subtype of protein arrays. These arrays comprise of multiplexed cell or tissue lysates spotted on a chip and are probed with a query antibody of interest to check target protein expression across multiple samples. Tissue microarrays emulate the principle of RPAs and comprise of microscopic tissue sections immobilized on a microarray chip to achieve similar goals [14–16]. For autoantibody detection, typically, an analytical microarray with full-length proteins are printed. Biospecimens such as serum and plasma from patients are used as sources of autoantibodies and act as probes that bind to their corresponding autoantigen [8]. Over the years, autoantibody screening has been performed using serum samples of patients from a wide range of malignancies [9, 11, 14]. This chapter focuses explicitly on autoantibody screening using serum samples of brain tumor patients.

The 2016 classification by WHO graded ~56 different subtypes of central nervous system (CNS) tumors [17]. While before this tumors were graded predominantly based on histopathology, the release of the 2016 WHO classification marked the incorporation of genetic biomarkers, such as IDH1/2, TERT, 1p/19q deletion,

and ATRX loss as diagnostic tools to brain malignancies like astrocytoma, oligodendroglioma, glioblastoma, and oligodendroglioma. Proteomic biomarkers complement the diagnostic and prognostic potential of genetic biomarkers and autoantibody profiling is a minimally invasive source of such markers. In the discussion of brain tumors, the blood-brain barrier (BBB) forms a critical component in understanding the biology of autoantibody generation [18]. The BBB is made up of endothelial cells that have unique properties that vascularize the brain. These tightly regulate the movement of ions, molecules, cells between the blood, and extracellular fluid circulating in the CNS. The semipermeable nature of the BBB ensures that large proteins such as antibodies and cellular components such as blood cells do not enter the brain. This regulation is critical for the maintenance of homeostasis. The BBB is intact at microtumor sites; however as the tumor enlarges in the brain, the properties of BBB change [19–21]. In cases of aggressive forms of brain tumors such as glioblastoma multiforme (GBM), the BBB is observed to rupture, disrupting its semipermeable nature and allowing access for the blood into the tumor site. This exposure aggravates immunological responses to tumor-associated antigens and can be instrumental in detecting abnormal proteins/protein expression using a protein microarray. It is intuitive to therefore question the efficacy of this technique on low-grade brain tumors where the BBB may remain intact. It is essential to note, therefore, that there are various other routes of priming the immune system. For instance, a mutated protein could adopt a secretory form and gain entry into blood plasma. This circulation would expose such proteins to the immune system. Tumor/antigen shedding may be also observed [7]. Therefore, an experimental design can incorporate grade-specific samples (low and high) to identify subtle differences in these autoantibody profiles to determine not only early diagnostic markers, but also prognostic markers, which have immense translational potential. In the case of brain tumors, besides serum, cerebrospinal fluid (CSF) is also a valuable source for autoantibody profiling [22]. Autoantibody profiles from diseased individuals can be compared to profiles of healthy individuals as controls. Aberrant autoantibody production can be determined upon a comparison between the two cohorts and a potential panel of biomarkers can be elucidated [23].

Protein arrays, right from their construction, assay execution, and ultimately data analysis, are extremely technology intensive. This aspect has been extensively reviewed by us previously [23]. This chapter overviews the protocol to perform autoantibody profiling from glioma serum samples (grades II, III, and IV) on HuProt™ chips harboring ~17,000 proteins, representing the human proteome, as published by Syed et al. [24]. This method can be suitably modified to incorporate a broader study design and can also be technically extrapolated to other protein microarray platforms based on recommendations from relevant technology providers [23].

2 Materials

2.1 Reagents

1. All solutions are prepared in ddH₂O, where applicable.
2. Blocking buffer: SuperBlock™ (PBS) Blocking Buffer from Thermo Fisher Scientific (catalog number: 37515) with 3% bovine serum albumin (BSA).
3. Sample dilution buffer: Tris-buffered saline with 0.1% Tween 20 (TBST) with 3% BSA.
4. Primary antibody cocktail: 1:500 diluted rabbit anti-GST antibody (EMD Millipore, catalog number AB3282) along with 1:500 diluted serum sample in TBST with 2% BSA.
5. Secondary antibody cocktail: Anti-human IgG conjugated with Cy5 (Jackson ImmunoResearch, catalog number 109-175-064) diluted 1:1000 in TBST with 2% BSA and anti-rabbit antibody conjugated with Cy3 (Invitrogen, catalog number A21429) diluted 1:5000 in TBST with 2% BSA.
6. Wash buffer: TBST.
7. This chapter describes the use of HuProt™ protein array chips. However, protein microarrays can be printed in-house. A wide range of surface chemistry options are available to users based on the intended application. These have been substantially reviewed by Gupta et al. [23].

2.2 Instruments and Software

1. Sorvall Legend X1R centrifuge (Thermo Fisher Scientific, catalog number 75004260).
2. Rockymax Rocking Shaker (Tarsons, catalog number 4080).
3. DWK Life Sciences Wheaton™ Glass 20-Slide Staining Dish with Removable Rack (Fisher Scientific, catalog number 08-812).
4. GenePix 4000B microarray scanner (Molecular Devices) and compatible software.
5. If users opt for in-house printing of high-density protein arrays, a suitable robotic arrayer would be required. A number of commercial ready-to-use protein array chips are available. In this chapter, we describe the use of HuProt™ protein array chips.

2.3 Materials

1. HuProt™ v2.0: Human proteome microarrays are protein microarrays with approximately 17,000 unique full-length proteins printed in duplicates on glass slides that were purchased from CDI Labs (Baltimore, MD, USA). Alternatively, protein arrays can be printed in-house or obtained from other commercial resources.
2. Serum samples: Serum samples from healthy volunteers and glioma patients were collected only after obtaining informed

written consent from the donors. The study described here, was approved by the institutional review board (ACTREC-TMC IEC No. 15, Advanced Centre for Treatment, Research and Education in Cancer and Tata Memorial Centre, Mumbai, India) in accordance with approved guidelines and results were published [24]. In brief these include serum samples from low-grade (II) and high-grade (III and IV) glioma. Serum samples were stored in aliquots at -80°C until further use.

3. General lab equipment: Pipettes, fine-needle tweezers, and microscope slide coverslips.

3 Methods

3.1 Recombinant Protein Production and Purification

1. The HuProt™ chips incorporate approximately 17,000 GST-tagged proteins that are cloned and expressed in a *Saccharomyces cerevisiae* expression system. The HuProt™ also comprises positive controls that include histones (H2A, H2B, H3, and H4) and GST printed in various concentrations, along with BSA, HeLa cell lysates, and p300-BHC which act as negative controls.
2. Detailed protocols describing the recombinant protein production and purification can be found in the study published by Heng Zhu and colleagues [26]. Briefly, Gateway recombinant cloning system (Invitrogen, CA) was used to shuttle human ORFs to yeast high-copy expression vector (pEGH-A). The expression vector pEGH-A expressing glutathione *S*-transferase-polyhistidine (GST-His₆) fusion proteins are then used for transformation into yeast for protein purification. A high-throughput GST-His₆ fusion protein purification is described by Zhu et al. [26]. A general workflow of Gateway recombinational cloning is represented in Fig. 2.

3.2 Printing Protocol

1. As mentioned previously, this chapter describes the use of commercial HuProt™ protein arrays that were developed by Zhu and colleagues [26] for autoantibody screening of glioma serum samples. For the construction of such a high-density protein microarray with ~17,000 full-length proteins, printed in duplicates, Zhu and colleagues used NanoPrint LM210 system (ArrayIT, Sunnyvale, CA) (**step 2**, Subheading 3.3).
2. Users can opt to use other commercially available protein chips or optimize a printing protocol suited for downstream application as reviewed by Gupta et al. [23]. A general schematic representation of chip printing is shown in Fig. 3. Points of caution during chip printing are further elaborated (*see Note 1*).

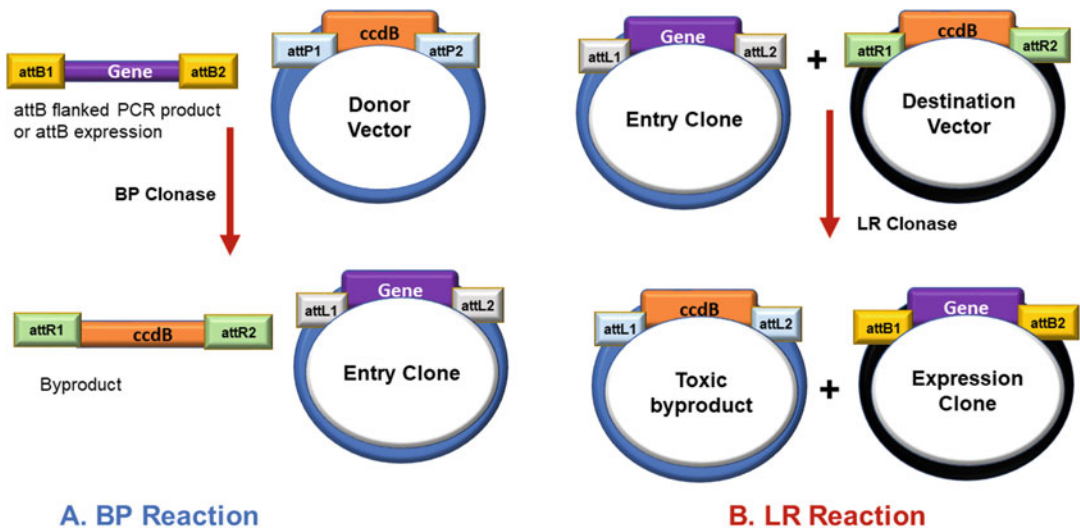


Fig. 2 Schematic representation of Gateway cloning

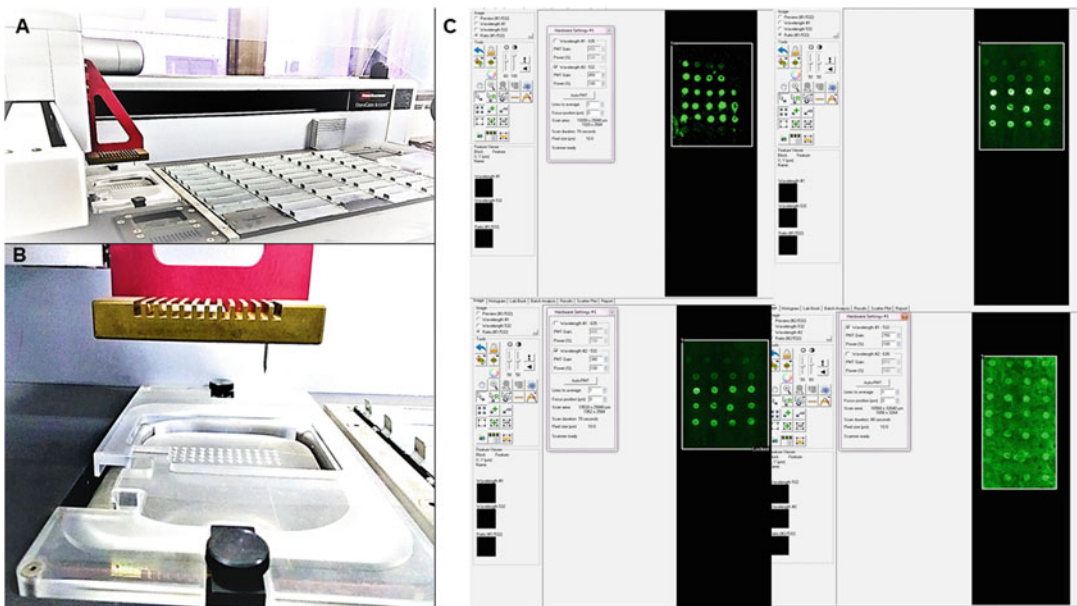


Fig. 3 Schematic representation of the steps involved in the microarray slide printing: (a) showcases the Omnigrid Ascent 100, Digilab, Inc., as a representative arrayer, (b) shows the arrayer needle which maintains precision during protein printing. (c) Represents optimization of a printing protocol on the slides such that accurate printing with minimal background is obtained

3.3 Assay Protocol

1. While protein microarray chips are stored in ultracold temperatures (-80°C), autoantibody profiling using protein microarray chips is performed at room temperature (RT). User must wear gloves while performing the assay and must always handle the microarray slides using tweezers near the bar-coded area or section of the slide where no proteins are printed to avoid scratching the printed proteins or leaving fingerprints.
2. The microarray chips are therefore placed in a plastic box and thawed in ice for 10 min.
3. 10 ml of blocking solution is then added to another plastic box (2% BSA in 10 ml SuperBlock™).
4. Tweezers are used to place the microarray slide carefully in the blocking solution by keeping the active (printed) side faced up. Care must be taken to have the slide evenly submerged in the blocking solution for 2 h with gentle shaking at RT.
5. The blocking solution is then gently removed using a pipette and slides are carefully placed in a Wheaton glass chamber using tweezers.
6. In this chamber, the slides are washed three times with $1\times$ TBST for 4–5 min each time with a magnetic bead placed in the chamber. This apparatus is placed on a magnetic stirrer such that washing is facilitated; however care must be taken to ensure that the bead does not touch the chips as it would lead to damage to the chip (*see Note 2*).
7. The slides are next rinsed with distilled water and centrifuged at 900 rpm for 2 min to remove TBST.
8. After this, the slides are submerged in 10 ml of primary antibody cocktail containing diluted anti-GST antibody and serum sample for 2 h with gentle shaking at RT. Serum samples in this cocktail must be appropriately preprocessed to avoid aggregates (*see Note 3*).
9. After primary antibody incubation, the solution is aspirated using a pipette and **steps 6** and **7** are repeated.
10. 10 ml of secondary antibody cocktail is next used to submerge the microarray chips for 2 h with gentle shaking at RT in the dark (*see Note 4*).
11. Secondary antibody solution is aspirated using a pipette and **steps 6** and **7** are repeated.

General schematic representation of the workflow is shown in Fig. 4.

3.4 Data Collection

1. A $40\mu\text{m}$ preview scan is used to select the scan area of the slide, which is then scanned at a higher resolution for acquiring the image that would be used for extracting data.

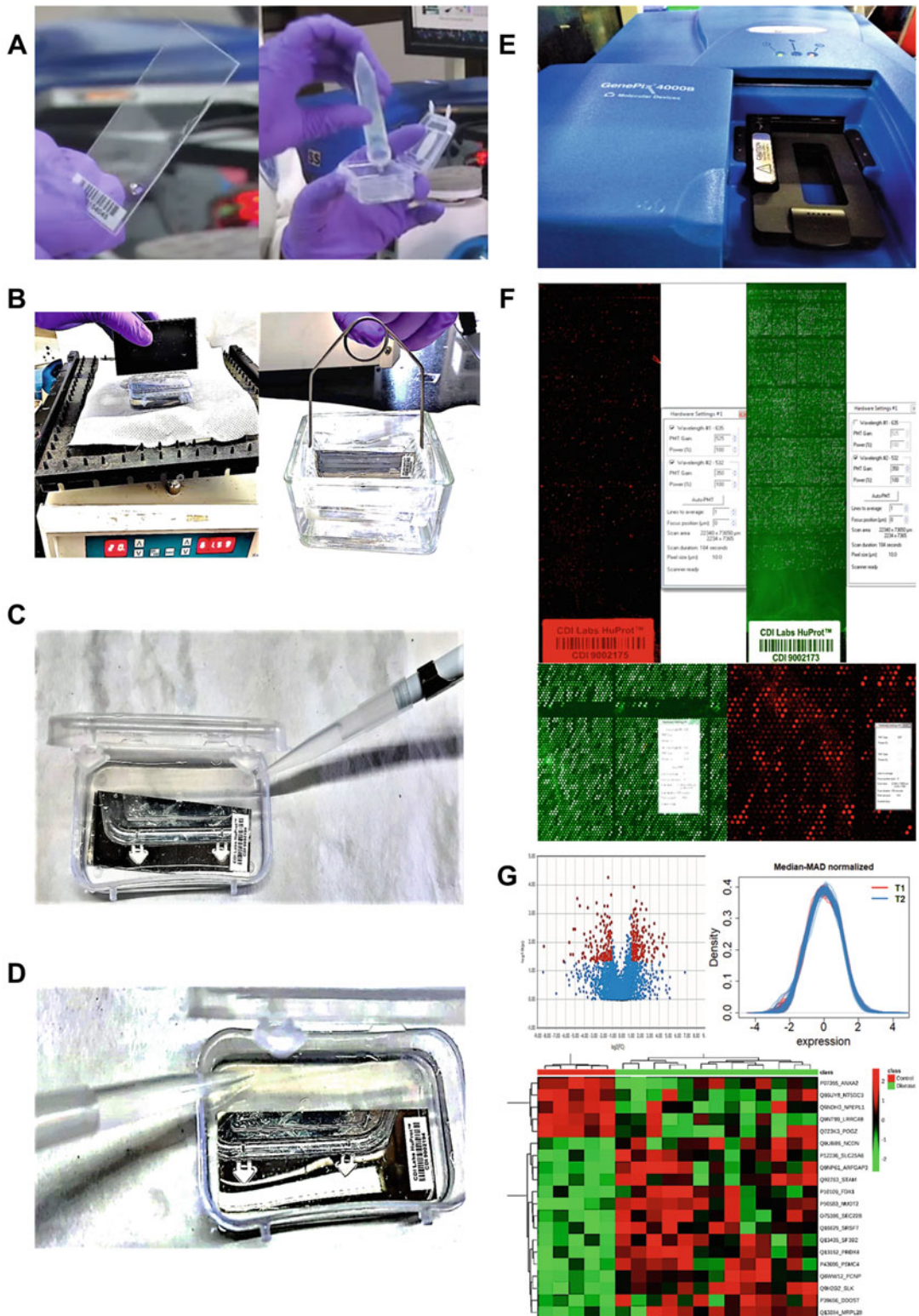


Fig. 4 Schematic representation of protein microarray protocol: (a) represents commercially available protein array chip and blocking; (b) represents incubation in the dark with agitation and washing the incubated slides in Wheaton glass chambers; (c) illustrates incubation with primary antibody(1:500 serum); (d) showcases 2-h

2. Optimized scan parameters for the above protocol include a pixel size of 10 μ m, 100% laser power, red (Cy5) channel PMT = 525 nm, and green (Cy3) channel PMT at 450 nm. These settings are subject to change based on any change in the type of sample, reagents, materials, or instrument.
3. The acquired image is then exported as a .tiff file. The .gal file is then overlaid onto the image to identify the printed features and their location using the GenePix Pro analysis software.
4. The .gal file, once overlaid, appears as a grid that can be correctly aligned to match the printed features. Once aligned, the settings can be saved to a .gps file.
5. Cumulative intensities for each feature are converted to numeric pixel intensities that can be saved in a .gpr file.
6. At this point, the processed slides can be stored in the dark in a dehumidified, airtight box at -20°C .

3.5 Analysis

1. Numeric pixel intensities exported in the .gpr files are normalized based on control spot intensities after subtracting background noise (*see Note 4*).
2. Normalized signal intensities are further subjected to appropriate statistical tests. Based on the aim of the experiment, the statistically significant proteins can be further subjected to recursive elimination model. As in the case of biomarker discovery-based experiments like this, such data processing strategies could yield a panel of classifier proteins that are capable of distinguishing healthy and patient cohorts with optimality. Differentiation between grades, viz. low-grade (II) against high-grade (III and IV) or intergrade analysis between two subtypes of tumor populations, is also possible, as it has been previously published [24].
3. Power calculations, data processing and visualization strategies, stringency criteria, etc. for such high-throughput translational studies with considerable day-to-day variations are best performed in consultation with a statistician. Commonly, the open-source R platform is used to perform data analysis. It also allows visualization of such complex high-throughput data.

Fig. 4 (continued) incubation at RT followed by washing and incubation with the secondary antibody(1:1000 anti-human IgG-Cy5/Cy3, incubated at RT for 2 h followed by washing and drying); (e) is an image of GenePix microarray scanner; (f) represents scanning of the chip in red channel and green channel for different fluorophores (Cy5/Cy3); (g) is a representative image of secondary data analysis using R programming

4 Concluding Remarks

Protein microarrays are incredibly versatile, customizable high-throughput platforms for screening protein-protein interactions (PPIs) [23]. This has helped researchers mold this technology into a powerful autoantibody screening tool for biofluids like serum and CSF. Autoantibodies are known to be produced while a “diseased” individual may be asymptomatic. Autoantibodies are therefore present in high circulating levels even before a diseased individual may think of subjecting themselves to any diagnostic screen [7, 27]. Autoantibody screening platforms have been extensively used to identify early biomarkers of translational importance in a range of malignancies [28–30]. Perhaps, an application that remains understated in this pipeline is its role in understanding disease pathobiology. Autoantibody production in cancers could be due to one or more of the following reasons: altered protein structure due to underlying mutation/aberrant PTMs, clonal selection defects and inflammation, defects in protein expression level and their cellular location, secreted tumor-associated antigen into circulation, presentation on the cell surface, etc. [7]. Therefore, common autoantibody targets in a well-constructed disease cohort can shed light on basic biochemical alterations observed in disease pathobiology stemming from the aforementioned causes. In addition to this, it also helps profile immune responses which can be subsequently used to grade tumors on their severity and identify prognostic targets. If refined, this methodology can be used to personalize diagnosis and treatment [31, 32], although there are several challenges to protein arrays being directly implemented in a clinical setting. This includes training of technicians in a specialized technique, cost constraints, low throughput of sample processing (one sample per each high-density chip), and more importantly data analysis. Due to these challenges, protein microarrays, to date, have been used in cohort-based studies in research laboratories and their findings have often been deconstructed into a simplified ELISA or immunoblot-based diagnostic tests in a clinical setting [23, 24, 30]. Despite this, given their flexibility, protein microarrays are perhaps the only high-throughput platform to perform autoantibody screening, making them indispensable in this area of translational research. With innovation in instrumentation, data analysis tools, and more importantly research protocols as the one described in this chapter, one can hope to make these technologies high throughput not only in PPI screening but also in sample screening to realize its enormous potential in personalized treatment.

5 Notes

1. Precession in chip printing: Accurate printing of the chip is an important determinant governing accurate detection of targets during a protein microarray experiment. It is observed that the features printed on commercially available protein array chips are sometimes misaligned or misprinted or lie “off” the grid layout. Errors can more occasionally arise from unoptimized settings when chips are printed in-house. One can manually align the .gal file to ensure that they obtain the true signal intensities from a printed spot. In other cases, the user may flag overlapping or merged spots. Merged spots are generally observed when the protein is printed on the chip that has a high fluid content (effective volume being printed). Large volumes can result in coalescence of two or more protein spots. Similar issues can also arise with increased humidity in the array printing chamber. Another commonly observed error in the protein arrays is that of “missing” spots. Missing spots result from fundamental hardware issues in chip printing. They mainly arise due to needles not making enough contact with the chip surface or because of the consumption of the protein buffer rendering the remaining volume for chip printing as suboptimal. Another error commonly observed in the protein array chips is doughnut structures. Doughnut structures arise from damaged pins, or because of un-optimized humidity condition in the arrayer. This leads to a ringlike structure being printed around the damaged pin or drying of spots which eventually looks like a doughnut spot. Thus, while designing an experiment, few chips from the batch should always be kept for quality control and testing of the protein array chips. In an experimental design, one should also monitor the functionality of the printed protein. These protein array chips can be tested for several number of proteins for printing quality. For instance, the HuProt™ chip harboring GST-tagged proteins can be probed using an anti-GST antibody tagged with a suitable fluorophore. Such QC testing is always advisable before the usage of actual biological samples to ensure that the batch of protein chips that would be used for an experiment does not harbor the commonly described errors arising from poor chip printing. The protocol mentioned above describes the steps for performing the above quality control test of the chip. If, however, the user is using cell-free extract (CFE)-based chip, they should perform the quality control testing for the printed DNA, using PicoGreen staining. Based on the surface

chemistry and nature of proteins printed on a chip, various QC tests can be devised to capture any error arising from substandard chip printing before performing the experiment. Once QC results from a batch of chips are satisfactory, one can proceed with their experiments, thus making optimal use of clinically precious biofluid specimens being tested for autoantibody screening.

2. Overcoming background issues: Nonspecific signals contribute to background issues that can suppress protein spot intensity, thereby skewing true numerical values. There are many reasons due to which one observes high backgrounds in the chip. Few of them can be broadly categorized into the following:
(a) *Concentration*: High concentration of protein while the chip is printed can contribute to the background significantly.
(b) *Excess of primary and secondary antibodies used*: During the experimental procedure if an excess of antibodies is used, this can lead to saturation and nonspecific binding, which eventually results in an uneven background. If the overall image profile of the protein array chip exhibits high background, one can decrease the concentration of primary and secondary antibody. It is for this reason that optimization of antibody concentrations before batch processing of clinical samples is instrumental before starting autoantibody profiling experiments.
(c) *Blocking*: It plays a crucial role in preventing nonspecific binding.
(d) *Time duration, temperature, and physical condition during the incubation*: One should optimize the optimum length, optimum temperature, and physical conditions such as agitation and shaking for uniform distribution of solvent. It is generally suggested that the blocking can usually be done for 1 h at room temperature or 4 °C overnight.
(e) *Improper washing*: Volume of the washing buffer used, duration of the wash, and number of times the chips are washed significantly influence the observed background signals. Overwashing of the chip can result in loss of the signal intensity of the spots while suboptimal washing can result in high background signals. Therefore, these parameters are imperative to be optimized before batch running the protocol.
(f) *Handling of the chip*: One should be careful while handling the chip. One should avoid touching the active/printed area of the protein array chip and care must be taken to ensure that buffers and solvents are applied gently to the chip to avoid any sheer stress arising from the fluid. Many proteins and fluorophores tagged to the proteins are light sensitive. Therefore, one should carry out experiments in the dark or with minimal light exposure.

Scratches, smudges, or fingerprints can be observed on the chip if handled with bare hands or gloves. One should use tweezers and hold chip from the extremities to avoid it. (g) *Washing and drying the chip*: One should wash and dry the chips before spectral scanning; washing helps in removal of unwanted background contributed due to various buffers and drying helps us in nullifying the background coming from washing buffer. (h) *PMT gain*: One can also optimize the instrument parameter such as PMT gain. The signal-to-noise ratio is neutralized by allowing the control spots to reach the saturation. (i) *Surface chemistry*: Surface chemistry of protein array chip also plays a crucial role in background contribution. It is observed that the nitrocellulose chip generally contributes to less background than epoxy-coated chips.

3. Spotting aggregates present in the chip: Aggregates present on the chip indicate a faulty buffer preparation, where proteins or analytes have not dissolved in the buffer. It could also imply the presence of particulate impurities on the chip before spotting. Furthermore, it has been observed that clinical samples, such as a serum, plasma, urine, and CSF, often contain particulate impurities. Care must be taken to filter such biological samples appropriately before using them for protein microarray experiments.
4. Quality control testing of the antibodies used: Reverse-phase protein array and other protein microarrays mainly depend on antibodies for the detection of the target analyte. Commercially manufactured protein arrays undergoes rigorous quality control analysis; hence batch variation in chip printing will be minimal in such cases. Batch-to-batch variability may include factors such as missing protein spots or errors arising from if the chip is not transported correctly or due to proper storage and handling. Even if the same antibody is used in two different chips; batch-to-batch differences in antibodies may lead to varied efficacies. Hence they need to be kept consistent throughout the experiment. Such batch-to-batch variations must be accounted and normalized for using control spots such as IgG and GST (Fig. 5) during data analysis. A schematic representation of these issues (*see* **Notes 1–4**) is illustrated in Fig. 6.

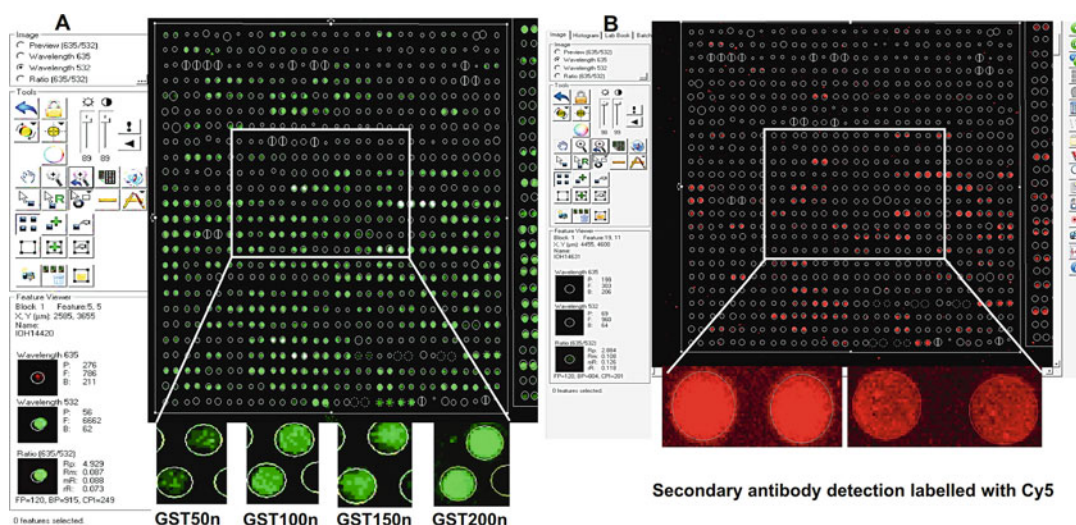


Fig. 5 (a) Represents the grid layout of a HuProt™ v2 overlaid on a chip imaged at a wavelength of 532 nm, secondary antibody labeled with Cy3, and QC profiles of green sulfo tag-labeled control spots loaded at different concentrations (GST50n, GST100n, GST150n, GST200n); (b) represents the HuProt™ v2 chip scanned at a wavelength of 635 nm and secondary antibody labeled with Cy5

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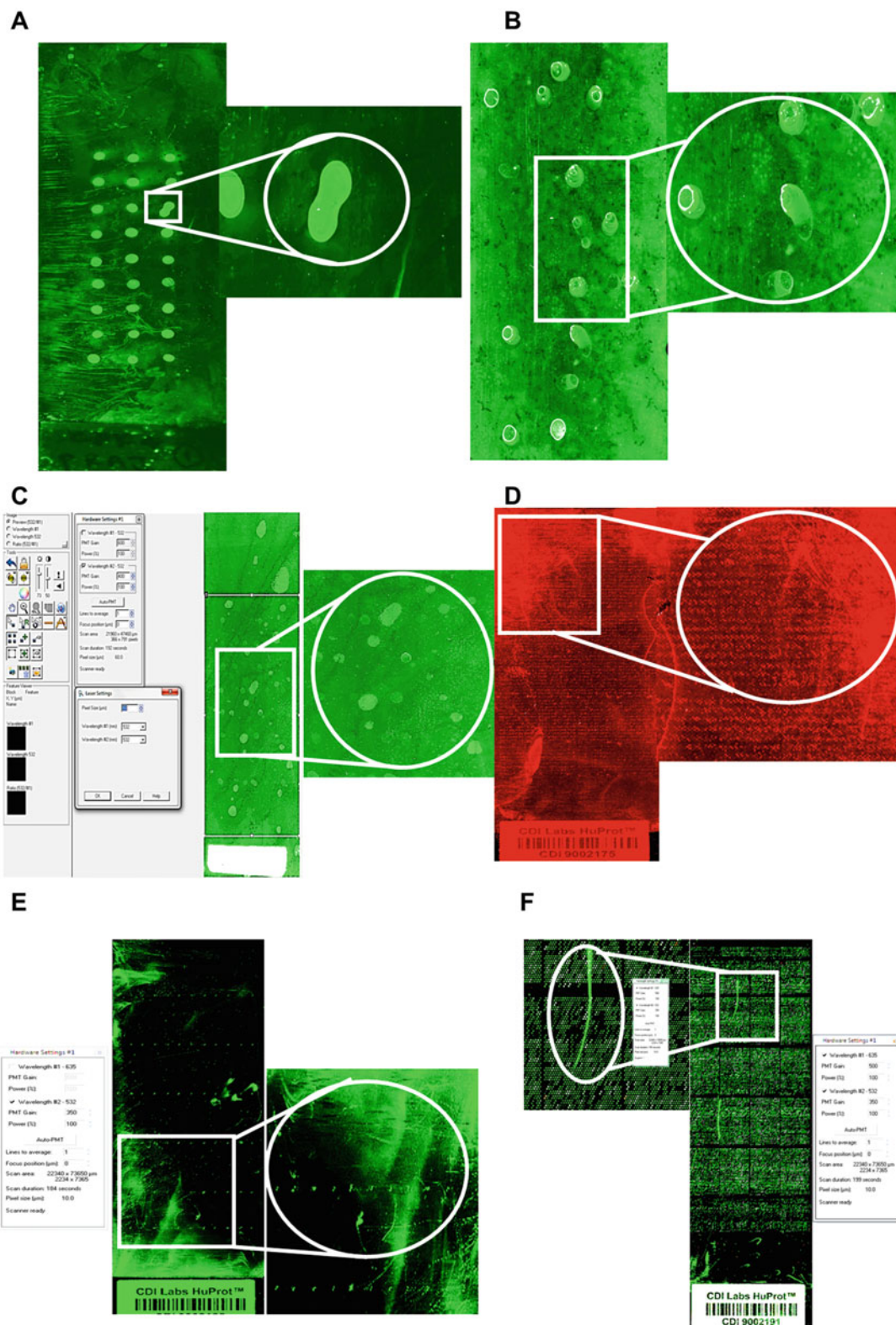


Fig. 6 Common issues requiring troubleshooting in protein microarray experiments: (a) represents overlapped or merged spots. (b) Represents doughnut structures, (c) showcases missing spots, (d and e) showcase background issues arising from nonspecific signals contributing to the background, (f) showcases handling errors such as scratches, smudges, or fingerprints

References

1. Neal RD, Tharmanathan P, France B et al (2015) Is increased time to diagnosis and treatment in symptomatic cancer associated with poorer outcomes? Systematic review. *Br J Cancer* 112:S92–S107
2. (2018) Early detection: a long road ahead. *Nat Rev Cancer* 18:401
3. Sahm F, Schrimpf D, Jones DTW et al (2016) Next-generation sequencing in routine brain tumor diagnostics enables an integrated diagnosis and identifies actionable targets. *Acta Neuropathol* 131:903–910. <https://doi.org/10.1007/s00401-015-1519-8>
4. Gupta S, Ray S, Talukdar A et al (2016) Geographic pervasiveness of cancer: prospects of novel biomarker and therapeutic research in developing countries using OMICS approaches. In: Biomarker discovery in the developing world: dissecting the pipeline for meeting the challenges. Springer, India, pp 9–17
5. Delves PPJ (2015) Overview of the immune system - immune disorders - MSD manual consumer version. In: MSD Manuals. <https://www.msmanuals.com/home/immune-disorders/biology-of-the-immune-system/overview-of-the-immune-system>. Accessed 15 Oct 2020
6. Gonzalez S, González-Rodríguez AP, Suárez-Álvarez B et al (2011) Conceptual aspects of self and nonself discrimination. *Self Nonself* 2:19–25
7. Zaenker P, Gray ES, Ziman MR (2016) Autoantibody production in cancer-the humoral immune response toward autologous antigens in cancer patients. *Autoimmun Rev* 15:477–483
8. Gupta S, Srivastava S (2019) Prospects of translational proteomics and protein microarrays in oligodendroglioma. In: Oligodendroglioma: clinical presentation, pathology, molecular biology, imaging, and treatment. Elsevier, Amsterdam, pp 117–127
9. Liotta LA, Espina V, Mehta AI et al (2003) Protein microarrays: meeting analytical challenges for clinical applications. *Cancer Cell* 3:317–325. [https://doi.org/10.1016/S1535-6108\(03\)00086-2](https://doi.org/10.1016/S1535-6108(03)00086-2)
10. Hall DA, Ptacek J, Snyder M (2007) Protein microarray technology. *Mech Ageing Dev* 128:161–167. <https://doi.org/10.1016/j.mad.2006.11.021>
11. Hu S, Xie Z, Qian J et al (2011) Functional protein microarray technology. Wiley Interdiscip Rev Syst Biol Med 3:255–268. <https://doi.org/10.1002/wsbm.118>
12. Bertone P, Snyder M (2005) Advances in functional protein microarray technology. *FEBS J* 272:5400–5411
13. Sauer U (2017) Analytical protein microarrays: advancements towards clinical applications. *Sensors (Switzerland)* 17(2):256
14. VanMeter A, Signore M, Pierobon M et al (2007) Reverse-phase protein microarrays: application to biomarker discovery and translational medicine. *Expert Rev Mol Diagn* 7:625–633
15. Pjawaletz CP, Charboneau L, Bichsel VE et al (2001) Reverse phase protein microarrays which capture disease progression show activation of pro-survival pathways at the cancer invasion front. *Oncogene* 20:1981–1989. <https://doi.org/10.1038/sj.onc.1204265>
16. Mueller C, Liotta LA, Espina V (2010) Reverse phase protein microarrays advance to use in clinical trials. *Mol Oncol* 4:461–481. <https://doi.org/10.1016/j.molonc.2010.09.003>
17. Louis DN, Perry A, Reifenberger G et al (2016) The 2016 World Health Organization classification of tumors of the central nervous system: a summary. *Acta Neuropathol* 131:803–820
18. Takeshita Y, Ransohoff RM (2015) Blood-brain barrier and neurological diseases. *Clin Exp Neuroimmunol* 6:351–361
19. Shimizu F, Nishihara H, Kanda T (2018) Blood–brain barrier dysfunction in immunomediated neurological diseases. *Immunol Med* 41:120–128
20. Platt MP, Agalliu D, Cutforth T (2017) Hello from the other side: how autoantibodies circumvent the blood-brain barrier in autoimmune encephalitis. *Front Immunol* 8:442
21. Mader S, Brimberg L, Diamond B (2017) The role of brain-reactive autoantibodies in brain pathology and cognitive impairment. *Front Immunol* 8:1101
22. Roche S, Dauvilliers Y, Tiers L et al (2008) Autoantibody profiling on high-density protein microarrays for biomarker discovery in the cerebrospinal fluid. *J Immunol Methods* 338:75–78. <https://doi.org/10.1016/j.jim.2008.07.002>
23. Gupta S, Manubhai KP, Kulkarni V, Srivastava S (2016) An overview of innovations and industrial solutions in protein microarray technology. *Proteomics* 16:1297–1308

24. Syed P, Gupta S, Choudhary S et al (2015) Autoantibody profiling of glioma serum samples to identify biomarkers using human proteome arrays. *Sci Rep* 5. <https://doi.org/10.1038/srep13895>
25. Hu S, Xie Z, Onishi A et al (2009) Profiling the human protein-DNA interactome reveals ERK2 as a transcriptional repressor of interferon signaling. *Cell* 139:610–622
26. Zhu H, Bilgin M, Bangham R et al (2001) Global analysis of protein activities using proteome chips. *Science* 293:2101–2105. <https://doi.org/10.1126/science.1062191>
27. Burbelo PD, Gordon SM, Waldman M et al (2019) Autoantibodies are present before the clinical diagnosis of systemic sclerosis. *PLoS One* 14. <https://doi.org/10.1371/journal.pone.0214202>
28. Ayoglu B, Schwenk JM, Nilsson P (2016) Antigen arrays for profiling autoantibody repertoires. *Bioanalysis* 8:1105–1126
29. Duarte JG, Blackburn JM (2017) Advances in the development of human protein microarrays. *Expert Rev Proteomics* 14:627–641
30. Atak A, Mukherjee S, Jain R et al (2016) Protein microarray applications: autoantibody detection and posttranslational modification. *Proteomics* 16:2557–2569
31. Da Syu G, Dunn J, Zhu H (2020) Developments and applications of functional protein microarrays. *Mol Cell Proteomics* 19:916–927
32. Neagu M, Bostan M, Constantin C (2019) Protein microarray technology: assisting personalized medicine in oncology (review). *World Acad Sci J* 1:113–124. <https://doi.org/10.3892/wasj.2019.15>

Part V

Antibody Microarrays for the Characterization of Disease-Specific Protein Profiles



Deciphering Intracellular Signaling Pathways in Tumoral Pathologies

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Abstract

The heterogeneity of diseases such as cancer makes it necessary to use high-throughput screening techniques to obtain the maximum number of parameters and characteristics of tumors. These obtained biomarkers can be used for the prediction, prognosis, and treatment or search for new therapeutic targets. In this sense, microarray technology allows exhaustive analysis in a short time and from a great variety of biological samples, becoming a fundamental tool in biomedical research projects. Here, operational process of protein microarrays based on the antibody-antigen interaction is described, emphasizing their application in intracellular signaling pathways in tumoral pathologies. In addition, a final validation using nucleic acid programmable protein array (NAPPA) technology in a simple ELISA assay was included to decipher functional characterization of featured proteins from microarray screening.

Key words Biomarkers, Proteins, Intracellular signaling, Tumoral pathologies, Microarray technology

1 Introduction

The word cancer includes a group of very heterogeneous diseases characterized by cells with abnormal behavior, which divide very quickly and have the ability to spread to other parts of the body. These cells acquire genomic, chromosomal, and/or molecular mutations that are reflected in the aberrant expression of proteins [1–5]. Detecting high levels of these differential proteins or tumor-specific antigens could be the key to controlling certain types of cancers, as they may be susceptible to being therapeutic targets [6].

However, to identify and validate biomarkers related to onco-pathologies, massive and simultaneous analysis techniques are necessary capable of generating a large amount of information for the

Alicia Landeira-Viñuela and Pablo Juanes-Velasco contributed equally with all other contributors.

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treatment and stratification of cancer patients. Another fundamental aspect in this area will be the integration of genomic, transcriptomic, and proteomic information generated to determine possible therapeutic targets from various complementary perspectives [7, 8].

In recent years, one of the research challenges has been and continues being the search for target biomarkers to determine prediction, prognosis, early diagnosis, and treatment of tumoral pathologies [9]. For this, techniques based on the simultaneous and massive study of thousands of proteins are necessary. In this complex search, where high content and high-throughput methodologies are required, a key tool is protein microarrays, which are capable of identifying potential biomarkers related to altered intracellular signaling pathways, in a faster manner, with a minimum requirement of sample and reagents and without being necessary to have in-depth knowledge of the progression of the disease [7, 10]. Microarray technology is suitable for biomarker discovery and validation mostly of the human proximal body fluids (such as plasma, serum, synovial fluid ...) with a wide dynamic range and avoiding sample pre-fraction or selective enrichment. For these reasons, among others, protein arrays are an extraordinary technology for high-throughput screening in multiple biomedical research projects [11].

Regarding protein microarrays, it is quite relevant to know the principal features for biomarker discovery and deciphering altered cell signaling pathways. In general, there are three types of protein microarrays according to the nature of the capture agent [12]. They can be classified into (1) analytical protein microarrays, in which antibodies are printed on their surface to detect and quantify proteins in complex biological samples [7, 12–14]; (2) functional protein arrays, in which recombinant proteins are printed on their surfaces in order to identify the interaction of proteins with other molecules such as other proteins, DNA, and lipids [12]; and (3) reverse-phase protein arrays, in which biological samples (i.e., human proximal fluids such as serum and CSF) or cell lysates are printed on their surface to detect the proteins with specific antibodies and then with another fluorescent antibody [12] (Fig. 1).

In this chapter, we describe detailed relevant aspects involved in all the operational process in analytical protein microarrays (also called antibody microarrays), based on the antibody-antigen interaction, because they are very useful to describe intracellular signaling pathways in tumoral pathologies [7, 12, 14] (Fig. 2). The great advantage of performing antibody microarray assays is based on the inherent specificity and selectivity of antigen-antibody recognition; multiple interactions can be simultaneously tested at the same time. In this assay, certain analytes can be detected in highly heterogeneous mixtures with a wide dynamic range (protein in mg/mL to protein in pg/mL). This type of array is usually used to identify

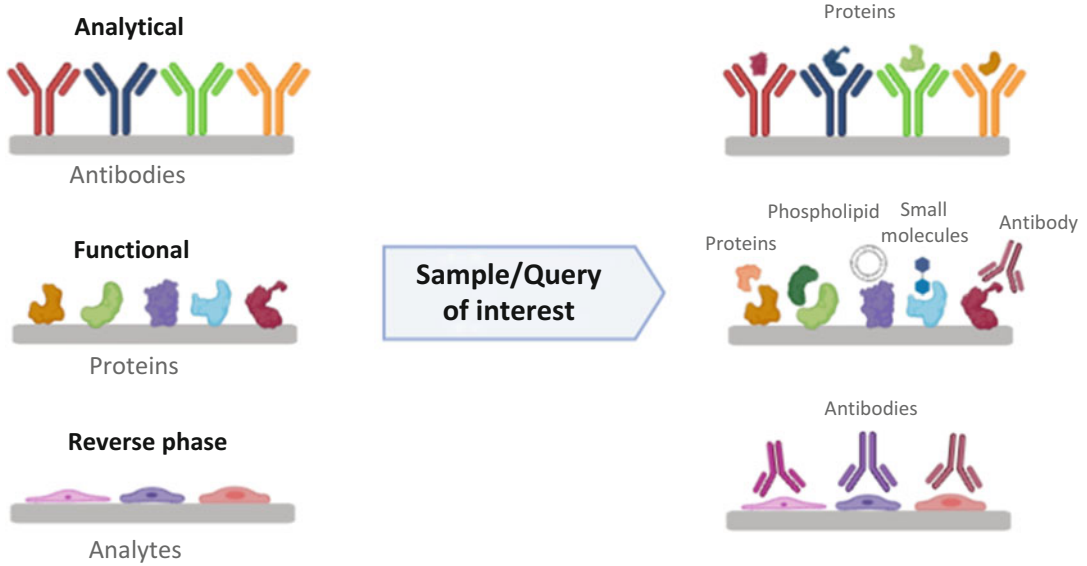


Fig. 1 Different types of arrays according to their content. (Created with BioRender.com)

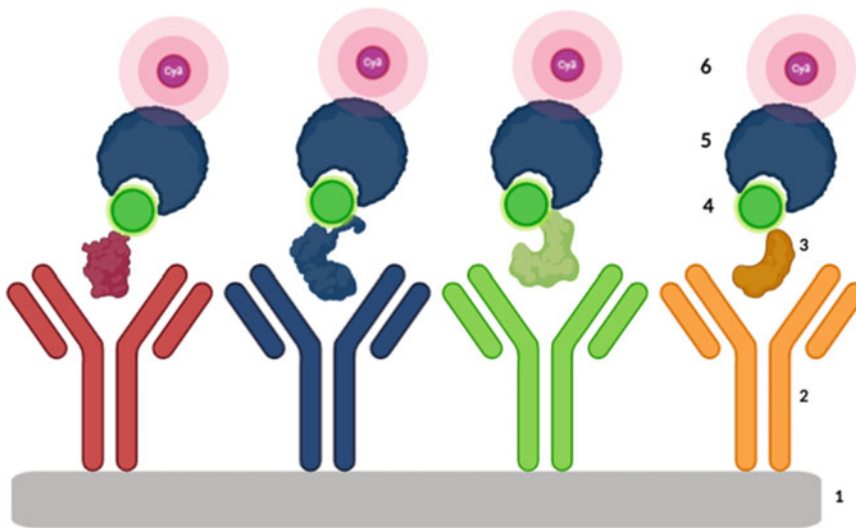


Fig. 2 Nanometric scheme of an antibody microarray assay. Each number points to a part of the assay: (1) Functionalized microarray surface. (2) Printed antibody. (3) Capture protein. (4) Biotin. (5) Streptavidin. (6) Fluorophore, for example, Cy3. (Created with BioRender.com)

biomarkers that predict a biological condition such as healthy or pathological [12]. Therefore, this shows that antibody microarrays are feasible to perform multiplex assays with high sensitivity to characterize complex proteomes with limited sample availability. Furthermore, several studies have shown that only a fraction of the antibodies can work correctly and this may be due to the loss of antibody functional activity by degradation or denaturation

during storage or during the printing process, or due to an inappropriate orientation of the antibody onto the array surface [10]. Then, this is a critical point which could improve protein array performance; hence, human protein atlas initiative is working on developing and validating antibodies for protein microarrays [15].

In addition, protein microarrays are compatible to be combined with targeted screening in order to decipher functional characterization of the protein of interest, and to validate the obtained results. For this goal, a NAPP-ELISA (self-assembled protein array) is the most suitable approach to confirm the functional activity of the differential proteins identified by antibody microarray experiments.

NAPP is a label-based technology that introduces the simple process of cloning the cDNAs of interest with a common epitope tag to all of the proteins at their C- or N-terminus (to facilitate protein purification and detection) into plasmids. This technology allows to produce the targeted proteins in situ with a high purity and in a short period of time. In this case, the in vitro translation/transcription (IVTT) takes places in 96-well plates using the NAPP technology as the base for an ELISA assay [16–19].

Combining both technologies, intracellular signaling pathways mediated by novel antitumoral compounds can be extensively described and tested. Here, antibody-array and NAPP-ELISA methods are developed in detail as well as results from screening and validation of new drugs are commented.

2 Materials

2.1 Cell Culture

1. MG-63 (ATCC CRL-1427).
2. 75 cm² Flask.
3. RPMI.
4. FBS.
5. Penicillin.
6. Streptomycin.

2.2 Sample Preparation for Protein Microarrays

2.2.1 Cell Lysis and Protein Extraction

1. 1× PBS Na⁺/K⁺.
2. Protease inhibitor cocktail (Sigma-Aldrich, P8340).
3. Lysis buffer (5 mM HEPES, 10 mM MgCl₂, 140 mM NaCl, 0.1% v/v Tween 20 at pH 8.0, and 1% v/v protease inhibitor cocktail). Prepare lysis buffer: Dilute 0.119 g of HEPES in 50 mL of Milli-Q water and add 0.20 mL of MgCl₂ (5 M), 2.8 mL of NaCl (5 M), 0.1 mL of 10% Tween 20, and 1 mL of protease inhibitor cocktail. Finally, make up to a volume of 100 mL with Milli-Q water.

4. 10% Octyl- β -D-glucopyranoside: To prepare 10% octyl- β -D-glucopyranoside dilute 1 g of octyl- β -D-glucopyranoside in 10 mL of Milli-Q water.
5. Ultrasound sonicator.

2.2.2 IgG Depletion and Protein Quantification

1. Protein G agarose beads (PD Mini TRAP G-25, GE Healthcare, 28-9225-29 AB).
2. Lowry quantification kit (RC Reagents Package, BioRad, #5000119).

2.2.3 Dye Labeling

1. 0.1 M Sodium carbonate/sodium bicarbonate pH 9.3.
2. Cy3 Mono-Reactive CyDye (GE Healthcare, PA23001).
3. Cy5 Mono-Reactive CyDye (GE Healthcare, PA25001).
4. Gel filtration columns (Amicon[®] Ultra-0.5 centrifugal filter 3K, Millipore-Merck, UFC505096).
5. NanoDrop 1000 (Thermo Fisher Scientific).

2.3 Preparation of Antibody Microarrays

The microarray of antibody used was the same that was reported previously by Ignacio E. León et al. [20, 21]. This microarray consists of 224 human monoclonal antibodies whose targets are involved in intracellular signaling pathways.

2.3.1 Blocking the Microarray Surface

1. SuperBlock[™] Blocking Buffer (Thermo Fisher Scientific, Cod: 37535) is diluted in $1 \times$ PBS Na^+/K^+ . For one microarray we need 6 mL of blocking buffer: 0.6 mL of SuperBlock[™] Blocking Buffer and 5.4 mL of $1 \times$ PBS Na^+/K^+ (*see Note 1*).
2. Microarray washing chamber.
3. Orbital shaker.

2.3.2 Wash Microarray

1. Distilled water.

2.3.3 Microarray System Incubation

1. Incubation chamber ($W \times L$ 7 mm $\times$ 16 mm, with Delrin[®] snap clips, wells, 8) and clips for microarray systems (Grace Bio-Labs ProPlate[®] microarray system, slide module, Sigma Aldrich).

2.4 Incubation of Dye-Conjugated Cell Lysates on Antibody Microarrays

Each sample dye with Cy3 or Cy5 is of 1 mg/mL concentration. For each subarray, we need 100 μL and it can detect at least a protein concentration of 1 $\mu\text{g}/\mu\text{L}$. For one subarray we need a mix of sample dye Cy3/Cy5: 25 μL of sample dye Cy3 (1 mg/mL), 25 μL of sample dye Cy5 (1 mg/mL), and 50 μL of blocking buffer.

2.4.1 Preparation of Sample Dilution

2.4.2 Incubation in Microarray

1. Transparent film.
2. Rotatory mixer.

2.4.3 Wash and Drying Microarray

1. $1\times$ Phosphate-buffered saline Tween 20 (PBST): 0.05% Tween 20 to PBS.

2.5 Scan of Microarrays and Data Analysis

1. Microarray scanner Imaging Reader Sensovation (Miltényi Imaging GmbH).
2. GenePix Pro 4.0 software (Axon).

2.6 Self-Assembled (NAPPA-ELISA) Protein Arrays

2.6.1 cDNA Preparation

1. LB Broth (Miller) medium.
2. Ampicillin: Dissolve ampicillin powder at 100 mg/mL in deionized water. Filter through 0.2 μ M filter. Aliquot in 1 mL portions.
3. Kanamycin: Dissolve kanamycin powder at 50 mg/mL in deionized water. Filter through 0.2 μ M filter. Aliquot in 1 mL portions. Thawed aliquots should be recently diluted 1000-fold into medium or agar.
4. Human genes in pANT7_cGST which allows in situ expression of GST-tagged proteins (Center for Personalized Diagnostics Arizona, USA, and www.dnasu.org).
5. Pure yield plasmid miniprep system (Promega).

2.6.2 In Situ Protein Expression

1. SuperBlock™ Blocking Buffer in PBS (Thermo Fisher Scientific, Ref 37535).
2. Corning™ 96-Well Solid Black Polystyrene Microplates (Thermo Fisher Scientific).
3. Phosphate-buffered saline (PBS) pH 7.4: 3.6 g Na_2HPO_4 , 0.2 g KCl, 0.24 g KH_2PO_4 , 8 g NaCl in 1 L.
4. Phosphate-buffered saline Tween 20 (PBST): 0.1% Tween 20 to PBS.
5. Carbonate-buffered saline pH 9.6: 0.159 g Na_2CO_3 , 0.293 NaHCO_3 in 1 L.
6. Milk powder.
7. Mouse anti-GST antibody (Cell Signaling Technologies Inc.).
8. TnT® Coupled Reticulocyte Lysate System (Promega, Ref. L4610).

2.6.3 ELISA Test

1. 3,3',5,5'-Tetramethylbenzidine (TMB) Liquid Substrate System for ELISA (Sigma-Aldrich).
2. HRP-linked antihuman antibody (Jackson ImmunoResearch Laboratories).
3. HRP-linked anti-mouse antibody (Jackson ImmunoResearch Laboratories).

3 Methods

The following protocol describes the procedures for incubation of protein solution by direct dye in microarrays of antibodies (Fig. 3) and self-assembled (NAPPA-ELISA) protein arrays. This protocol pays attention for easy implementation of the method in other laboratories.

3.1 Cell Culture

3.1.1 Cell Culture Conditions

MG-63 cells are grown in a 75 cm² flask for 24 h in RPMI with 10% FBS and 100 U/mL penicillin and 100 µg/mL streptomycin at 37 °C in a 5% CO₂ atmosphere.

3.2 Preparation of Samples

3.2.1 Extraction of Protein

1. Cells are harvested and washed 3× with 1× PBS Na⁺/K⁺.
2. Cells are resuspended in 3 volumes of lysis buffer (5 mM HEPES, 10 mM MgCl₂, 140 mM NaCl, 0.1% (v/v) Tween 20) at pH 8.0, and 1% (v/v) protease inhibitor cocktail (*see Note 2*).
3. Lysed cells are centrifuged for 15 min at 15,000 × *g* at 4 °C.
4. Supernatant is collected in a new tube and 10% (w/v) octyl-β-D-glucopyranoside is added.
5. Solution is sonicated three times with 3-s bursts.
6. Samples are incubated on ice for 30 min.
7. Tube is centrifuged as in **step 4** to obtain the cytoplasmic fraction.

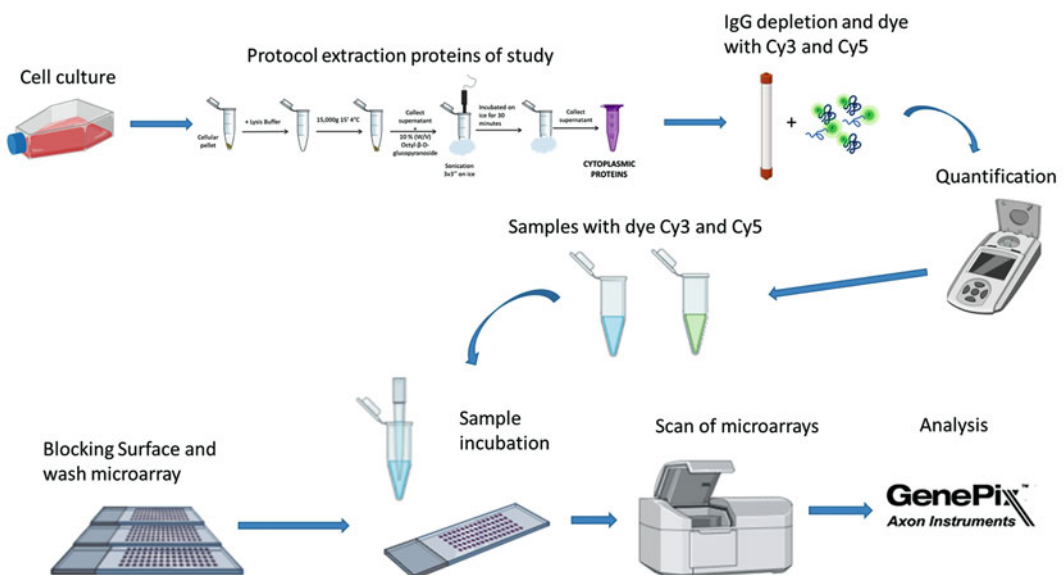


Fig. 3 Preparation and incubation of samples in an antibody microarray using the direct dye method. (Created with BioRender.com)

3.2.2 IgG Depletion, Quantification

1. IgG depletion is made by using protein G agarose beads.
2. Purified proteins are quantified by Lowry following the manufacturer's instruction (*see* **Note 3**).

3.2.3 Dye Labeling

1. After sample quantification we prepare two aliquots of 1 mL with a final concentration of protein of 1 mg/mL for each sample of study. Sample is diluted in 0.1 M sodium carbonate/sodium bicarbonate pH 9.3.
2. One aliquot of sample is transferred to a vial of Cy3 Mono-Reactive CyDye.
3. Same procedure of **step 2** is made for a vial of Cy5 Mono-Reactive CyDye.
4. Gently vortex the vials.
5. Incubate for 30 min with gentle vortex each for 10 s at room temperature and in darkness.
6. Eliminate unconjugated dye by gel filtration columns following the manufacturer's instruction.
7. Eluted proteins are quantified at 280 nm by NanoDrop.

3.3 Antibody Microarray Blocking

3.3.1 Blocking the Microarray Surface

1. 6 mL of blocking buffer is added to each microarray washing chamber (*see* **Note 4**).
2. The microarray is immersed in the blocking buffer and incubated in agitation for 1 h at room temperature with orbital shaker (*see* **Note 5**).

3.3.2 Wash Microarray

1. Remove the blocking buffer and add distilled water (*see* **Note 6**).
2. Manually shake the wash chamber microarray.
3. Remove the distilled water and add new.
4. Repeat **steps 2** and **3** twice.
5. Remove the distilled water and keep the microarrays in clean distilled water until they are incubated.

3.3.3 Microarray System Incubation

1. Remove the microarray from the washing chamber and dry very carefully and without touching the surface on absorbent paper (gently tapping on the edge) (*see* **Note 7**).
2. Place the incubation chamber on the surface of the microarray.
3. Put the clips for microarray systems.

3.4 Incubation of Dye-Labeled Protein Lysates in Antibody Microarrays

3.4.1 Microarray Incubation

1. Prepare 100 μ L of each mix of sample dye Cy3/Cy5 (1 μ g/ μ L).
2. Add 100 μ L of a mix of sample dye Cy3/Cy5 (1 μ g/ μ L) in each subarray (*see Note 8*).
3. Wrap the microarray system incubation with transparent film and put in rotatory mixer.
4. Incubate overnight at 4 °C in rotatory mixer (*see Note 9*).

3.4.2 Washing and Drying Microarray

1. Remove the microarray system incubation and sample.
2. Add 1 $\times$ PBS Na⁺/K⁺-0.05% Tween 20 and put the microarray in washing chamber.
3. Strongly shake in an orbital shaker for 5 min.
4. Remove the 1 $\times$ PBS Na⁺/K⁺-0.05% Tween 20 and add new.
5. Repeat **steps 3** and **4** four more times.
6. We took the microarrays out of the 1 $\times$ PBS Na⁺/K⁺-0.05% Tween 20.
8. Put each microarray in a falcon of 50 mL with a hole in the bottom and centrifuge at room temperature for 3 min at 1000 $\times g$.

3.5 Microarray Scanning and Data Analysis

1. Microarrays are scanned with Scanner GenePix 4000B (Axon) using Cy3- and Cy5-like settings.
2. The images are analyzed with GenePix Pro 4.0 software (Axon). The GenePix Array List file (.gal) generated after printing of array is used as a reference to locate and identify each spot on the microarray surface.
3. Background correction for each spot is locally made by applying standardized settings of GenePix software.
4. Mean values of negative control set of spots in each fluorescence channel (Cy3/Cy5) are subtracted from individual spot values.
5. Each normalized slide is made according to Ignacio E. León et al. [20, 21] and based on Diez P. et al.'s study [22]. For this, after obtaining the raw intensity data, a reference protein (housekeeping) with a highly expressed fluorescence intensity value is selected.
6. After reference protein selection, fluorescence intensity value for each protein is divided for fluorescence intensity value for a housekeeping protein selected.
7. In this case, z-score estimated was a value >2 and for replicated proteins the mean of fluorescence values obtained was used to obtain a single value for this protein.

3.6 Self-Assembled (NAPPA-ELISA) Microtiter Protein Arrays

A GST fused to the protein genes was expressed in vitro using a reticulocyte lysate, as described here:

3.6.1 cDNA Preparation

1. *Escherichia coli* was grown for 24 h at 37 °C in 100 mL of LB medium supplemented with ampicillin for cGST clones according to the previous reports [23].
2. Plasmid DNA was purified using the PureYield Plasmid Mini-prep System according to the manufacturer's instructions.

3.6.2 In Situ Protein Expression

96-Well Plate Activation for NAPPA-ELISA

1. Prepare a solution of anti-GST antibody in carbonate buffer at 4 µg/mL.
2. Add 100 µL of the antibody solution to each well.
3. Cover the 96-well plate and incubate overnight at 4 °C.
4. Prepare a blocker buffer: PBST and milk powder 5%.
5. Turn over the plate to discard the antibody supernatant.
6. Add 200 µL of the blocker buffer to each well and incubate overnight at 4 °C.

In Situ Protein Expression (IVTT) [24, 25]

1. Turn over the plate to discard the blocker solution.
2. Wash three times the blocked plates with 200 µL PBS, removing the solutions by turning over the plate.
3. Take an appropriated cDNA volume and the TnT[®] Coupled Reticulocyte Lysate System kit (see **Notes 10** and **11**). Perform the procedure according to the manufacturer's instructions to obtain the dye-labeled proteins (see **Notes 12** and **13**).
4. Add the total volume of IVTT-expressed protein to each well.
5. Incubate overnight with agitation at 4 °C.
6. Wash well plate with blocking buffer (200 µL/well) and remove the supernatant by turning over the plate, trying to eliminate the buffer as much as possible. Repeat this step five times.

Incubation with Dye-Labeled Protein Lysates

1. Prepare the samples with the blocking buffer and add 100 µM (final concentration) to each well.
2. For cellular lysate samples, prepare a 1:300 dilution (v/v).
3. Mouse anti-GST monoclonal antibody at 1:1000 dilution (v/v) to detect the protein expressed in IVTT system.
4. Negative control: IVTT without cDNA and mouse anti-GST antibody at 1:1000 dilution (v/v).
5. Incubate for 2 h at room temperature (RT) with agitation.
6. Wash the plates three times with 200 µL blocker buffer, removing the solutions by turning over the plate.

3.6.3 ELISA Assay of IVTT Protein

1. Prepare the secondary antibody solutions:
 - (a) Samples of cellular lysate: HRP-anti-human antibody in blocker solution (dilution 1:1000 (v/v)).
 - (b) Positive and negative controls: HRP-linked anti-mouse antibody in blocker solution (dilution 1:1000 (v/v)).
2. Incubate for 1 h at RT with agitation.
3. Wash the plates three times with 200 μ L PBS, removing the solutions by turning over the plate.
4. Add 100 μ L TMB solution to each well and protect from light.
5. Incubate for 30 min at RT with agitation.
6. Read the results at 450 nm.

4 Results

These described antibody microarrays allow to decipher differential altered cell signaling pathways in a particular tumoral cell line at different conditions, which could provide relevant functional information and discovery target molecules. Here, it is reviewed, as an example of small molecule, as a therapeutic drug, to identify differential expressed pathways as potential protein candidates for drug-targeted therapy (Fig. 4). In particular, a drug (oxovanadium-IV complex with the flavonoid chrysin (VO(CQ)₂) in MG-63 (human osteosarcoma cell lines))) was simultaneously evaluated in several conditions (i.e., drug concentration, incubation times) and 220 targeted proteins covering mostly relevant intracellular cell signaling pathways [20, 21].

At first glance, several differential expressed proteins were observed (*see* Table 1), where canonical cell signaling pathways were significantly differentially expressed. Results obtained after studying global differential relative protein expression profiles on the alteration in the intracellular cell signaling pathways due to the drug in different concentrations were (1) apoptosis, (2) cell cycle, (3) cell stress, and (4) signaling transduction.

For conditions 2.5, 25, and 100 μ M of VO(CQ)₂ for 6 h, the complex induced the increase of relative levels of antiapoptotic proteins (Bcl-x) and pro-apoptotic proteins (Bcl-10, CASP6, CASP7, CASP10, and CASP11). Moreover, Ignacio León et al. [21] obtained with 2.5 μ M of VO(CQ)₂ for 6 h a Cy5/Cy3 ratio of Bcl-x higher than 10 for 10 μ M of VO(CQ)₂ for 6 h. This effect of the complex concentration could be explained by the upregulation level of active CASP3 for a condition of 10 μ M of VO(CQ)₂ for 6 h and its cross-regulation with Bcl-x.

Moreover, these authors describe for all different conditions of concentration of drug (except 25 μ M of VO(CQ)₂ for 3 h) that VO(CQ)₂ seems to induce the upregulation of several proteins

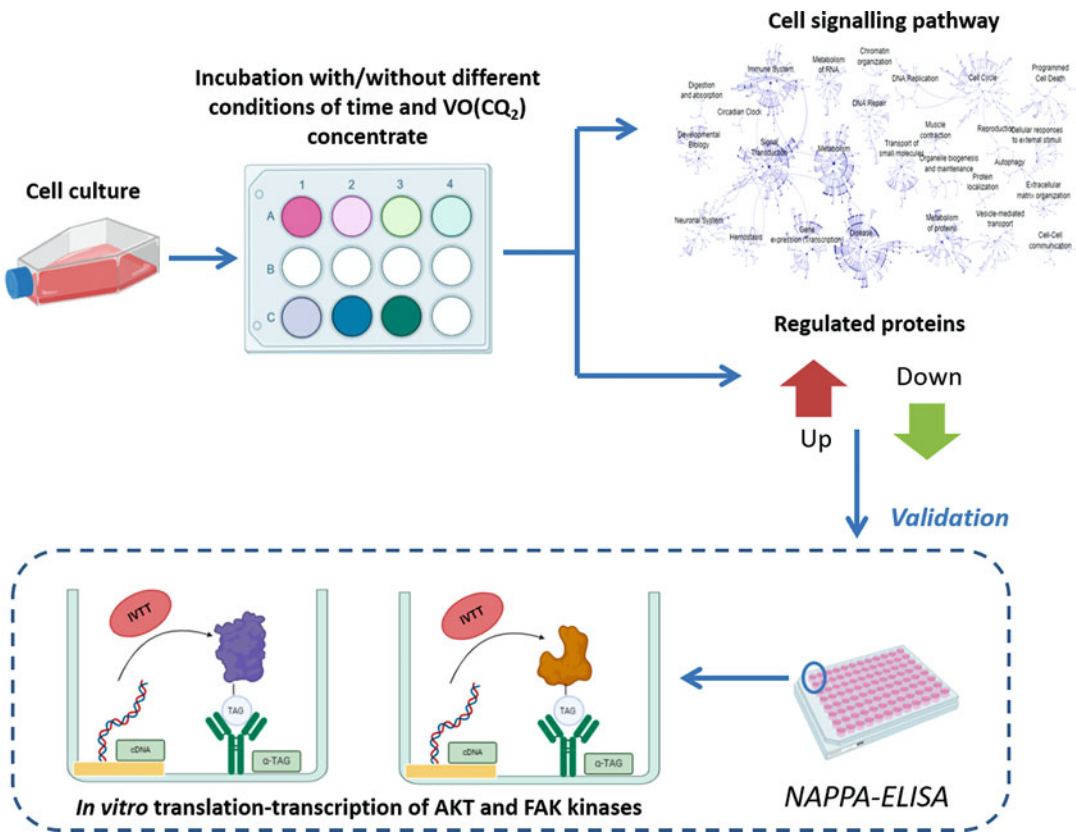


Fig. 4 Study of cell signalling pathway and type regulated of protein in a tumoral cell line (MG-63) under different time and concentration of oxovanadium IV complex with the flavonoid chrysin (VO(CQ₂)) conditions. (Created with BioRender.com)

Table 1
Conditions of study

Condition	Incubation time (h)	Upregulated proteins	Downregulated proteins
Control	3	—	—
Control	6	—	—
2.5μM of VO(CQ ₂)	6	49	11
10μM of VO(CQ ₂)	6	20	0
25μM of VO(CQ ₂)	3	22	2
25μM of VO(CQ ₂)	6	27	8
100μM of VO(CQ ₂)	6	82	9

involved in the cell cycle regulation, such as c-Myc, E2F1, cyclin D3, p21Waf-1, SMAD4, Ap-1/cjun, ATF2, HADC4, and PCAF, among others, suggesting the key role of these transcription factors in cell cycle progress and apoptosis. Also, samples had an enrichment in FAK (Tyr⁵⁷⁷) suggesting the importance of this site of phosphorylation in the response of tumor against chemotherapeutic drug actions.

Only samples treated with 100 mM of VO(CQ)₂ presented low levels of Cdc6 and cyclin D1, while cyclin A and D3, Cdc25 and CDK4, and 6 and 7 were upregulated confirming an increment in cell cycle activity in direct relation with the function of the concentration of VO(CQ)₂.

AP2 family (alpha, beta, and gamma) was upregulated in conditions of 2.5 and 10 μM of VO(CQ)₂ for 6 h. These suggest that this family of transcription factors could be involved in the anticancer activity of VO(CQ)₂.

In both cases, functional characterization was using the bioinformatics software DAVID 6.7 for these examples and other protein studies in the articles [20, 21].

On the other hand, the down- and upregulated proteins identified in each group of treatment (drug's different concentrations) were used for identification of potential novel targets by determination of differential expression profiles.

One example of the molecular targets detected in both reports is the PKC family (PKCb, PKCg) and FAK as proteins common to all treatments; this suggests that they play an important role associated with anticancer effects of VO(CQ)₂, in addition to confirming the complexity of the interaction network of different kinases involved in the antitumor activity of VO(CQ)₂. Once the relative protein expression was determined and the correlation with the altered cell signaling pathways was identified, then AKT and FAK kinases were functionally validated by a NAPPA-ELISA (Fig. 4). In this case, an in situ cell-free protein expression and in vitro transcription-translation (IVTT) of recombinant kinases AKT1 and FAK tagged with GST in COOH terminus were carried out [20, 21].

As quality controls of the assay, the expression of the complete protein was corroborated thanks to the signal associated to the α-GST antibody. In the same way, the results showed the compatibility of vanadium compounds with the IVTT system.

With this strategy, the pattern of tyrosine phosphorylation sites induced by studied compounds was studied by determining the phospho-tyrosine/GST signal ratio. In both cases, vanadium complexes decrease the level of tyrosine phosphorylation of AKT1 and FAK in the majority of conditions tested. These results also correlate with the data obtained in the antibody array study, where it was found that one of the vanadium complexes inhibited selectively the autophosphorylation activity (Tyr397) of FAK kinase [20].

To finalize the functional validation of the method, the expression level of FAK kinase was evaluated. In this case, the treatment with 50 and 100 mM generated a significant decrease of translation of the protein (40% and 95%, respectively).

As conclusions to both studies, thanks to array technology and cell-free IVTT system, vanadium complexes were tested and their intracellular signaling was extensively described at the same time that their potential pharmacological targets were functionally validated.

5 Notes

1. Blocking buffer must be well mixed; for this, you should give it a gentle vortex.
2. Depending on the needs of the study, we will use different protein extraction protocols. In this chapter, we use a cytoplasmic sub-fractionation protocol.
3. Detergents, surfactants, and other reagents present in the lysis buffer may interfere with the quantification. For this reason, according to protocol extraction and needs of the study, other quantification methods can be used such as Bradford or NanoDrop.
4. The microarray must be completely covered by the blocking buffer.
5. We must be careful when introducing the microarray. Surface should never be touched because we could spoil spot printing. Pay special attention to which side the spots are printed.
6. Tilt the washing chamber of microarrays to remove solution. Do not add on top of the microarray the different solutions necessary for washing process, always in areas of washing chamber that are not occupied by the microarray.
7. Lift the microarray out of the washing chamber very carefully with a pair of tweezers. Never touch the printed area of the slide with the tweezers or any other object or hand. Hold the microarray by the side edges and place it vertically. Once the microarray has been secured, gently tap on several sheets of absorbent paper to remove any excess solution.
8. Be very careful not to generate bubbles in the hatchery when the sample is added. If they have occurred, move the chamber from left to right and up and down carefully and always keep it on a surface.
9. Ensure that the sample is distributed evenly throughout the chamber. Modify, if necessary, the inclination of the rotatory mixer.

10. cDNAs corresponding to the recombinant proteins to be expressed must be found in the vectors required by the selected in situ expression system.
11. Take a sufficient volume of cDNA to obtain the minimum amount necessary to perform the expression (0.5 µg but ideally 1 µg). Normally, the amount of cDNA is established in the specific guidelines/protocol for each kit.
12. If the volume to be taken of cDNA exceeds the recommended volume to carry out the expression, according to the manufacturer's instructions, the samples will be concentrated by evaporating the elution buffer in the thermoblock at a temperature of 37 °C or better, precipitating the DNA with isopropanol and sodium acetate, and resuspending in the required volume with DEPC water.
13. Expression kits are typically stored at −80 °C and must be thawed gradually on ice.

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References

1. Gattei V, Bulian P, Del Principe MI et al (2008) Relevance of CD49d protein expression as overall survival and progressive disease prognosticator in chronic lymphocytic leukemia. *Blood* 111:865–873
2. Chiorazzi N (2012) Implications of new prognostic markers in chronic lymphocytic leukemia. *Hematology Am Soc Hematol Educ Program* 2012:76–87
3. Damm F, Mylonas E, Cosson A et al (2014) Acquired initiating mutations in early hematopoietic cells of CLL patients. *Cancer Discov* 4:1088–1101
4. Brachtl G, Piñón Hofbauer J, Greil R, Hartmann TN (2014) The pathogenic relevance of the prognostic markers CD38 and CD49d in chronic lymphocytic leukemia. *Ann Hematol* 93:361–374
5. Schick M, Habringer S, Nilsson JA, Keller U (2017) Pathogenesis and therapeutic targeting of aberrant MYC expression in haematological cancers. *Br J Haematol* 179:724–738
6. Burger JA, Chiorazzi N (2013) B cell receptor signaling in chronic lymphocytic leukemia. *Trends Immunol* 34:592–601
7. Matarraz S, González-González M, Jara M et al (2011) New technologies in cancer. Protein microarrays for biomarker discovery. *Clin Transl Oncol* 13:156–161

8. Díez P, Góngora R, Orfao A, Fuentes M (2017) Functional proteomic insights in B-cell chronic lymphocytic leukemia. *Expert Rev Proteomics* 14:137–146
9. Díez P, Dégano RM, Ibarrola N et al (2015) Genomics and proteomics for biomarker validation. In: *Biomarker validation*. Wiley, Hoboken, NJ, pp 231–242
10. Gonzalez-Gonzalez M, Jara-Acevedo R, Matarraz S et al (2012) Nanotechniques in proteomics: protein microarrays and novel detection platforms. *Eur J Pharm Sci* 45:499–506
11. Lourido L, Díez P, Dasilva N et al (2014) Protein microarrays: overview, applications and challenges. In: Marko-Varga G (ed) *Genomics and proteomics for clinical discovery and development*. Springer Netherlands, Dordrecht, pp 147–173
12. Juanes-Velasco P, Carabias-Sanchez J, Garcia-Valiente R, et al (2018) Microarrays as platform for multiplex assays in biomarker and drug discovery. *Rapid Test - Advances in Design, Format and Diagnostic Applications*
13. Casado-Vela J, Fuentes M, Franco-Zorrilla JM (2014) Chapter 8: Screening of protein–protein and protein–DNA interactions using microarrays: applications in biomedicine. In: Donev R (ed) *Advances in protein chemistry and structural biology*. Academic, Cambridge, pp 231–281
14. Fernández-García J, García-Valiente R, Carabias-Sánchez J et al (2019) A systematic workflow for design and computational analysis of protein microarrays. In: Narayan R (ed) *Encyclopedia of biomedical engineering*. Elsevier, Oxford, pp 213–222
15. Uhlén M, Fagerberg L, Hallström BM et al (2015) Proteomics. Tissue-based map of the human proteome. *Science* 347:1260419
16. Ramachandran N, Raphael JV, Hainsworth E et al (2008) Next generation high density self assembling functional protein arrays. *Nat Methods* 5:535–538
17. Wong J, Sibani S, Lokko NN et al (2009) Rapid detection of antibodies in sera using multiplexed self-assembling bead arrays. *J Immunol Methods* 350:171–182
18. Henjes F, Lourido L, Ruiz-Romero C et al (2014) Analysis of autoantibody profiles in osteoarthritis using comprehensive protein array concepts. *J Proteome Res* 13:5218–5229
19. Manzano-Román R, Fuentes M (2019) A decade of nucleic acid programmable protein arrays (NAPPA) availability: news, actors, progress, prospects and access. *J Proteomics* 198:27–35
20. León IE, Díez P, Etcheverry SB, Fuentes M (2016) Deciphering the effect of an oxovanadium(IV) complex with the flavonoid chrysin (VOChrys) on intracellular cell signalling pathways in an osteosarcoma cell line. *Metallomics* 8:739–749
21. León IE, Díez P, Etcheverry SB, Fuentes M (2017) Decoding the anticancer activity of VO-clioquinol compound: the mechanism of action and cell death pathways in human osteosarcoma cells. *Metallomics* 9:891–901
22. Díez P, Dasilva N, González-González M et al (2012) Data analysis strategies for protein microarrays. *Microarrays* 1:64–83
23. Manzano-Román R, Díaz-Martín V, González-González M et al (2012) Self-assembled protein arrays from an *Ornithodoros moubata* salivary gland expression library. *J Proteome Res* 11:5972–5982
24. Barba-Vicente V, Parra MJA, Boyero-Benito JF et al (2020) Detection of human p53 in-vitro expressed in a transcription-translation cell-free system by a novel conjugate based on cadmium sulphide nanoparticles. *Nanomaterials* 10:984
25. Camacho-Encina M, Balboa-Barreiro V, Rego-Perez I et al (2019) Discovery of an autoantibody signature for the early diagnosis of knee osteoarthritis: data from the osteoarthritis initiative. *Ann Rheum Dis* 78:1699–1705



Combination of Antibody Arrays to Functionally Characterize Dark Proteins in Human Olfactory Neuroepithelial Cells

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Abstract

The completion and annotation of the human proteome require the availability of information related to protein function. Currently, more than 1800 human genes constitute the “dark proteome,” which include missing proteins, uncharacterized human genes validated at protein level, smORFs, proteins from lncRNAs, or any uncharacterized transcripts. During the last years, different experimental workflows based on multi-omics analyses, bioinformatics, and in vitro and in vivo studies have been promoted by the Human Proteome Project Consortium to enhance the annotation of dark proteins. In this chapter, we describe a method that utilizes recombinant proteins and antibody arrays to establish a straightforward methodology in order to rapidly characterize potential functional features of dark proteins associated to intracellular signaling dynamics and extracellular immune response in human cell cultures. Further validating the method, this workflow was applied to probe changes in the activation patterns of kinases and transcription factors as well as in cytokine production modulated by the dark C1orf128 (PITHD1) protein in human olfactory neuroepithelial cells.

Key words PITHD1, Olfactory cells, Antibody arrays, Human Proteome Project

1 Introduction

After the previous success of the Human Genome Project (HGP) providing an accurate fingerprint of the genes encoding the human genome [1], the Human Proteome Project (HPP) was designed to map the entire human proteome in order to understand human biology at cellular level [2]. The initial goal of the HPP was to identify at least one representative protein encoded by each of the approximately 20,000 human genes defined in the HGP in a systematic effort using currently available and emerging techniques. For that, efforts of international teams have been dedicated aiming to characterize proteins taking into account tissue localization,

major isoforms, posttranslational modifications (PTMs), and use of quantitative mass spectrometry and antibody reagents as main standardized approaches. On the other hand, another commitment of the HPP working group was to publicly share all data through coordinated proteomic databases such as UniProtKB/Swiss-Prot [3], PRIDE [4], PeptideAtlas [5], and ProteomeXchange [6]. Another of these resources is neXtProt [7], an online knowledge platform on human proteins gathering information regarding function, subcellular location, interactions, expression, and even role in diseases. Annually, neXtProt incorporates new information from a big variety of sources (UniprotKB, Bgee [8], PeptideAtlas [9], Ensembl [10], COSMIC [11], among others) with particular emphasis on expression, proteomic, and variant data. According to the last neXtProt release, the predicted number of human proteins is now 20,350 (release 2020-01-17) [12]. These can be divided into five classes depending on their type of evidence. The proteins from PE1 level have credible evidence of protein expression and identification by mass spectrometry, immunohistochemistry, amino acid sequencing, and 3D structure. The proteins from PE2 level recognize transcript expression evidence, without evidence of protein expression. Those from PE3 level represent the lack of protein or transcript evidence but with presence of a homologous protein in related species. Finally, the proteins from PE4 level hypothesize from gene models and in silico predictions and proteins from PE5 are hypothetical gene products, pseudogenes, or proteins from other dubious information. Among this distribution, proteins belonging to PE2 to PE4 are considered missing proteins, since they lack sufficient experimental evidence for their existence supported by mass spectrometry. Most of them have known or predicted function by homology with protein family members. However, it is worth to mention that among neXtProt database, 1937 proteins, accounting for approximately 10% of the total number of human proteins, have no annotated specific function and are considered “dark proteins.” Interestingly, 1254 belong to the PE1 group, meaning that although experimental information regarding these proteins has been annotated, there is no information concerning their biological function. In this sense, a new pilot project called next-CP50 Pilot Project was launched by HPP in the 2017 Annual Meeting of HUPO in Dublin in order to characterize the function of 50 uncharacterized proteins (uPE1) within 3 years.

As part of the Spanish HPP initiative, our group has been focused on the evaluation of this uncharacterized proteome within the human olfactory system. Specifically, we have focused our attention on the uncharacterized protein PITH (C-terminal proteasome-interacting domain of thioredoxin-like) domain containing protein 1 (PITHD1; Clorf128). Although some functional features have been described for this protein mainly related to

megakaryopoiesis and ovarian carcinoma [13–16], actual function for the PITHD1 protein is still unknown. Previous reports of our group have revealed altered protein levels for PITHD1 at the level of the olfactory bulb in both Alzheimer’s disease and Parkinson’s disease [17, 18]. So, aiming to increase the knowledge of PITHD1 function and its potential implication in the neurodegenerative process occurring within the olfactory system, we have built an exhaustive workflow combining neuropathological diagnosis, co-regulation maps, physical and functional interaction data, and intracellular and extracellular proteomics combining mass spectrometry and antibody arrays.

The array technology is a term that refers to the miniaturization of thousands of assays on one small plate. Concerning protein arrays, the most representative model of analytical protein arrays is the antibody arrays. Providing a high sensitivity, the sandwich assay format is now widely used. This format uses two different antibodies to detect the targeted proteins [19]. Thus, the capture antibodies are placed on the solid phase where they immobilize the targeted proteins or analytes. Then, the other antibodies, called the reporter or detection antibodies, generate a signal for the detection system. Thus, using two antibodies significantly increases the specificity and sensitivity of this “sandwich” format [19]. In this sense, the great versatility of this technology has led to the development of commercially available protein arrays in many different formats. Thus, it provides commercial access to researchers who are not familiar with this technology allowing them to study phosphorylation events, autophagy, apoptosis, or stress biomarkers and importantly cytokines and growth factors. In addition, other advantages of this technology are its speed and the small amount of sample required. Hence, the results can be obtained as soon as 8–9 h and as much as 4 days and for instance cytokine arrays can be performed using 50–1000 µg of protein. Last but not least, this procedure can be developed using different sample types, from fluids such as serum or cell culture medium, to cell or tissue protein extracts, depending on the aim of the experiment. On the other hand, it is important to take into account that there are many protein array formats, with different number of targets to study, different solid supports (membrane or glass slides), and different types of results (quantitative or semiquantitative).

This chapter focuses on the utility of antibody arrays to establish a straightforward methodology in order to rapidly characterize potential functional features of PITHD1 associated to signaling dynamics and immune response in human olfactory neuroepithelial cells. This workflow can be adapted by the biology/disease-HPP research teams as a guidance to decipher functional features of dark proteins (Fig. 1).

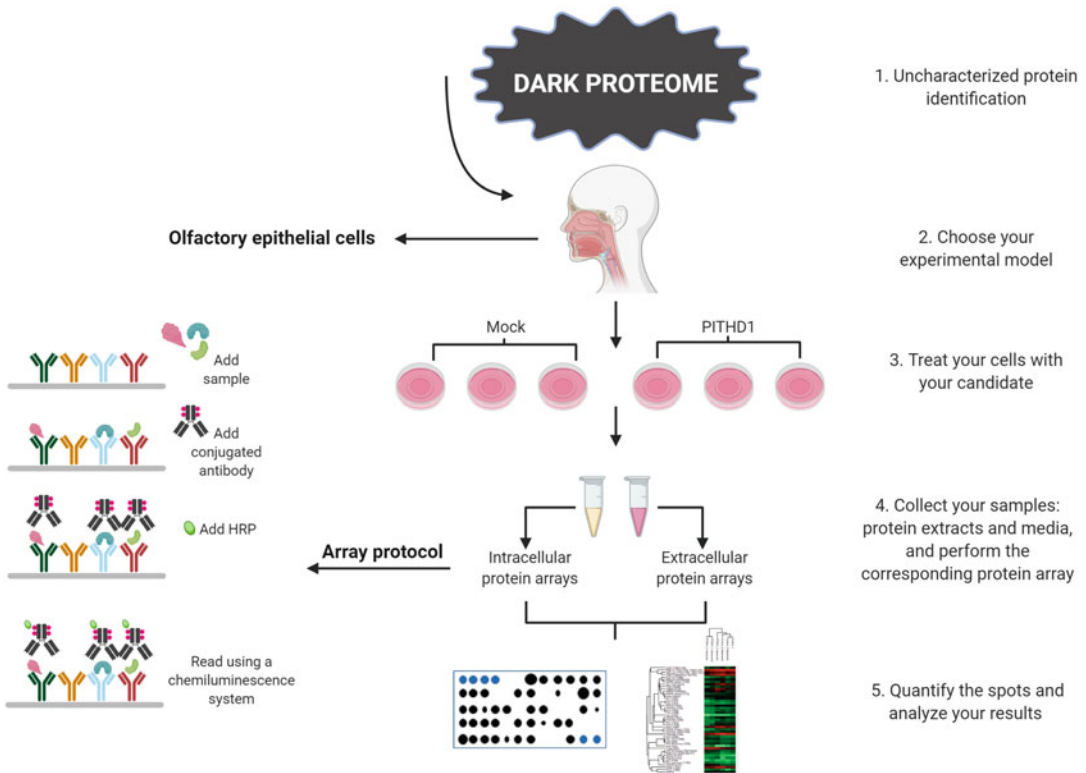


Fig. 1 Technical workflow based on antibody arrays used to characterize functional features of PITHD1 protein in olfactory epithelial cells

2 Materials

2.1 Reagents

2.1.1 Cell Culture

1. DMEM high glucose, pyruvate (Gibco).
2. Fetal bovine serum (Gibco).
3. Antibiotic-antimitotic (Gibco).
4. Trypsin-EDTA (0.25%), Phenol Red (Gibco).
5. Phosphate-buffered saline 1×: 6.7 mM PO₄ without calcium and magnesium.
6. Flasks, plates, pipettes, etc.
7. rPITHD1: PITHD1 recombinant protein (*E. coli*) (MyBioSource).
8. Lysis buffer: 7 M Urea, 2 M thiourea, 50 mM DTT.

2.1.2 Cytokine and Phosphokinase Arrays

1. Cytokine or phosphokinase antibody array membranes (Abcam and R&D Systems).
2. 8-Well plastic tray.
3. Plastic sheets.
4. 1× Blocking buffer (provided by the kit).

5. 20× Wash buffer I (provided by the kit).
6. 20× Wash buffer II (provided by the kit).
7. 2000× Biotin-conjugated anti-cytokines.
8. 1000× HRP-conjugated streptavidin.
9. Detection buffer C.
10. Detection buffer D.
11. Tweezers.
12. Small plastic boxes/containers/centrifuge tubes (50 ml).
13. Tissue paper/blotting paper/chromatography paper.

2.2 Equipment

1. Centrifuge 5427 R (Eppendorf).
2. Orbital shaker/oscillating rocker/roller.
3. Chemidoc MP Imaging System (BioRad).
4. ImageLab Software (BioRad).
5. Perseus software [20].

3 Methods

3.1 Harvest, Isolation, Culture, and Maintenance of Human Olfactory Neuroepithelial Cells

1. Sterilize forceps for 2 min at 200 °C (or autoclave them).
2. Prepare the necessary Eppendorf tubes to collect the samples inside a cabin with 500 µl of complete medium. Put them on ice.
3. Also prepare an autoclaved pipette tip.
4. Keep complete medium at 37 °C.
5. Nasal biopsies are then obtained using a giraffe forceps and immediately transferred to culture medium for transport to the laboratory.
6. Clean the Eppendorf tubes with ethanol before putting them in the cabin.
7. Hold the brush with tweezers or a forceps and pass it through the cut tip on the Eppendorf tube so that the sample stays on the Eppendorf tube and not on the brush.
8. With a P1000 pipette, add 250 µl of medium inside the tip to lower what may be left of the sample.
9. With a P200 pipette, pipet up and down with the cut tip of the Eppendorf tube to clean it and make the whole sample stay in the middle.
10. Disintegrate with P1000 pipette (avoid bubbles). Disaggregate with P200 and P10 pipettes.
11. Cultivate in P24 wells. Cell growth should be monitored by microscope during the next days.

12. Change medium after a week. Carefully aspirate the old medium from the side of the well and add 500 μ l of new complete medium.
13. Incubate for 3 weeks or until confluence and perform first passage (*see Note 1*).

3.2 Treatment with Recombinant PITHD1

1. Culture cells in P10 plates (*see Note 2*).
2. The next day, remove medium in order to remove nonattached cells.
3. In new medium, add the rPITHD1 recombinant protein (1 μ g/ml) (*see Note 3*).
4. Cultivate cells for 24 h.

3.3 Protein Extraction

1. Remove medium and do not discard it (*see Note 4*).
2. Wash cells with PBS (*see Note 5*).
3. Trypsinize cells for 3–5 min at 37 °C and gently collect detached cells into a centrifuge tube.
4. Centrifuge cells during 5 min at 500 $\times g$ to remove trypsin solution and discard supernatant.
5. Resuspend pellet with lysis buffer containing 7 M urea, 2 M thiourea, and 50 mM DTT.
6. Incubate samples on ice for 30 min, vortex, and spin every 10 min (*see Note 6*).
7. Centrifuge samples for 1 h at 20,000 $\times g$ at 15 °C.
8. Quantify proteins using a protein quantification protocol such as Bradford and Lowry.

3.4 Sample Incubation on Cytokine/Phosphokinase Antibody Membranes (See Note 7)

1. Grasp membranes by the edges using tweezers and place each membrane into the 8-well tray printed side up (*see Note 8*).
2. Block membranes incubating with 2 ml 1 $\times$ blocking buffer in a shaker at room temperature for 30 min.
3. Prepare samples. Dilute them if it is necessary with blocking buffer (*see Note 9*).
4. Aspirate 1 $\times$ blocking buffer from each well (*see Note 10*).
5. Pipette 1 ml of sample into each well and incubate for 1.5–2 h at room temperature (RT) or overnight (o/n) at 4 °C using a shaker or oscillating rocker (*see Note 11*).
6. Aspirate samples from each well.
7. After o/n incubation: wash membranes in separate containers with 20–30 ml of 1 $\times$ wash buffer I (*see Note 12*) per membrane for 30–45 min at RT on a shaker. Then wash membranes for 5 min at RT on a shaker for a total of three washes.

8. Alternatively, after 1.5–2-h incubation, pipette 2 ml of 1× wash buffer I into each well and incubate for 5 min at RT on a shaker. Repeat this step twice for a total of three washes.
9. Wash membranes using 1× wash buffer II. Pipette 2 ml of 1× wash buffer II into each well and incubate for 5 min at RT on a shaker. Repeat this step for a total of two washes.
10. Pipette 1 ml of 1× biotin-conjugated anti-cytokines into each well and incubate for 1.5–2 h at RT or o/n at 4 °C using a shaker (*see* **Note 13**).
11. Aspirate the 1× biotin-conjugated anti-cytokines from each well.
12. Wash membranes as indicated in **steps 7–9**.
13. Pipette 2 ml of 1× HRP-conjugated streptavidin into each well and incubate on a shaker for 2 h at RT or o/n at 4 °C on a shaker (*see* **Note 14**).
14. Aspirate the 1× HRP-conjugated streptavidin from each well.
15. Wash membranes as indicated in **steps 7–9**.

**3.5 Cytokines and
Phosphokinase
Detection (See
Note 15)**

1. Transfer each membrane printed side up into a sheet/chromatography/blotting paper (absorbent paper) on a flat surface. Remove any excess of washing buffer.
2. Transfer each membrane printed side up onto one of the plastic sheets provided with the kit on a flat surface.
3. Prepare equal volumes of detection buffer C and detection buffer D (*see* **Note 16**).
4. Pipette 500 µl of the detection mixture onto each membrane and incubate for 2 min at RT (*see* **Note 17**).
5. Carefully place another plastic sheet on top of your membrane starting from one end and gently rolling the sheet across the surface to the opposite end to prevent bubble from forming.
6. Using forceps transfer the sandwiched membrane to the imaging system (*see* **Note 18**).
7. Let the exposure begin with the following parameters (*see* **Note 19**):
Using ImageLab software (BioRad):
 - (a) New protocol.
 - (b) High resolution.
 - (c) Select the size of the image (*see* **Note 20**).
 - (d) Membrane exposure: automatic (*see* **Note 21**).
 - (e) Save the image.
8. After exposure, remove the upper plastic sheet and place the membrane in the corresponding well of the tray. Clean both plastic sheets with water and dry them. Repeat the process from **step 4** for all of the membranes (*see* **Note 22**).

9. It is possible to store membranes. Sandwich them between the plastic sheets provided with the kit. It is possible to store six membranes in each sandwich. Afterwards, tape the sheets together or wrap in plastic wrap to secure them and store them at $\leq -20^{\circ}\text{C}$ (*see Note 23*).

3.6 Cytokines and Phosphokinase Quantification (ImageLab Software)

1. Use the “Volume tools” option to measure the density of each protein spot (*see Note 24*).
2. Click on “Analysis table” to get the quantitative density values of each spot.
3. Export table as Excel table format.

3.7 Statistical Analysis of the Differential Proteins

1. Name each spot with the corresponding protein.
2. Use the Adjust volume data.
3. Data normalization (Excel):
 - (a) Calculate the average of the positive values of each membrane: Posn.
 - (b) Calculate the correction factor for each membrane based on the reference membrane (*see Note 25*):
 - $\text{Posn}/\text{Posref} = \text{Correction factor (CFn)}$.
 - (c) Correct values of each membrane based on its correction factor: $\text{Prot.Spotn}/\text{FCn}$.
 - (d) Save the normalized protein matrix as txt (*see Note 26*).

3.8 Data Statistics (Perseus Software)

1. Import the txt matrix to Perseus and place data in its corresponding category:
 - (a) Protein spot values in the “Main category”.
 - (b) Label (protein names) in the “Categorical category”.
2. Transform the standardized values to Log2.
3. Using the “Categorical annotation row” option, group your samples as desired, e.g., control and treated.
4. Check how your samples are distributed visualizing on graphs such as histograms, dendrograms, or boxplot.
5. Perform a *t*-test: two-sample test.
6. If desired, filter rows applying a desired cutoff for fold change (FC) and *p* value. For example:
 - (a) For $-\log p$ value, $x \geq 1.3$ to filter proteins with a *p* value less than 0.05 (*see Note 27*). Select Add categorical column.
 - (b) For FC, $x \geq 0.38$ and $x \leq -0.38$ to filter proteins with a differential expression of 30%. Select Add categorical column.

7. Select the differential protein based on p value and/or FC (*see Note 28*).
8. Visualize the data using the scatterplot (volcano plot):
 - (a) X-axis: FC.
 - (b) Y-axis: $-\log p$ value.
9. You can select the differential protein according to the previously established criteria (p value and FC) selecting those with the “Keep_Keep” label. Once selected, you can export them in a matrix clicking on the tab “points” and choosing “Export selection (reduced matrix).”
10. (Optional): You can add annotations related to the proteins using defined information packages that Perseus software offers (*see Note 29*).
11. To save any matrix, click on “Generic matrix export.”
12. Save the session (*see Note 30*).

4 Notes

1. Perform as many cell passages as desired although it is highly recommended to perform the experiment during short cell passages.
2. Cells should be about 50% confluent.
3. The optimum concentration of your analyte should be addressed before performing this experiment. Moreover, treat at least other three wells with the protein buffer as a control.
4. Changes in the proteostasis of your media may be happening after your treatment, so further analysis of this sample may be of interest. Store at -20°C .
5. Add the PBS carefully; cells may get detached.
6. You might need to sonicate your samples due to a high presence of DNA.
7. Kit reagents including membranes should be stored at $\leq -20^{\circ}\text{C}$ till use. Kit must be used within the first 6 months after delivery.
8. The printed side of each membrane is denoted by a dash mark (-) in the upper left corner. It is recommended to use a permanent marker to write on the lid and on the side of the tray the name of the sample that will be placed in each of the wells. At the end of the assay, you can clean them with ethanol. Use tweezers every time you handle the membranes.
9. If samples have a consistent protein concentration among them ($\leq 20\%$), you can dilute the samples using equal volumes of $1\times$ blocking buffer for each sample. In the case that samples

present wider protein concentration variation among them, dilute each sample with 1× blocking buffer to equal the concentrations.

10. To avoid damaging the membrane, tilt the tray to pick up the different reagents with the help of a pipette tip, from a corner.
11. Avoid spreading the reagents you use in this assay directly on the membrane, in order to avoid damaging it.
12. Both wash buffers I and II have to be 20-fold diluted with distilled or deionized water. These 1× washing buffers can be stored at 4 °C for up to 3 months.
13. This antibody cocktail is concentrated (2000×) as small liquid bead, so by using one vial you can test two membranes. Briefly centrifuge each vial prior to reconstruction as the concentrate can adhere to the inside walls and cap during transit. Reconstitute by pipetting 1 ml of 1× blocking buffer into the vial, and mix the cocktail by softly pipetting up and down. Then pipette 500 µl of blocking buffer and 500 µl of 1× antibody cocktail, into each well (=1 ml).
14. The HRP-conjugated streptavidin has to be 1000-fold diluted with 1× blocking buffer.
15. Array kits will retain full activity although you store them for up to 24 h at room temperature.
16. 500 µl of detection buffer is needed for each membrane.
17. The exposure should start within 5 min after adding the detection mixture, and should last as much as 20 min. Otherwise chemiluminescent signals will fade over time. For this reason, it is strongly recommended to do this step, and the following ones, in each membrane separately.
18. Do not allow membranes to dry out during detection and avoid sliding the plastic sheet along the membranes' printed surface.
19. Expose the membranes one by one making sure that all the cytokine spots will be visible.
20. The size of the imaging area must always be the same, so all the images have the same size and can be easily compared.
21. Typical exposure times take between few seconds and 2 min, but, if the imaging system allows it, expose the membranes in auto-expose mode. This ensures the detection of most of the cytokine spots, because it detects little abundant proteins without saturating those with the most abundant expression.
22. If the image has low quality, repeat the ECL incubation.
23. Once thawed, membranes and 1× blocking buffer can be stored at ≤ -20 °C while all other components can be stored at 4 °C for no more than 3 months.

24. Use the same area (circle) on all the membranes you want to compare (use copy and paste). If the size of the spots is too different, select three different sizes (small, medium, and large) that fit better the size of your spots.
25. The reference membrane can be any of them, although it is recommended to use one of the controls.
26. The Excel matrix should contain the header: Label each cytokine/GF name and the sample names.
27. The p value is represented in $-\text{Log}_{10}$, so $-\text{Log } p \text{ value } x \geq 1.3$ means $p \text{ value } \leq 0.05$. The FC chosen here is just an example, and you should use the one that best fits your experiment data or criteria. Take into account that the FC is represented in Log_2 . In case of a 30% FC, $x \geq 0.38$ means a $\text{FC} \geq 1.3$ and $x \leq -0.38$ means a $\text{FC} \leq 0.7$.
28. If you choose both criteria to select the differential proteins, you should follow the option Rearrange—Combine categorical columns.
29. Go to Annotation columns and choose Add annotation. First you must have downloaded the database with the information you want to have, and save it in the annotations folder where the program is. Select the annotations that you want to add.
30. Save the Perseus session and carefully annotate in which program version you have worked. Perseus session should always be opened in the same program version as the one in which they were created.

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References

1. Craig Venter J, Adams MD, Myers EW et al (2001) The sequence of the human genome. *Science*. <https://doi.org/10.1126/science.1058040>
2. Rabilloud T, Hochstrasser D, Simpson RJ (2010) A gene-centric human proteome project: HUPO—the Human Proteome organization. *Mol Cell Proteomics*. <https://doi.org/10.1074/mcp.H900001-MCP200>
3. Bateman A (2019) UniProt: a worldwide hub of protein knowledge. *Nucleic Acids Res*. <https://doi.org/10.1093/nar/gky1049>
4. Vizcaíno JA, Csordas A, Del-Toro N et al (2016) 2016 update of the PRIDE database and its related tools. *Nucleic Acids Res*. <https://doi.org/10.1093/nar/gkv1145>
5. Schwenk JM, Omenn GS, Sun Z, Campbell DS, Baker MS, Overall CM, Aebersold R, Moritz RL, Deutsch EW (2017) The Human Plasma Proteome draft of 2017: building on the human plasma PeptideAtlas from mass spectrometry and complementary assays. *J Proteome Res*. <https://doi.org/10.1021/acs.jproteome.7b00467>
6. Deutsch EW, Csordas A, Sun Z et al (2017) The ProteomeXchange consortium in 2017: supporting the cultural change in proteomics public data deposition. *Nucleic Acids Res*. <https://doi.org/10.1093/nar/gkw936>
7. Gaudet P, Michel PA, Zahn-Zabal M et al (2017) The neXtProt knowledgebase on human proteins: 2017 update. *Nucleic Acids Res*. <https://doi.org/10.1093/nar/gkw1062>
8. Bastian F, Parmentier G, Roux J, Moretti S, Laudet V, Robinson-Rechavi M (2008) Bgee: integrating and comparing heterogeneous transcriptome data among species. *Lect Notes Comput Sci*. https://doi.org/10.1007/978-3-540-69828-9_12
9. Deutsch EW (2010) The PeptideAtlas project. *Methods Mol Biol*. <https://doi.org/10.1093/nar/gkj040>
10. Zerbino DR, Achuthan P, Akanni W et al (2018) Ensembl 2018. *Nucleic Acids Res*. <https://doi.org/10.1093/nar/gkx1098>
11. Forbes SA, Beare D, Boutselakis H et al (2017) COSMIC: somatic cancer genetics at high-resolution. *Nucleic Acids Res*. <https://doi.org/10.1093/nar/gkw1121>
12. Zahn-Zabal M, Michel PA, Gateau A et al (2020) The neXtProt knowledgebase in 2020: data, tools and usability improvements. *Nucleic Acids Res*. <https://doi.org/10.1093/nar/gkz995>
13. Lu B, Sun X, Chen Y, Jin Q, Liang Q, Liu S, Li Y, Zhou Y, Li W, Huang Z (2015) Novel function of PITH domain-containing 1 as an activator of internal ribosomal entry site to enhance RUNX1 expression and promote megakaryocyte differentiation. *Cell Mol Life Sci*. <https://doi.org/10.1007/s00018-014-1704-2>
14. Engqvist H, Parris TZ, Kovács A, Rönnerman EW, Sundfeldt K, Karlsson P, Helou K (2020) Validation of novel prognostic biomarkers for early-stage clear-cell, endometrioid and mucinous ovarian carcinomas using immunohistochemistry. *Front Oncol*. <https://doi.org/10.3389/fonc.2020.00162>
15. Kondo H, Matsumura XT, Kaneko M, Inoue K, Kosako H, Ikawa M, Takahama Y, Ohigashi I (2020) PITHD1 is a proteasome-interacting protein essential for male fertilization. *J Biol Chem*. <https://doi.org/10.1074/jbc.RA119.011144>
16. Engqvist H, Parris TZ, Kovács A, Nemes S, Werner Rönnerman E, De Lara S, Biermann J, Sundfeldt K, Karlsson P, Helou K (2019) Immunohistochemical validation of COL3A1, GPR158 and PITHD1 as prognostic biomarkers in early-stage ovarian carcinomas. *BMC Cancer*. <https://doi.org/10.1186/s12885-019-6084-4>
17. Lachén-Montes M, González-Morales A, Zelaya MV, Pérez-Valderrama E, Ausín K, Ferrer I, Fernández-Irigoyen J, Santamaría E (2017) Olfactory bulb neuroproteomics reveals a chronological perturbation of survival routes and a disruption of prohibitin complex during Alzheimer's disease progression. *Sci Rep*. <https://doi.org/10.1038/s41598-017-09481-x>
18. Lachén-Montes M, González-Morales A, Iloro I, Elortza F, Ferrer I, Gveric D, Fernández-Irigoyen J, Santamaría E (2019) Unveiling the olfactory proteostatic disarrangement in Parkinson's disease by proteome-wide profiling. *Neurobiol Aging*. <https://doi.org/10.1016/j.neurobiolaging.2018.09.018>
19. Sutandy FXR, Qian J, Chen CS, Zhu H (2013) Overview of protein microarrays. *Curr Protoc Protein Sci*. <https://doi.org/10.1002/0471140864.ps2701s72>
20. Tyanova S, Temu T, Sinitcyn P, Carlson A, Hein MY, Geiger T, Mann M, Cox J (2016) The Perseus computational platform for comprehensive analysis of (prote)omics data. *Nat Methods*. <https://doi.org/10.1038/nmeth.3901>



Protein Microarrays for Ocular Diseases

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Abstract

The eye is a multifaceted organ organized in several compartments with particular properties that reflect their diverse functions. The prevalence of ocular diseases is increasing, mainly because of its relationship with aging and of generalized lifestyle changes. However, the pathogenic molecular mechanisms of many common eye pathologies remain poorly understood. Considering the unquestionable importance of proteins in cellular processes and disease progression, proteomic techniques, such as protein microarrays, represent a valuable approach to analyze pathophysiological protein changes in the ocular environment. This technology enables to perform multiplex high-throughput protein expression profiling with minimal sample volume requirements broadening our knowledge of ocular proteome network in eye diseases.

In this review, we present a brief summary of the main types of protein microarrays (antibody microarrays, reverse-phase protein microarrays, and protein microarrays) and their application for protein change detection in chronic ocular diseases such as dry eye, age-related macular degeneration, diabetic retinopathy, and glaucoma. The validation of these specific protein changes in eye pathologies may lead to the identification of new biomarkers, depiction of ocular disease pathways, and assistance in the diagnosis, prognosis, and development of new therapeutic options for eye pathologies.

Key words Protein microarrays, Ocular pathology, Dry eye, Glaucoma, Age-related macular degeneration, Diabetic retinopathy

1 Introduction

Ocular diseases are a major topic for public health. Comprehensive approaches are required to expand eye research in order to understand molecular pathway determinants in eye pathologies' progress and improve ocular diagnosis and treatment. Most of these ocular diseases are age-related conditions and their incidence has rapidly risen consistently with the world's population aging [1, 2].

The small sample volumes available and the sensitivity of traditional immunoassays (enzyme-linked immunosorbent assay or Western blot) have hampered ocular proteomic profiling analyses.

Protein microarrays support the simultaneous detection of large number of proteins with minimal small volume requirements, offering an interesting combination of sensitivity and cost-effective multiplexing ability. Consequently, protein microarrays are an affordable technical strategy that has emerged as a promising platform for ocular research. Moreover, this high-throughput proteomic approach can be particularly useful to gain insight into ocular disorders since eye pathologies are commonly multifactorial and complex diseases. Additionally, data analysis and verification are less time consuming than in mass spectrometry-based proteomic studies since the identities of detected proteins in microarray assays are known. The selection of a microarray panel for analyzing protein/antibody expression in ocular specimens depends on the scientist's proposed application. In order to find new prognostic biomarkers or novel pathological molecular pathways the panel should include a rationally large diversity of targets. In contrast, to evaluate more specific events such as inflammation or angiogenic processes in ocular diseases, chemokines, cytokines, and their receptors or angiogenic factors should be included.

Ocular fluids (tears, aqueous and vitreous humors) and eye tissues [3–5] are the main subjects of protein studies of the eye. Both represent valuable sources of molecular signatures for diagnostic and prognostic purposes of eye diseases. Tears reveal the status of cornea, conjunctiva, lacrimal, and meibomian glands on ocular surface diseases. They are also used for anterior chambers' ocular pathology analysis since tear protein changes have been observed between control subjects and eye disease patients. Aqueous humor provides information about underlying tissues such as trabecular meshwork, iris, ciliary body, and cornea endothelium. Vitreous sample studies focused on proteins involved in retina pathophysiology. This ocular fluid is frequently obtained at the time of surgery. However, since most of the proteins secreted into the vitreous diffuse into aqueous humor, protein levels in aqueous humor can be related with its corresponding concentration in the vitreous [6]. This finding can be of particular interest as aqueous humor can be extracted by corneal puncture in the outpatient clinic, thus allowing for an easier biological sample collection for the study of retinal eye disease. In this sense, tears are the most easily accessible ocular fluid that could be collected by simple and barely invasive collection procedures. For protein array analysis the most common tear fluid retrieval method is by microcapillary tube [7]. Alternatively, Schirmer strips (with or without anesthesia) [8] and polyurethane minisponges [9] followed by protein elution steps are also used to collect tears.

On the other hand, in proteomic eye research, some studies have analyzed blood proteome alterations in ocular pathologies associated with humoral immune response evaluation.

This review is focused on the description of the most relevant proteomic findings achieved in the field of ocular disease using protein microarrays. Firstly, a classification about protein arrays is provided, and then the main types of protein microarrays used in ocular studies are described. Particularly, a summary of the target proteins and potential biomarkers identified by protein microarrays in ocular chronic pathological processes such as dry eye, glaucoma, age-related macular degeneration, or diabetic retinopathy is provided. As discussed, these protein array data can provide insight about eye disease molecular mechanisms, identify new biomarkers for detection or classification of ocular disorders, and open the door to define alternative therapeutic options.

2 Main Types and Characteristics of Protein Arrays Used for Ocular Disease Research

According to their format, microarrays can be classified as bead-based arrays or planar (glass or membrane arrays) (Fig. 1). In the bead-based arrays the molecules are covalently attached to microspheres with a diameter usually ranging from 0.1 to 6 μm [10]. Microspheres with different ligands are labeled with different fluorochromes alone or in combination. The detection of these bead-based protein microarrays with different color codes is then performed by flow cytometry that makes a fast and highly sensitive quantitative-qualitative evaluation of a high number of proteins possible. This bead-based microarray approach has been expanded in the last years, since commercial microspheres (i.e., Luminex[®]) have become readily available allowing for the immobilization and exploration in the same test of up to 384 different proteins.

On a planar array, the proteins of interest (mainly recombinant or natural proteins, or antibodies) are printed on the surface of a glass slide, which can be made of nitrocellulose or coated with functional chemical groups (amino-, epoxy- ...). Indirect or direct labeling sample can be used in planar protein microarrays. In the case of indirect labeling, samples are incubated with the microarrays and with an HRP-labeled antibody that develops the signal or with a biotinylated antibody that needs a successive incubation with labeled streptavidin for signal uncovering. The use of two antibodies, the capture and the detection antibody, confers specificity and reduces background, improving the detection method. However, its development is not easy for high-density microarrays and direct labeling sample is the method of choice for these microarrays. In the direct labeling method, all proteins in a sample are labeled with biotin or fluorescent dyes such as cyanines and Alexa dyes and then applied to the planar array. The captured proteins are then detected using laser scanning or fluorescence-labeled streptavidin.

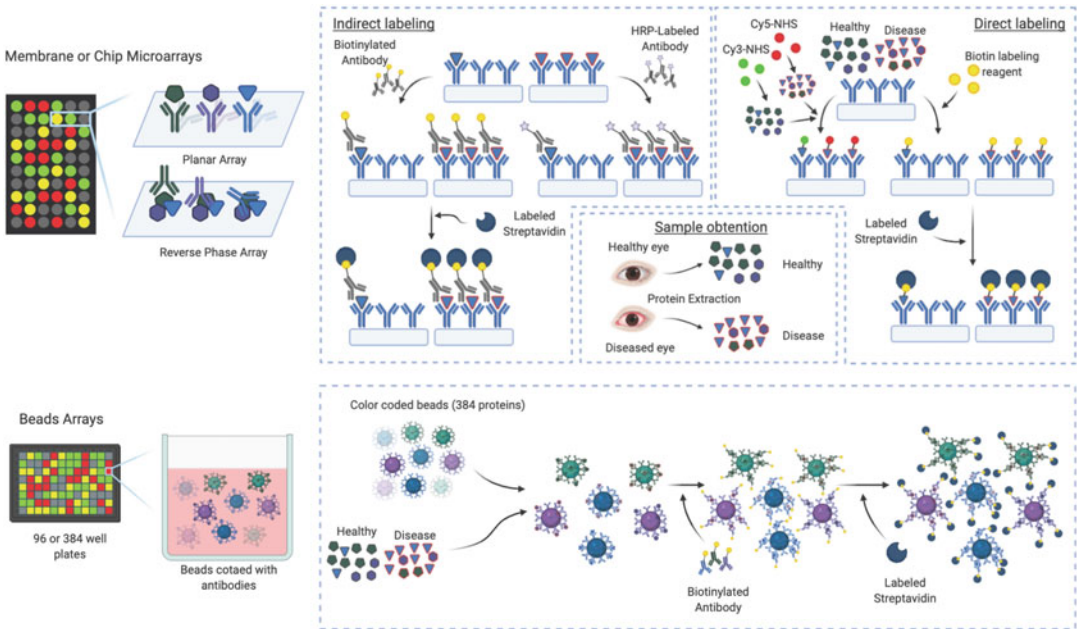


Fig. 1 Protein microarrays used in ocular research. Different types of multiplexed assay systems such as planar arrays or bead-based arrays are shown. For planar protein microarrays, indirect or direct labeling can be performed. For indirect labeling, samples are incubated with the microarrays and incubated with a biotinylated antibody that needs a subsequent incubation with labeled streptavidin for signal detection. Alternatively, samples are incubated with the planar microarrays and incubated with an HRP-labeled antibody that develops the signal. For the bead-based protein microarrays, samples are incubated with color-coded beads and a detection antibody labeled with biotin. An incubation step with labeled streptavidin is required for signal acquisition and quantification. (Figure created with biorender.com)

On the other hand, protein microarrays can also be categorized based on the molecule printed or covalently attached to their surface. This classification is the one that will be considered in this review to describe protein arrays in ocular disease research. In this sense, we can distinguish antibody microarrays, reverse-phase protein microarrays, and protein microarrays. The applications for which each method is more suited will be extensively discussed below. Briefly, antibody microarrays are the most adequate technique to identify altered protein levels in eye-related cells, tissue lysates, or biological fluids. Conversely, reverse-phase protein microarrays are selected for validation in large numbers of samples of altered proteins. Finally, protein microarray approaches (using printed natural or recombinant protein microarrays) are currently employed for humoral immune response characterization.

3 Antibody Microarrays

Antibody arrays are mostly used for the determination of protein profiles differentially expressed in cell or tissue lysates, or body fluids under pathological conditions [11–16]. The parallel analysis of multiple proteins in small sample volumes is applied to identify alterations in the amount of hundreds to thousands of proteins for the discovery of key protein associated with ocular disease processes. The elucidation of key proteins associated with pathologies reveals new disease biomarkers and provides potential targets for future drug discovery and therapeutic intervention.

The common principle of this approach is based on the highly specific recognition between an antibody, printed on a solid support, and its target antigen present in the biological samples. The interaction between the antibody and its specific antigen is then visualized by common indirect or direct labeling detection methods.

Commercial antibody microarrays containing more than 1000 antibodies against human proteins (RayBiotech) have been developed [17, 18] that make the discovery of altered proteins in a single experiment possible at an affordable price [18–20]. Additionally, there are antibody microarrays containing fragments of antibodies (scFvs, Fabs ...) [21, 22]. However, these antibody fragments usually have between 10 and 100 times less affinity than complete antibodies, and, moreover, require the development of a specific “framework” for its correct immobilization on the surface of the slides [22, 23]. These concerns have hindered their development in recent years compared to complete antibody-based microarrays. Consequently, an important challenge for antibody microarray platform development is the availability of validated antibodies that are indispensable for successful antibody array application. To address this point, high-throughput approaches like phage display may contribute to solve this issue.

3.1 Antibody Microarray Approaches in Ocular Surface Diseases: Dry Eye

Dry eye is an ocular surface multifactorial disorder characterized by an unstable and hyperosmolar tear film. Increased tear osmolarity is produced by tear-deficient production (aqueous-deficient dry eye), excessive evaporation with normal lacrimal function (evaporative dry eye), or a combination of both events [24, 25]. Discomfort, tear film instability, irritation, vision disturbance, ocular surface damage, and neurosensory disturbances are common clinical symptoms, but there does not exist a direct relation between these symptoms and clinical signs [26, 27]. This inconsistent relation between symptoms and signs precludes precise dry eye diagnosis and monitoring and draws out new pharmacological therapies by the lack of objective tests to evaluate outcomes in clinical trials. In this context, protein microarrays can be a useful tool for unbiased

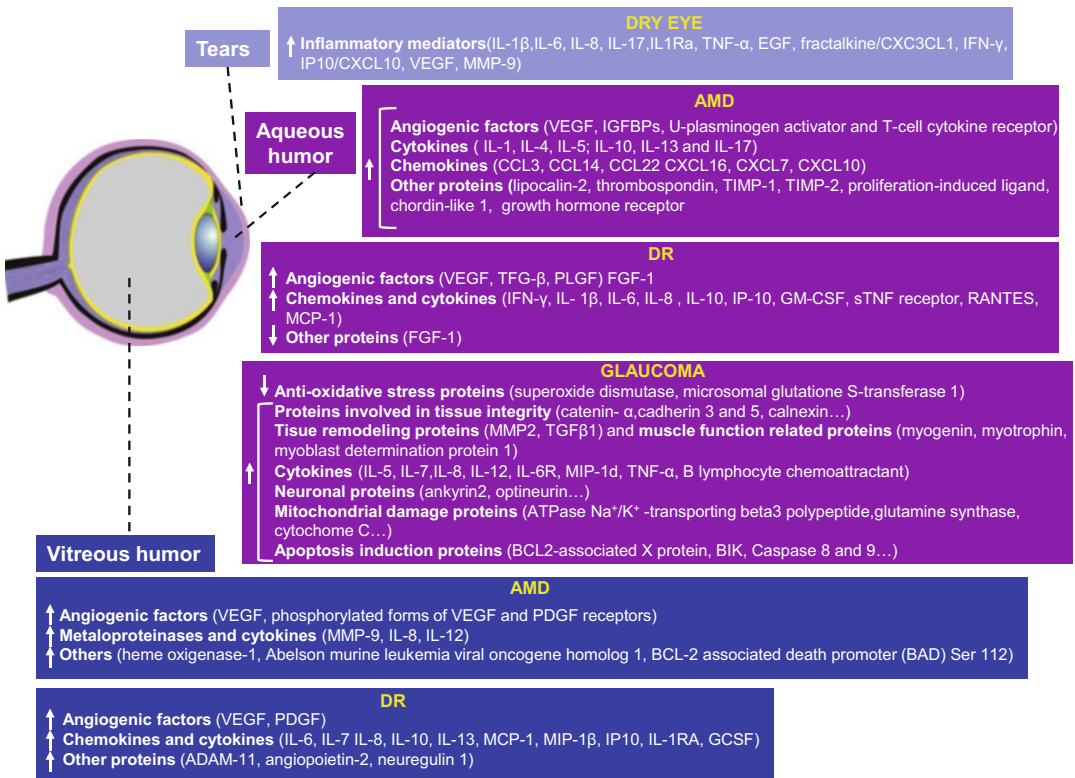


Fig. 2 Protein changes detected by antibody microarrays in tears, aqueous, and vitreous humor from dry eye, age-related macular degeneration (AMD), diabetic retinopathy (DR), and glaucoma patients as compared to normal subject samples

dry eye biomarker identification with diagnostic purposes, or for treatment efficacy monitoring. Although the pathogenesis of dry eye is not fully clarified, it is known that hyperosmolar stress damages the ocular surface both directly and indirectly by triggering a pathologic inflammatory condition [24, 28, 29]. Taking into account that inflammation is an integral event in dry eye, the vast majority of antibody microarray studies have been focused on the elucidation of inflammatory mediator (cytokines, chemokines ...) profile in tears of dry-eye patients (Fig. 2). Multiplex bead arrays were mostly used for this purpose, among which the Luminex method was the most frequently employed. Membrane antibody planar microarrays have also been used, although more occasionally. When both methods are compared [30], Luminex assay seems to exhibit a higher sensitivity to inflammatory markers related to dry eye than membrane antibody planar microarrays, whereas more significant changes between the study groups were detected using membrane antibody planar microarrays.

Using membrane antibody planar microarrays, increased proinflammatory levels of tear cytokines were measured in dry-eye patients with aqueous deficiency or with both lipid (associated with higher tear evaporation) and aqueous deficiencies, as compared to healthy subjects [31]. Differences in tear cytokine profiles between dry-eye and control subjects have also been analyzed by multiple bead analysis and correlations with ocular surface parameters have been investigated. Higher concentrations of proinflammatory cytokines interleukin (IL)-1 β , IL-6, IL-8, IL-17 A, IL-1 receptor antagonist (IL-1Ra), and tumor necrosis factor- α (TNF- α) have been detected in several reports and a good correlation between the levels of these molecules and clinical severity parameters of dry eye (symptom severity, Schirmer test, tear breakup time, and corneal fluorescein staining) has been found [32–36]. Likewise, in tears of evaporative dry-eye patients the levels of inflammatory molecules epidermal growth factor (EGF), fractalkine/CX3CL1, interferon- γ (IFN- γ)-inducible protein-10 (IP-10)/CXCL10, and vascular endothelial growth factor (VEGF) were significantly elevated as compared to control subjects and correlated with pain and clinical parameters such as cornea and conjunctiva staining and tear stability/production [37].

Interestingly, quantified tear inflammatory mediators do not show significant inter-day variation [32, 38] and the intra-subject reproducibility is good [38]. These results emphasize the reliability of the use of these molecules as dry-eye biomarkers.

Dry eye is particularly severe in Sjögren's syndrome, a chronic autoimmune disease characterized by lymphocytic infiltration and destruction of exocrine (lacrimal and salivary) glands. Exposure of Sjögren's syndrome patients to low-humidity conditions, to recreate daily indoor adverse environment, produced a significant increase in tear levels of some inflammatory molecules (IL-1RA, IL-6, IL-8, and matrix metalloproteinase (MMP)-9) as determined by multiplex immunobead assay, indicating an exacerbation of inflammatory state for these patients. Bead-based antibody microarray systems have also been used to analyze altered cytokine profiles in blood samples of Sjögren's patients [39, 40]. Plasma cytokine profiles allowed to distinguish between Sjögren's patients and healthy subjects and correlated with altered clinical parameters such as hypergammaglobulinemia [39], elevated erythrocyte sedimentation rate, or extra-glandular manifestation [40], indicating its relevance in Sjögren's syndrome diagnosis and management.

Furthermore, diverse subtypes of dry eye have been demonstrated to have different altered cytokine profiles. In a study using planar antibody microarrays aqueous-deficient dry-eye patients had the highest level of total tear cytokine, followed by lipid-deficient dry eye and patients with both aqueous and lipid deficiencies [41]. Statistically significant differences have also been observed for IL-2, IL-4, IL-8, IL-17, and TNF- α among Sjögren's syndrome

dry eye and non-Sjögren's syndrome tears [42, 43]. Additionally, tear concentrations of IL-8 and TNF- α for both dry-eye subpopulations (Sjögren's syndrome and non-Sjögren's syndrome aqueous tear-deficiency subjects) were significantly different from those detected in meibomian gland dysfunction patients, an alteration leading to evaporative dry eye [43]. These findings could suggest the existence of diverse underlying pathophysiologic mechanisms and point to the relevance of panel cytokine determination for the diagnosis of dry-eye subclasses.

Multiplex arrays have been used to evaluate new dry-eye treatments identifying cytokine profile modulation in response to pharmacological therapy in experimental animal models [44] and clinical trials [45, 46]. In patients with meibomian gland dysfunction, oral therapy with antibiotic minocycline combined with artificial tears induced a significant reduction in tear cytokine levels (IL-1, IL-6, IL-12p70, IL-17, and TNF- α) that was not found after treatment with sole artificial tears [46]. Immunomodulatory ability of tofacitinib was also evaluated in moderate-to-severe dry-eye patients. Bead-based arrays showed a marked decrease in tear levels of cytokines and other inflammatory mediators (IL-1 β , IL-12p70, IL-15, IL-17A, MMP-3, and MMP-9) after topical ophthalmic application of this Janus kinase inhibitor [45].

**3.2 Antibody
Microarray Application
in Intraocular
Diseases: Age-Related
Macular Degeneration**

Age-related macular degeneration (AMD) is the leading cause of irreversible blindness in old people in the Western world [47]. This progressive disease affects the central portion of the retina (macula). AMD is classified into non-neovascular (non-exudative or dry) and neovascular AMD. The former results from atrophy of the retinal pigment epithelial layer below the retina with the consequent photoreceptor loss, while the latter is characterized by chorioidal neovascularization [48].

The etiology of this disease is still unknown and several factors such as aging, diet, genetic, inflammation, or oxidative stress have been related to AMD progression. VEGF inhibition is one of the therapies currently used to treat exudative AMD due to the relevant role of VEGF and its receptor VEGFR-1 in its pathogenesis [49]. However, patient response to anti-VEGF therapy is heterogeneous. A significant visual improvement is only achieved in one-third of patients treated and one-sixth of treated patients still progress to blindness [50]. Consequently, VEGF is not the only protein responsible for this disease and the research of alternative targets is imperative.

With this aim, protein alterations in aqueous humor of patients with wet AMD compared to control subjects have been analyzed by antibody arrays in several papers (Fig. 2). Increased protein levels of U-plasminogen activator and T-cell cytokine receptor (TCCR/WSX-1) were found. Furthermore, *in vitro* treatment of human umbilical vein endothelial cells with the extracellular domain of TCCR/WSX-1 showed the angiogenic activity of this protein [51].

Using biotin label-based human antibody array higher levels of lipocalin-2, thrombospondin, tissue inhibitors of metalloproteinases (TIMP-1 and TIMP-2), proliferation-induced ligand, chordin-like 1, glucocorticoid-induced tumor necrosis factor receptor family-related gene ligand, growth hormone receptor 3, and insulin-like growth factor-binding proteins (IGFBP-2, IGFBP-6, and IGFBP-7) were also detected in exudative AMD patients [52]. IGBPs are members of IGF family, which modulate VEGF-induced proliferation, migration, and tube formation of retinal endothelial and tumor angiogenesis [53, 54]. Their altered content in aqueous humor from exudative AMD eyes suggests a potential involvement in AMD pathogenesis.

Evidence for the role of inflammation in AMD progression has been reported in histopathological studies. Consistent with the contribution of inflammation in AMD pathogenesis, elevated levels of serum proinflammatory cytokines (IL-1, IL-4, IL-5, IL-10, IL-13, and IL-17) were determined by multiplex human cytokine arrays in AMD patients as compared to control subjects [55]. In a study by Nassar et al. [55] the evolution of the cytokine profile of AMD patients under anti-VEGF therapy was evaluated. It was concluded that patients with high serum IL-17 and TNF- α concentrations were more likely to recover with the anti-VEGF treatment.

Increased protein expression of the chemokines CCL22 and CCL3 [56] as well as CXCL10, CCL14, CXCL16, and CXCL7 [57] has also been demonstrated in AMD aqueous humor analyses, while inconsistent results were found for growth-regulated oncogene (GRO) chemokine. A significant decrease in GRO chemokine was measured using Quantibody Human Chemokine Array (Ray-Biotech) [57], whereas higher levels were determined by Luminex platform in AMD aqueous humor [56]. Interestingly, an increase in CCL22 expression was associated with wet AMD pathological signs such as intraretinal fluid or hyperreflective foci [57].

Plasma levels of the aforementioned cytokines were not significantly altered between healthy and pathological conditions, indicating that dysregulation of immune factors is rather local than systemic. Confirming this notion, multiplex bead assays demonstrated significant changes in monocyte chemotactic protein (MCP)-1/CCL2 and vascular cell adhesion molecule (VCAM) concentrations in aqueous humor from AMD patients compared with controls whereas systemic differences were not found [58].

In AMD, chronic inflammation in the outer retina seems to stimulate AMD pathogenesis progression before symptom onset. Consequently, anti-inflammatory strategies to early AMD handling have been explored. Vinpocetine (vinca alkaloid extracted from periwinkle plant) treatment significantly reduced cytokine concentration (IL-1, IL-6, IL-18, macrophage inflammatory protein-3 α ,

and TNF- α) in vitreous humor from animal model of subclinical stage of AMD [59], showing its potential as an anti-inflammatory agent.

Another central pathological event in AMD, particularly in wet AMD, is retinal neovascularization and the antiangiogenic properties of different compounds have been evaluated using antibody microarrays. Using a multiplex bead-based assay, administration of bisphosphonates (alendronate and etidronate) revealed an antiangiogenic potential of these agents inhibiting the expression of eotaxin and basic fibroblast growth factor in primary human retinal pigment epithelium [60].

A herbal medicine, *Cnidium officinale*, and its bioactive compound butylidenephthalide downregulated the retinal levels of the angiogenesis-related factors (amphiregulin, angiogenin, delta-like ligand 4, and VEGF) and suppressed retinal neovascularization in an oxygen-induced retinopathy mouse model [61]. In addition, butylidenephthalide markedly reduced IL-1 α concentration in treated mice. Antibody microarray analysis performed in the same animal model showed that Guibi-tang (a herbal formula composed of 12 ingredients) significantly decreased the expression of pro-angiogenic molecules such as plasminogen activator 1 (PAI-1), insulin-like growth factor-binding protein 1 (IGFBP-1), fibroblast growth factor 2 (FGF-2), and chemokine monocyte chemoattractant protein (MCP)-1 [62]. The ability of these compounds to reduce not only intraocular angiogenic factors but also inflammatory markers such as IL-1 α or MCP-1 is noteworthy, since conventional treatment with ranibizumab only modifies intraocular VEGF levels whereas inflammatory cytokine factors and other pro-angiogenic factors remained invariable [58]. Future therapies or treatment combinations able to target angiogenic but also inflammatory components in AMD may contribute to improving visual outcomes in this disease.

3.3 Antibody Microarray Technology in Intraocular Pathologies: Diabetic Retinopathy

Diabetic retinopathy (DR) is a common neurovascular complication of diabetes occurring in around 1/3 of patients diagnosed with this pathology [63] and it is the leading cause of blindness in adult working population [64]. DR is classified into nonproliferative DR (NPDR) (divided into mild, moderate, and severe stages that may include diabetic macular edema) and proliferative DR (PDR) characterized by retinal neovascularization [65].

Chronic hyperglycemia triggers key mechanisms such as increased oxidative stress and proinflammatory mediators and promotion of angiogenesis through VEGF and IGF-1 activation whose end result is neural and vascular retinal damage [66].

Vitreous fluid is considered as an indirect information source on the retinal tissue due to its nearness to the retina. Consequently, angiogenic growth factors, cytokines, and chemokine profile have been investigated in the vitreous fluid from patients with diabetic

retinopathy (Fig. 2). A significant increase in IL-6, IL-8, IL-10, IL-13, MCP-1, macrophage inflammatory protein (MIP)-1 β , platelet-derived growth factor (PDGF), and VEGF levels was detected in DR vitreous samples as compared to control group using an array system with beads conjugated with antibodies [67]. Similarly, greater vitreous concentrations of IL-6, IL-8, and VEGF could be quantified in advanced proliferative DR patients and oxygen tension in the mid-vitreous was directly correlated with VEGF concentration [68]. In addition to these identified cytokines, elevated levels of ADAM11 (disintegrin and metalloproteinase domain-containing protein 11) were found by cytometric bead analysis in office-based vitreous aspirates from proliferative DR patients [69]. It has been speculated that this protein could be involved in the neuropathic alterations associated with diabetic retinopathy [69]. On the other hand, tyrosine phosphorylation status of some of these vitreous angiogenic and inflammatory factors was reduced in DR samples as revealed by microarray analysis, suggesting a potential alteration in protein function [70].

Vitreous protein profile associated with epiretinal fibrovascular membrane formation, a severe complication of proliferative DR inducing retinal traction and detachment, was studied using semi-quantitative planar arrays containing 507 antibodies [71]. Higher vitreous levels of angiopoietin-2 correlated with retinal fibrosis degree while fibrovascular membrane appearance and elevated vitreous concentrations of neurotrophic factors (platelet-derived growth factor and neuregulin 1) were associated with neurite growth, which happens in fibrovascular membrane formation in proliferative DR [71]. Cytometric bead array studies showed that vitreous levels of the cytokines MCP-1 and IL-8 significantly correlated with the density of inflammatory cells (macrophages and T lymphocytes) in fibrovascular membranes of proliferative DR patients, suggesting an important role of these cytokines in proliferative DR progress [72].

Frequently, pars plana vitrectomy in an operating room is the technique used to extract vitreous sample. In an attempt to circumvent this invasive procedure, serum cytokine and growth factor profile was compared with that present in vitreous samples from proliferative DR patients [73]. The percent detectable and levels of cytokines were significantly lower in serum; particularly IL-4, IL-10, IL-17, IL-21, and IL-22 were only measured in the vitreous humor, indicating that serum does not reliably reflect cytokine levels detected in vitreous [73].

Alternatively, a less disturbing procedure has been developed based on vitreous reflux collection at the end of routine intravitreal anti-VEGF injection [74]. Using this technique, a significant upregulation of IL-1RA, IL-6, IL-7, IL-8, granulocyte colony-stimulating factor (GCSF), and IP-10 was found in diabetic macular edema (DME) patients and high-risk proliferative diabetic retinopathy patients as compared with no-DR subjects. In addition,

IL1RA/IL-1b ratio was 13 times higher in the DME group as compared with high-risk proliferative diabetic retinopathy patients [74]. An interesting advantage of this procedure is the possibility of repeated vitreous reflex collection from patients undergoing continuous intravitreal injection that could ease therapy monitoring. In this sense, a significant decrease in osteopontin, IL-6, and VEGF levels was detected on anti-VEGF-treated DME patients after the third anti-VEGF intravitreal injection [75].

Aqueous humor is also recurrently used as a surrogate fluid of vitreous since its sampling is less complicated than vitreous extraction. Similar to vitreous humor, higher levels of inflammatory markers have been identified in aqueous humor of both nonproliferative and proliferative DR. By means of multiplex bead cytokine analysis, transforming growth factor- β (TGF- β) and placental growth factor (PLGF) were found to be higher in aqueous humor of nonproliferative DR patients whereas fibroblast growth factor 1 (FGF-1) was lower as compared to controls and correlated with total retinal blood flow [76]. Moreover, it has been demonstrated that aqueous humor levels of PLGF are negatively correlated with axial length in NPDR patients; this negative correlation may justify why myopia eyes exhibit a lower DR prevalence [77]. Elevated levels of the cytokines IFN- γ , IL-10, IP-10, granulocyte-macrophage colony-stimulating factor, soluble TNF- α receptor, and RANTES (regulated and normal T cell expressed and secreted) were also detected in mild DR patients as compared to control [78]. As determined by Luminex measurement, IP-10 and MCP-1 were significantly elevated in aqueous humor from severe NPDR and PDR in comparison with less severe DR and control subjects [79]. Consistent with this observation, IL-2, IL-5, and VEGF concentrations were significantly higher in PDR patients than those in the NPDR patients [80]. Elevated levels of several cytokines (IL-1 β , IL-6, MCP-1, IP-10, IL-8, and VEGF) were identified in the aqueous humor of diabetic patients with macular edema and associated with retinal macular volume and thickness [81]. Overall, these results indicate that intraocular inflammation is positively correlated with DR progression and severity.

Although these data can provide valuable biochemical information, we should be cautious since some differences can exist in cytokine levels in aqueous humor and vitreous fluid. Takeuchi et al. [73] reported that IL-17 was detected in the aqueous humor from PDR patients and the level correlated with IL-17 in vitreous fluid; however, the mean level and percent detectable were significantly lower than those in the vitreous humor.

Laser retinal photocoagulation is a recurrent treatment for DR; however, it only diminishes the risk of moderate visual loss by approximately 50% with no significant vision recovery and it is linked with ocular side effects. Furthermore, new approaches such as intravitreal injections of the anti-VEGF have emerged to delay the proliferative diabetic phase. Multiplex cytokine kit and Luminex

technology showed that ranibizumab treatment significantly decreased aqueous humor VEGF concentrations as well as MCP-1, TNF- α , IL-1 β , IL-7, IL-8, IL-10, IL-12, and IL-17 levels [82]. This finding is particularly interesting since the manifold evidence points toward a role for chronic subclinical inflammation in DR pathogenesis.

In contrast, therapy with the anti-VEGF agent bevacizumab only induced a decrease in VEGF concentration, whereas the levels of other inflammatory mediators remained unaffected (IL-6, MCP-1) [83] or even increased (IL-8) [84] as determined by multiplex arrays. These opposed results could be due to a more potent effect of ranibizumab on intraocular cytokine levels or it could be related with other factors such as the different DR severity in patient cohort studied or the timing of analysis.

In order to reduce these cytokine levels, an alternative treatment including the combination of the corticosteroid triamcinolone with bevacizumab was evaluated [85]. As revealed by Luminex multiplex array assay the combined treatment reduced VEGF, MCP-1, and PDGF-AA (platelet-derived growth factor AA) levels whereas aqueous humor concentration of IL-8 was even higher after combined therapy [85]. The high concentration of this cytokine could be related to the insensitivity or reappearance of diabetic macular edema after bevacizumab treatment [86].

**3.4 Antibody
Microarray
Approaches
in Intraocular
Diseases: Glaucoma**

Glaucoma consists of a group of eye diseases showing a broad spectrum of clinical presentation and unknown etiologies that lead to a permanent loss of visual function as a consequence of optical nerve damage [87, 88]. Glaucoma pathology is currently classified into two different categories, the angle-closure glaucoma and the primary open-angle glaucoma (POAG), the most common form. High intraocular pressure is considered as a critical risk factor in glaucoma pathogenesis. Intraocular pressure (IOP) is generated and maintained by the aqueous humor circulation system in the anterior eye. Aqueous humor is secreted by the ciliary body and exits the eye in the iridocorneal angle mainly through the trabecular meshwork (TM) and Schlemm's canal [89]. Imbalance between aqueous humor secretion and outflow induces high IOP and pharmacological glaucoma treatment is focused on lowering it. Surgical procedures like trabeculectomy are assigned to those patients whose pharmacological treatment is insufficient.

The pathological events that compose glaucomatous disease are not yet fully understood. Likewise, glaucoma diagnosis demands new approaches since IOP is subjected to fluctuations in a patient along the day due to circadian rhythm influence and reliable measurement can be masked by increased corneal thickness. Moreover, glaucoma can be normotensive. Collectively, these observations point to the demand of new diagnostic biomarkers and pathologic molecular pathway determination.

Antibody microarrays are useful tools to confront this purpose. Significant variations of 31 proteins were found in samples from aqueous humor of primary open-angle glaucoma patients labeled with cyanine and hybridized with antibody microarrays (Fig. 2). These protein alterations were related with pathogenic mechanisms including mitochondrial damage (increases in ATPase Na^+/K^+ transporting beta-3 polypeptide and cytochrome C proteins), apoptosis, and neurodegeneration (elevations in caspases, BCL2-associated X protein, Fas (TNFRSF6) associated via death domain, optineurin, and ankyrin 2) [90]. Oxidative stress also contributes to glaucoma progression by damaging TM and retinal ganglion cells. Modifications in oxidative biomarker levels were detected in this work as well as in a later study [91]. Thus, reduced levels of antioxidant enzymes like superoxide dismutase and glutathione S-transferase were measured whereas the amounts of pro-oxidant enzymes (nitric oxide synthase 2 and glutamine synthase) were significantly increased in primary open-angle patients in comparison to control subjects [91].

Changes in proteins involved in the maintenance of tissue integrity (catenin alpha, cadherins 3 and 5, calnexin) were also identified in glaucomatous aqueous humor which would indicate tissue degeneration process in TM [90]. Supporting this notion, higher levels of proteins involved in muscle function (myogenin, myotrophin, myoblast determination protein 1) have been detected by antibody-spotted glass microarrays in primary open-angle glaucomatous aqueous humor [92]. The contractile component of muscle tissue is vital to appropriate TM aqueous humor drainage function and the presence of high levels of these proteins in aqueous humor could reflect TM damage. Additionally, aqueous humor protein alterations also revealed a substantial endothelial damage that probably concerns the endothelial barrier of TM [92].

Other experimental evidence that reinforces TM damage hypothesis is the upregulation of tissue remodeling proteins (MMP2, VEGF, transforming growth factor beta 1) observed in human glaucomatous TM by antibody microarray [93].

Neuroinflammation is an event described in chronic neurodegenerative disorders like glaucoma. Looking for the inflammatory characteristics of glaucoma, the concentration of 40 cytokines was evaluated using multiplex quantitative antibody arrays in aqueous humor from glaucoma secondary to Sturge-Weber syndrome patients [94]. The levels of interleukins IL-5, IL-7, IL-12, interleukin-6 receptor, MIP-1d, TNF- α , and B lymphocyte chemoattractant were increased in glaucoma secondary samples compared to control samples. Elevations in IL-7 and TNF- α were positively correlated with IOP parameter [94]. On the contrary, in aqueous humor of primary open-angle glaucoma patients, only IL-8 concentration was significantly upregulated as determined by bead-

based multiplex cytokine arrays [95]. This finding suggests that changes in aqueous humor inflammatory cytokines can depend on the glaucoma types.

The proinflammatory environment not only circumscribes to aqueous humor fluid, but also affects two target tissues in glaucoma: TM and retina [93, 96]. In glaucomatous TM specimens, cytokines IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-10, and TNF- α and chemokines (vascular cell adhesion molecule 1, macrophage inflammatory protein MIP 1 δ/α) were remarkably elevated [93]. Higher levels of proinflammatory cytokines (IL-1 β , IL-6, IL-8, and TNF- α) were also found in glaucomatous retina compared to non-glaucoma controls [96].

Some members of the cytokine family play a key role in fibrotic processes [97]. Since scarring and fibrosis are detrimental side effects of trabeculectomy surgery, aqueous humor cytokine profile was compared between patients with positive or negative outcome after trabeculectomy by means of commercial cytokine antibody arrays [98]. A strong upregulation of pro-fibrotic cytokines (VEGF, MCP-1, TGF- β , IL-1, IL-6, and TNF- α) and downregulation of IL-22 (with anti-fibrotic properties) were observed in the samples from failed trabeculectomy as compared to successful surgery [98].

4 Reverse-Phase Protein Arrays

In reverse-phase protein arrays (RPPA), cell or tissue lysates or body fluid samples are spotted on the surface of the plane microarrays, and probed with a primary antibody. Three to four times higher concentration of specific antibody than in western blot followed by an appropriate secondary antibody is required for the detection of picograms to femtograms of proteins onto RPPA arrays. Detection is accomplished by fluorescence, colorimetric method, or chemiluminescence. RPPA allows the analysis of multiple samples in the array with a single detection antibody. However, on the contrary to antibody arrays, RPPA arrays only evaluate one protein per slide. The main drawback of these arrays is their optimization to avoid intra-array and inter-array cross-reactivity. This RPPA arrays, although time consuming, are an interesting methodology to experimentally clarify pathological intracellular protein pathways in a high-throughput format. Nevertheless, analysis and detection of low-abundance proteins in complex samples can be challenging [99].

4.1 Reverse-Phase Protein Microarray Technology in Age-Related Macular Degeneration

The vitreous humor bathes the retina making it a noteworthy fluid to examine potential indicators of the AMD pathology. Several papers have shown the efficacy and safety of small vitreous extractions in an in-office setting to perform reverse-phase protein microarrays able to analyze angiogenic and other proteins [100, 101]. Using these reverse-phase protein microarrays,

expression of eight proteins (MMP-9, IL-8, IL-12, Abelson murine leukemia viral oncogene homolog 1, heme oxygenase-1, Musashi, platelet-derived growth factor receptor beta, and Bcl-2-associated death promoter) was found to be higher in vitreous from AMD patients with subretinal fluid as compared to AMD patients without subretinal fluid [101]. MMP-9 protein was the most significantly altered between both groups, and a positive correlation between vitreous MMP-9 content and subretinal fluid level was determined [101] that suggests its applicability as subretinal fluid predictive marker development in AMD patients.

Activated (phosphorylated) forms of VEGFR and PDGFR (platelet-derived growth factor receptor) were identified in 11 vitreous samples taken prior to intravitreal bevacizumab injection and immobilized on a nitrocellulose-coated slide [100]. Vitreous wet AMD patients evinced higher concentration of VEGFR Y1175 than vitreous fluid from control eyes without neovascular alterations. Moreover, the vitreous levels of phosphorylated forms were related with the response to bevacizumab administration [100]. A significant variance in VEGFR Y1175, VEGFR Y996, and PDGFR β Y751 levels, but not in PDGFR β Y716 or c-KIT Y703 concentration, was observed in successful responders to treatment compared with nonresponders and controls [100]. These results put forward that alternative signaling pathways can be involved in neovascularization and exudation processes in nonresponder patients. Reverse-phase protein microarrays also allowed the detection of low-concentration post-translationally modified proteins in exosomes shed by retinal pigment epithelial cells [102]. In a similar way to the results obtained in vitreous samples, phosphoproteins such as PDGFR (Y751 and Y7169), VEGFR (Y951), and C-KIT (Y703) were found in exosomes, supporting the concept that exosomes can mediate phosphoprotein secretion by retinal cells into the vitreous [102]. Furthermore, oxidative stress exposure modified the content of several phosphorylated signaling proteins linked to apoptosis/survival and cell metabolism pathways [102].

5 Protein Microarrays

Protein or antigen microarrays printed with either natural or recombinant proteins have been extensively used for humoral response characterization [103–112]. There are commercially available high-density protein microarrays covering ~87% of the human proteome (HuProt) [109, 110, 112–115]. Apart from full-length protein microarrays, microarrays of protein fragments that contain nearly the entire proteome considering one protein fragment for each gene have also been designed [116]. This technical approach has been developed in recent years by the Human Protein Atlas platform (www.proteinatlas.org) that has produced antibodies and fragments of proteins—PrESTs—for almost every human protein [117, 118].

5.1 Protein Microarray Technology in Age-Related Macular Degeneration and Glaucoma

Autoantibody production is associated with ocular pathologies such as AMD and glaucoma. The role of autoantibodies in eye diseases remains elusive and it is unclear whether they have a causative contribution in the disease or they are only a secondary consequence.

In AMD, it has been suggested that autoimmunity could contribute to drusen development and disease progression [119, 120]. Elevated levels of retinal antibodies to 28–49 kDa retinal proteins have been detected in 94% of patients with early-stage AMD compared to only 9% of normal subjects [121]. A protein microarray analysis of serum samples from AMD patients and control subjects revealed significantly higher immunoglobulin (Ig)G/IgM ratios for anti-phosphatidylserine (PS), anti-Fab D1.3, and anti-JO-1 in dry AMD sera compared to the control sera. The ratios for anti-Ro-52 and anti-PS were also significantly elevated in the wet AMD group as compared to dry AMD patients [122]. This last ratio for anti-PS showed the best correlation with AMD stage. Moreover, incubation of endothelial cells with IgGs from wet AMD patients increased endothelial cell tube formation [122], suggesting an angiogenic potential of IgG antibodies from wet AMD patients contributing to AMD pathogenesis.

Detection of autoantibody deposition in glaucomatous retina and modification of autoantigen profile found in aqueous humor and serum of glaucomatous patients have evidenced a role for autoimmunity in glaucoma [96, 123, 124].

Protein microarrays were performed to establish intraindividual comparison of aqueous humor and serum autoantibody reactivities. Importantly, the antibody pattern was not considerably different between both biological fluids. This strong positive correlation between aqueous humor and sera antibody profile facilitates autoantibody biomarker identification and glaucoma diagnosis due to the feasibility of blood sampling contrary to aqueous humor sampling. Moreover, significant elevated autoantibody reactivities (e.g., against heat-shock 70 and 27 kDa proteins, myelin basic protein, and α -1-antitrypsin) were identified in primary open-angle glaucoma patients as comparison to control subjects as well as decreased immunoreactivities (e.g., against glial fibrillary acidic protein and β -L-crystallin) [123]. Gramlich et al. [125] have proposed that downregulation of some autoantibodies in glaucoma patients could lead to a loss of immune protection and predispose for glaucoma development. Supporting a potential role of autoantibodies in glaucoma progression, elevated levels of two autoantibodies (anti-SPRY domain-containing SOCS box protein 3 and anti-adaptor protein 1 complex subunit mu-1) were determined in the serum of primary open-angle glaucoma patients with optic disk hemorrhage (a sign considered as an indicator of underway deterioration of glaucoma disease correlated with a worsened visual function [126]), as compared to glaucoma patients without optic disk hemorrhage [127].

Using high-density colony protein microarrays expressing His-tagged recombinant human proteins derived from a human fetal brain cDNA library, higher levels of seven serum antibodies to transmembrane and coiled domain-containing protein, stathmin-like 4, transmembrane protein 9 domain family member B, fibroblast growth factor receptor 3, cleft lip and palate transmembrane protein 1, EH-domain-containing protein 1, and eukaryotic translation elongation factor 2 were noticed in pseudoexfoliative glaucoma, the most common form of secondary open-angle glaucoma [124]. Increased serum autoantibodies previously detected in primary open-angle glaucoma were not found in pseudoexfoliative glaucoma that could be a consequence of divergent etiologic factors involved in glaucoma evolution.

Antigen microarray approaches have also been accomplished to analyze whether serum autoantibody profile changes are related with retinal ganglion cell damage and clinical glaucoma signs such as increased IOP. Higher levels of three serum autoantibodies (ab-immunoreactivities to heat-shock 27 kDa protein, tubulin-tyrosine ligase-like protein 12, and neuron-specific enolase) were assessed after sudden IOP rise in acute angle-closure glaucoma patients without previous signs of former glaucoma [128]. Similarly, significant increased serum autoantibody reactivities (against glutathione-S-transferase, spectrin, and transferrin) were observed after intermittent IOP elevations and were negatively associated to axonal survival in experimental glaucomatous animals [129].

Independently of the potential pathogenic role of autoantibodies or if they are merely secondary products in AMD and glaucoma, identification of antibody patterns could anticipate disease detection, before clinical signs' appearance. In this context, protein microarrays represent a valuable tool to detect autoantibodies for early diagnosis and prognosis of these neurodegenerative ocular diseases. Autoantibody repertoire associated with these pathologies could also have other potential benefits in the management of these diseases such as effective enrollment of patients to clinical trials and estimation of responses to treatments.

6 Advantages and Drawbacks of Protein Microarray Applications to Ocular Diseases

Protein arrays offer several advantages compared to other proteomics methods. This high-throughput technology enables the simultaneous analysis of several hundreds to dozens of thousands of proteins in a single and small sample and allows the identification of many proteins, including low-abundance proteins. This ability is important for ocular researchers working with unique samples of scarce volume like eye fluids. Likewise, the generation of these multiple data diminishes the possible freeze-thaw changes of samples. This technology is less time consuming and not highly trained

staff is required as compared to mass spectrometry approaches. Regarding their cost, protein microarrays are less expensive than mass spectrometry since minimal equipment is necessary, or in comparison to the amount of tested proteins by other methods such as ELISA.

Despite the abovementioned advantages of protein microarrays, they have several limitations. An essential issue concerning the dataset obtained from protein microarray analyses is the lack of validated datasets in many studies. A further validation and functional analysis of protein microarray data by other techniques including dot blot, western blot, or ELISA is mandatory to draw significant conclusions. High-quality validated bioanalytical data avoid false-positive protein identifications and contribute to ensure in subsequent studies their translation into clinical diagnostics and therapeutics.

Disparities in sensitivities of commercial kits from different companies and absence of standardized procedures can lead to inter-study disparity and low reproducibility. Advances in the automation of array processing and signal detection could contribute to mitigating reproducibility troubles. Standardization of bioinformatics analysis methods as well as data submission and storage are also needed to compare proficiently data from different laboratories.

Additionally, although the technique is reasonably cost effective as compared to other methods (mass spectrometry, ELISA testing the same amount of proteins), a higher affordability is required for a realistic clinical translational application.

Finally, ocular sample availability can partially limit the application of protein microarrays in some specific ocular studies. Tears, aqueous, and vitreous fluids are surrogate fluids for ocular structures and posttranslational protein modifications can be observed in secreted proteins present in these fluids but their detection for intracellular proteins is hampered since some ocular target tissues such as retina are non-collectable in living subjects. Moreover, the invasive nature of aqueous and vitreous humor extraction could preclude longitudinal studies' execution in patients.

7 Conclusions

We here summarized the use of different types of protein microarrays for the identification of proteins altered in several chronic eye pathologies. One of the most promising applications of protein microarrays in ocular diseases is diagnostic biomarker discovery. These biomarkers could simplify early diagnosis of eye pathologies and even help to support disease-related pathways of drug development process. The identification of proteins associated with eye disease progression provides potential therapeutic targets and represents other key applications of protein arrays in eye.

Consequently, protein microarrays should open the door to the identification of new diagnostic biomarkers as well as new options of management and treatment for eye disorders in clinical practice. Nevertheless, to reach the translation of the advances from the bench to clinical ophthalmology, validation on larger patients' cohorts is essential. A closer cooperation between basic and clinical researchers is critical to succeed in clinical translation. Translating proteomic eye research attainments to the clinical ophthalmology would result in ocular disease detection improvement and personalized therapeutic modality development.

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References

1. Akpek EK, Smith RA (2013) Overview of age-related ocular conditions. *Am J Manag Care* 19(5 Suppl):S67–S75
2. Klein R, Klein BE (2013) The prevalence of age-related eye diseases and visual impairment in aging: current estimates. *Invest Ophthalmol Vis Sci* 54(14):ORSF5–ORSF13. <https://doi.org/10.1167/iovs.13-12789>
3. Ahmad MT, Zhang P, Dufresne C, Ferrucci L, Semba RD (2018) The Human Eye Proteome Project: updates on an emerging proteome. *Proteomics* 18(5–6):e1700394. <https://doi.org/10.1002/pmhc.201700394>
4. Lam TC, Chun RK, Li KK, To CH (2008) Application of proteomic technology in eye research: a mini review. *Clin Exp Optom* 91(1):23–33. <https://doi.org/10.1111/j.1444-0938.2007.00194.x>
5. Semba RD, Enghild JJ (2014) Proteomics and the eye. *Proteomics Clin Appl* 8(3–4):127–129. <https://doi.org/10.1002/prca.201470024>
6. Fortmann SD, Lorenc VE, Shen J, Hackett SF, Campochiaro PA (2018) Mousetap, a novel technique to collect uncontaminated vitreous or aqueous and expand usefulness of mouse models. *Sci Rep* 8(1):6371. <https://doi.org/10.1038/s41598-018-24197-2>
7. Hagan S, Tomlinson A (2013) Tear fluid biomarker profiling: a review of multiplex bead analysis. *Ocul Surf* 11(4):219–235. <https://doi.org/10.1016/j.jtos.2013.04.004>
8. VanDerMeid KR, Su SP, Krenzer KL, Ward KW, Zhang JZ (2011) A method to extract cytokines and matrix metalloproteinases from Schirmer strips and analyze using Luminex. *Mol Vis* 17:1056–1063
9. Inic-Kanada A, Nussbaumer A, Montanaro J, Belij S, Schlacher S, Stein E, Bintner N, Merio M, Zlabinger GJ, Barisani-Asenbauer T (2012) Comparison of ophthalmic sponges and extraction buffers for quantifying cytokine profiles in tears using Luminex technology. *Mol Vis* 18:2717–2725

10. Gonzalez-Gonzalez M, Bartolome R, Jara-Acevedo R, Casado-Vela J, Dasilva N, Matarraz S, Garcia J, Alcazar JA, Sayagues JM, Orfao A, Fuentes M (2014) Evaluation of homo- and hetero-functionally activated glass surfaces for optimized antibody arrays. *Anal Biochem* 450:37–45. <https://doi.org/10.1016/j.ab.2014.01.002>
11. Garranzo-Asensio M, San Segundo-Acosta P, Martinez-Useros J, Montero-Calle A, Fernandez-Acenero MJ, Haggmark-Manberg A, Pelaez-Garcia A, Villalba M, Rabano A, Nilsson P, Barderas R (2018) Identification of prefrontal cortex protein alterations in Alzheimer's disease. *Oncotarget* 9 (13):10847–10867. <https://doi.org/10.18632/oncotarget.24303>
12. Ahn SB, Khan A (2014) Detection and quantitation of twenty-seven cytokines, chemokines and growth factors pre- and post-high abundance protein depletion in human plasma. *EuPA Open Proteom* 3:78–84
13. Stampolidis P, Ullrich A, Iacobelli S (2015) LGALS3BP, lectin galactoside-binding soluble 3 binding protein, promotes oncogenic cellular events impeded by antibody intervention. *Oncogene* 34(1):39–52. <https://doi.org/10.1038/onc.2013.548>
14. Yu XB, Wallstrom G, Magee DM, Qiu J, Mendoza DEA, Wang J, Bian XF, Graves M, LaBaer J (2013) Quantifying antibody binding on protein microarrays using microarray nonlinear calibration. *Biotechniques* 54 (5):257. <https://doi.org/10.2144/000114028>
15. Hospach I, Joseph Y, Mai MK, Krasteva N, Nelles G (2014) Fabrication of homogeneous high-density antibody microarrays for cytokine detection. *Microarrays (Basel)* 3 (4):282–301. <https://doi.org/10.3390/microarrays3040282>
16. Barderas R, Bartolome RA, Fernandez-Acenero MJ, Torres S, Casal JI (2012) High expression of IL-13 receptor alpha2 in colorectal cancer is associated with invasion, liver metastasis, and poor prognosis. *Cancer Res* 72(11):2780–2790. <https://doi.org/10.1158/0008-5472.CAN-11-4090>
17. Huang RP (2001) Detection of multiple proteins in an antibody-based protein microarray system. *J Immunol Methods* 255(1–2):1–13
18. Jaeger PA, Lucin KM, Britschgi M, Vardarajan B, Huang RP, Kirby ED, Abbey R, Boeve BF, Boxer AL, Farrer LA, Finch N, Graff-Radford NR, Head E, Hoffree M, Huang R, Johns H, Karydas A, Knopman DS, Loboda A, Masliah E, Narasimhan R, Petersen RC, Podtelevnikov A, Pradhan S, Rademakers R, Sun CH, Younkin SG, Miller BL, Ideker T, Wyss-Coray T (2016) Network-driven plasma proteomics expose molecular changes in the Alzheimer's brain. *Mol Neurodegener* 11:31. <https://doi.org/10.1186/s13024-016-0095-2>
19. Madoz-Gurpide J, Canamero M, Sanchez L, Solano J, Alfonso P, Casal JI (2007) A proteomics analysis of cell signaling alterations in colorectal cancer. *Mol Cell Proteomics* 6 (12):2150–2164. <https://doi.org/10.1074/mcp.M700006-MCP200>
20. Pena C, Garcia JM, Larriba MJ, Barderas R, Gomez I, Herrera M, Garcia V, Silva J, Dominguez G, Rodriguez R, Cuevas J, de Herreros AG, Casal JI, Munoz A, Bonilla F (2009) SNAI1 expression in colon cancer related with CDH1 and VDR downregulation in normal adjacent tissue. *Oncogene* 28 (49):4375–4385. <https://doi.org/10.1038/onc.2009.285>
21. Babel I, Barderas R, Pelaez-Garcia A, Casal JI (2011) Antibodies on demand: a fast method for the production of human scFvs with minimal amounts of antigen. *BMC Biotechnol* 11:61. <https://doi.org/10.1186/1472-6750-11-61>
22. Delfani P, Dexlin Mellby L, Nordstrom M, Holmer A, Ohlsson M, Borrebaeck CA, Wingren C (2016) Technical advances of the recombinant antibody microarray technology platform for clinical immunoproteomics. *PLoS One* 11(7):e0159138. <https://doi.org/10.1371/journal.pone.0159138>
23. Borrebaeck CA, Wingren C (2009) Design of high-density antibody microarrays for disease proteomics: key technological issues. *J Proteomics* 72(6):928–935. <https://doi.org/10.1016/j.jprot.2009.01.027>
24. Bron AJ, de Paiva CS, Chauhan SK, Bonini S, Gabison EE, Jain S, Knop E, Markoulli M, Ogawa Y, Perez V, Uchino Y, Yokoi N, Zoukhri D, Sullivan DA (2017) TFOS DEWS II pathophysiology report. *Ocul Surf* 15(3):438–510. <https://doi.org/10.1016/j.jtos.2017.05.011>
25. Nelson JD, Craig JP, Akpek EK, Azar DT, Belmonte C, Bron AJ, Clayton JA, Dogru M, Dua HS, Foulks GN, Gomes JAP, Hammitt KM, Holopainen J, Jones L, Joo CK, Liu Z, Nichols JJ, Nichols KK, Novack GD, Sangwan V, Stapleton F, Tomlinson A, Tsubota K, Willcox MDP, Wolffsohn JS, Sullivan DA (2017) TFOS DEWS II introduction. *Ocul Surf* 15(3):269–275. <https://doi.org/10.1016/j.jtos.2017.05.005>

26. Begley CG, Chalmers RL, Abetz L, Venkataraman K, Mertzanis P, Caffery BA, Snyder C, Edrington T, Nelson D, Simpson T (2003) The relationship between habitual patient-reported symptoms and clinical signs among patients with dry eye of varying severity. *Invest Ophthalmol Vis Sci* 44 (11):4753–4761
27. Nichols KK, Nichols JJ, Mitchell GL (2004) The lack of association between signs and symptoms in patients with dry eye disease. *Cornea* 23(8):762–770
28. Hessen M, Akpek EK (2014) Dry eye: an inflammatory ocular disease. *J Ophthalmic Vis Res* 9(2):240–250
29. Stevenson W, Chauhan SK, Dana R (2012) Dry eye disease: an immune-mediated ocular surface disorder. *Arch Ophthalmol* 130 (1):90–100. <https://doi.org/10.1001/archophthalmol.2011.364>
30. Dionne K, Redfern RL, Nichols JJ, Nichols KK (2016) Analysis of tear inflammatory mediators: a comparison between the microarray and Luminex methods. *Mol Vis* 22:177–188
31. Boehm N, Riechardt AI, Wiegand M, Pfeiffer N, Grus FH (2011) Proinflammatory cytokine profiling of tears from dry eye patients by means of antibody microarrays. *Invest Ophthalmol Vis Sci* 52 (10):7725–7730. <https://doi.org/10.1167/iovs.11-7266>
32. Huang JF, Zhang Y, Rittenhouse KD, Pickering EH, McDowell MT (2012) Evaluations of tear protein markers in dry eye disease: repeatability of measurement and correlation with disease. *Invest Ophthalmol Vis Sci* 53 (8):4556–4564. <https://doi.org/10.1167/iovs.11-9054>
33. Lam H, Bleiden L, de Paiva CS, Farley W, Stern ME, Pflugfelder SC (2009) Tear cytokine profiles in dysfunctional tear syndrome. *Am J Ophthalmol* 147(2):198–205.e191. <https://doi.org/10.1016/j.ajo.2008.08.032>
34. Liu R, Gao C, Chen H, Li Y, Jin Y, Qi H (2017) Analysis of Th17-associated cytokines and clinical correlations in patients with dry eye disease. *PLoS One* 12(4):e0173301. <https://doi.org/10.1371/journal.pone.0173301>
35. Massingale ML, Li X, Vallabhajosyula M, Chen D, Wei Y, Asbell PA (2009) Analysis of inflammatory cytokines in the tears of dry eye patients. *Cornea* 28(9):1023–1027. <https://doi.org/10.1097/ICO.0b013e3181a16578>
36. Na KS, Mok JW, Kim JY, Rho CR, Joo CK (2012) Correlations between tear cytokines, chemokines, and soluble receptors and clinical severity of dry eye disease. *Invest Ophthalmol Vis Sci* 53(9):5443–5450. <https://doi.org/10.1167/iovs.11-9417>
37. Enriquez-de-Salamanca A, Castellanos E, Stern ME, Fernandez I, Carreno E, Garcia-Vazquez C, Herreras JM, Calonge M (2010) Tear cytokine and chemokine analysis and clinical correlations in evaporative-type dry eye disease. *Mol Vis* 16:862–873
38. Benito MJ, Gonzalez-Garcia MJ, Teson M, Garcia N, Fernandez I, Calonge M, Enriquez-de-Salamanca A (2014) Intra- and inter-day variation of cytokines and chemokines in tears of healthy subjects. *Exp Eye Res* 120:43–49. <https://doi.org/10.1016/j.exer.2013.12.017>
39. Giron-Gonzalez JA, Baturone R, Soto MJ, Marquez M, Macias I, Montes de Oca M, Medina F, Chozas N, Garcia-Perez S (2009) Implications of immunomodulatory interleukins for the hyperimmunoglobulinemia of Sjogren's syndrome. *Cell Immunol* 259 (1):56–60. <https://doi.org/10.1016/j.cellimm.2009.05.013>
40. Szodoray P, Alex P, Brun JG, Centola M, Jonsson R (2004) Circulating cytokines in primary Sjogren's syndrome determined by a multiplex cytokine array system. *Scand J Immunol* 59(6):592–599. <https://doi.org/10.1111/j.0300-9475.2004.01432.x>
41. Meadows JF, Dionne K, Nichols KK (2016) Differential profiling of T-cell cytokines as measured by protein microarray across dry eye subgroups. *Cornea* 35(3):329–335. <https://doi.org/10.1097/ICO.0000000000000721>
42. Lee SY, Han SJ, Nam SM, Yoon SC, Ahn JM, Kim TI, Kim EK, Seo KY (2013) Analysis of tear cytokines and clinical correlations in Sjogren syndrome dry eye patients and non-Sjogren syndrome dry eye patients. *Am J Ophthalmol* 156(2):247–253.e241. <https://doi.org/10.1016/j.ajo.2013.04.003>
43. Zhao H, Li Q, Ye M, Yu J (2018) Tear Luminex analysis in dry eye patients. *Med Sci Monitor* 24:7595–7602. <https://doi.org/10.12659/MSM.912010>
44. Li Z, Woo JM, Chung SW, Kwon MY, Choi JS, Oh HJ, Yoon KC (2013) Therapeutic effect of topical adiponectin in a mouse model of desiccating stress-induced dry eye. *Invest Ophthalmol Vis Sci* 54(1):155–162. <https://doi.org/10.1167/iovs.12-10648>
45. Huang JF, Yafawi R, Zhang M, McDowell M, Rittenhouse KD, Sace F, Liew SH, Cooper SR, Pickering EH (2012) Immunomodulatory effect of the topical ophthalmic Janus kinase inhibitor tofacitinib (CP-690,550) in patients with dry eye disease. *Ophthalmology*

- 119(7):e43–e50. <https://doi.org/10.1016/j.ophttha.2012.03.017>
46. Lee H, Min K, Kim EK, Kim TI (2012) Minocycline controls clinical outcomes and inflammatory cytokines in moderate and severe meibomian gland dysfunction. *Am J Ophthalmol* 154(6):949–957.e941. <https://doi.org/10.1016/j.ajo.2012.06.009>
 47. Wong WL, Su X, Li X, Cheung CM, Klein R, Cheng CY, Wong TY (2014) Global prevalence of age-related macular degeneration and disease burden projection for 2020 and 2040: a systematic review and meta-analysis. *Lancet Glob Health* 2(2):e106–e116. [https://doi.org/10.1016/S2214-109X\(13\)70145-1](https://doi.org/10.1016/S2214-109X(13)70145-1)
 48. Lambert NG, ElShelmani H, Singh MK, Mansergh FC, Wride MA, Padilla M, Keegan D, Hogg RE, Ambati BK (2016) Risk factors and biomarkers of age-related macular degeneration. *Prog Retin Eye Res* 54:64–102. <https://doi.org/10.1016/j.preteyeres.2016.04.003>
 49. Campa C, Harding SP (2011) Anti-VEGF compounds in the treatment of neovascular age related macular degeneration. *Curr Drug Targets* 12(2):173–181
 50. Takeda A, Baffi JZ, Kleinman ME, Cho WG, Nozaki M, Yamada K, Kaneko H, Albuquerque RJ, Dridi S, Saito K, Raisler BJ, Budd SJ, Geisen P, Munitz A, Ambati BK, Green MG, Ishibashi T, Wright JD, Humbles AA, Gerard CJ, Ogura Y, Pan Y, Smith JR, Grisanti S, Hartnett ME, Rothenberg ME, Ambati J (2009) CCR3 is a target for age-related macular degeneration diagnosis and therapy. *Nature* 460(7252):225–230. <https://doi.org/10.1038/nature08151>
 51. Sung HJ, Han JI, Lee JW, Uhm KB, Heo K (2012) TCCR/WSX-1 is a novel angiogenic factor in age-related macular degeneration. *Mol Vis* 18:234–240
 52. Cha DM, Woo SJ, Kim HJ, Lee C, Park KH (2013) Comparative analysis of aqueous humor cytokine levels between patients with exudative age-related macular degeneration and normal controls. *Invest Ophthalmol Vis Sci* 54(10):7038–7044. <https://doi.org/10.1167/iovs.13-12730>
 53. Azar WJ, Azar SH, Higgins S, Hu JF, Hoffman AR, Newgreen DF, Werther GA, Russo VC (2011) IGFBP-2 enhances VEGF gene promoter activity and consequent promotion of angiogenesis by neuroblastoma cells. *Endocrinology* 152(9):3332–3342. <https://doi.org/10.1210/en.2011-1121>
 54. Sun T, Cao H, Xu L, Zhu B, Gu Q, Xu X (2011) Insulin-like growth factor binding protein-related protein 1 mediates VEGF-induced proliferation, migration and tube formation of retinal endothelial cells. *Curr Eye Res* 36(4):341–349. <https://doi.org/10.3109/02713683.2010.545498>
 55. Nassar K, Grisanti S, Elfär E, Luke J, Luke M (2015) Serum cytokines as biomarkers for age-related macular degeneration. *Graefes Arch Clin Exp Ophthalmol* 253(5):699–704. <https://doi.org/10.1007/s00417-014-2738-8>
 56. Agrawal R, Balne PK, Wei X, Bijin VA, Lee B, Ghosh A, Narayanan R, Agrawal M, Connolly J (2019) Cytokine profiling in patients with exudative age-related macular degeneration and polypoidal choroidal vasculopathy. *Invest Ophthalmol Vis Sci* 60(1):376–382. <https://doi.org/10.1167/iovs.18-24387>
 57. Liu F, Ding X, Yang Y, Li J, Tang M, Yuan M, Hu A, Zhan Z, Li Z, Lu L (2016) Aqueous humor cytokine profiling in patients with wet AMD. *Mol Vis* 22:352–361
 58. Fauser S, Viebahn U, Muether PS (2015) Intraocular and systemic inflammation-related cytokines during one year of ranibizumab treatment for neovascular age-related macular degeneration. *Acta Ophthalmol* 93(8):734–738. <https://doi.org/10.1111/aos.12770>
 59. Liu RT, Wang A, To E, Gao J, Cao S, Cui JZ, Matsubara JA (2014) Vinpocetine inhibits amyloid-beta induced activation of NF-kappaB, NLRP3 inflammasome and cytokine production in retinal pigment epithelial cells. *Exp Eye Res* 127:49–58. <https://doi.org/10.1016/j.exer.2014.07.003>
 60. Or C, Cui J, Matsubara J, Forooghian F (2013) Pro-inflammatory and anti-angiogenic effects of bisphosphonates on human cultured retinal pigment epithelial cells. *Br J Ophthalmol* 97(8):1074–1078. <https://doi.org/10.1136/bjophthalmol-2013-303355>
 61. Lee YM, Lee YR, Kim CS, Jo K, Sohn E, Kim JS, Kim J (2016) Cnidium officinale extract and butylidenephthalide inhibits retinal neovascularization in vitro and in vivo. *BMC Complement Altern Med* 16:231. <https://doi.org/10.1186/s12906-016-1216-8>
 62. Lee YM, Lee YR, Kim CS, Jo K, Sohn E, Kim JS, Kim J (2015) Effect of Guibi-Tang, a traditional herbal formula, on retinal neovascularization in a mouse model of proliferative retinopathy. *Int J Mol Sci* 16(12):29900–29910. <https://doi.org/10.3390/ijms161226211>
 63. Wong TY, Cheung CM, Larsen M, Sharma S, Simo R (2016) Diabetic retinopathy. *Nat Rev*

- Dis Primers 2:16012. <https://doi.org/10.1038/nrdp.2016.12>
64. Yau JW, Rogers SL, Kawasaki R, Lamoureux EL, Kowalski JW, Bek T, Chen SJ, Dekker JM, Fletcher A, Grauslund J, Haffner S, Hamman RF, Ikram MK, Kayama T, Klein BE, Klein R, Krishnaiah S, Mayurasakorn K, O'Hare JP, Orchard TJ, Porta M, Rema M, Roy MS, Sharma T, Shaw J, Taylor H, Tielsch JM, Varma R, Wang JJ, Wang N, West S, Xu L, Yasuda M, Zhang X, Mitchell P, Wong TY (2012) Global prevalence and major risk factors of diabetic retinopathy. *Diabetes Care* 35 (3):556–564. <https://doi.org/10.2337/dc11-1909>
 65. Cai X, McGinnis JF (2016) Diabetic retinopathy: animal models, therapies, and perspectives. *J Diabetes Res* 2016:3789217. <https://doi.org/10.1155/2016/3789217>
 66. Pusparajah P, Lee LH, Abdul Kadir K (2016) Molecular markers of diabetic retinopathy: potential screening tool of the future? *Front Physiol* 7:200. <https://doi.org/10.3389/fphys.2016.00200>
 67. Suzuki Y, Nakazawa M, Suzuki K, Yamazaki H, Miyagawa Y (2011) Expression profiles of cytokines and chemokines in vitreous fluid in diabetic retinopathy and central retinal vein occlusion. *Jpn J Ophthalmol* 55 (3):256–263. <https://doi.org/10.1007/s10384-011-0004-8>
 68. Lange CAK, Stavarakas P, Luhmann UFO, de Silva DJ, Ali RR, Gregor ZJ, Bainbridge JWB (2011) Intraocular oxygen distribution in advanced proliferative diabetic retinopathy. *Am J Ophthalmol* 152(3):406–412.e403. <https://doi.org/10.1016/j.ajo.2011.02.014>
 69. Ghodasra DH, Fante R, Gardner TW, Langue M, Niziol LM, Besirli C, Cohen SR, Dedania VS, Demirci H, Jain N, Jayasundera KT, Johnson MW, Kalyani PS, Rao RC, Zacks DN, Sundstrom JM (2016) Safety and feasibility of quantitative multiplexed cytokine analysis from office-based vitreous aspiration. *Invest Ophthalmol Vis Sci* 57(7):3017–3023. <https://doi.org/10.1167/iops.15-18721>
 70. Reverter JL, Nadal J, Fernandez-Novell JM, Ballester J, Ramio-Lluch L, Rivera MM, Elizalde J, Abengoechea S, Guinovart JJ, Rodriguez-Gil JE (2009) Tyrosine phosphorylation of vitreous inflammatory and angiogenic peptides and proteins in diabetic retinopathy. *Invest Ophthalmol Vis Sci* 50 (3):1378–1382. <https://doi.org/10.1167/iops.08-2736>
 71. Klaassen I, de Vries EW, Vogels IMC, van Kampen AHC, Bosscha MI, Steel DHW, Van Noorden CJF, Lesnik-Oberstein SY, Schlingemann RO (2017) Identification of proteins associated with clinical and pathological features of proliferative diabetic retinopathy in vitreous and fibrovascular membranes. *PLoS One* 12(11):e0187304. <https://doi.org/10.1371/journal.pone.0187304>
 72. Urbancic M, Petrovic D, Zivin AM, Korosec P, Flezar M, Petrovic MG (2020) Correlations between vitreous cytokine levels and inflammatory cells in fibrovascular membranes of patients with proliferative diabetic retinopathy. *Mol Vis* 26:472–482
 73. Takeuchi M, Sato T, Tanaka A, Muraoka T, Taguchi M, Sakurai Y, Karasawa Y, Ito M (2015) Elevated levels of cytokines associated with Th2 and Th17 cells in vitreous fluid of proliferative diabetic retinopathy patients. *PLoS One* 10(9):e0137358. <https://doi.org/10.1371/journal.pone.0137358>
 74. Srividya G, Jain M, Mahalakshmi K, Gayathri S, Raman R, Angayarkanni N (2018) A novel and less invasive technique to assess cytokine profile of vitreous in patients of diabetic macular oedema. *Eye (Lond)* 32(4):820–829. <https://doi.org/10.1038/eye.2017.285>
 75. Cacciamani A, Esposito G, Scarinci F, Parravano M, Dinice L, Di Nicola M, Micera A (2019) Inflammatory mediators in the vitreal reflux of patients with diabetic macular edema. *Graefes Arch Clin Exp Ophthalmol* 257(1):187–197. <https://doi.org/10.1007/s00417-018-4169-4>
 76. Khuu LA, Tayyari F, Sivak JM, Flanagan JG, Singer S, Brent MH, Huang D, Tan O, Hudson C (2017) Aqueous humour concentrations of TGF-beta, PLGF and FGF-1 and total retinal blood flow in patients with early non-proliferative diabetic retinopathy. *Acta Ophthalmol* 95(3):e206–e211. <https://doi.org/10.1111/aos.13230>
 77. Hong F, Yang DY, Li L, Zheng YF, Wang XJ, Guo SRN, Jiang S, Zhu D, Tao Y (2020) Relationship between aqueous humor levels of cytokines and axial length in patients with diabetic retinopathy. *Asia Pac J Ophthalmol (Phila)* 9(2):149–155. <https://doi.org/10.1097/APO.0000000000000270>
 78. Vujosevic S, Micera A, Bini S, Berton M, Esposito G, Midena E (2016) Proteome analysis of retinal glia cells-related inflammatory cytokines in the aqueous humour of diabetic patients. *Acta Ophthalmol* 94(1):56–64. <https://doi.org/10.1111/aos.12812>
 79. Oh IK, Kim SW, Oh J, Lee TS, Huh K (2010) Inflammatory and angiogenic factors in the aqueous humor and the relationship to diabetic retinopathy. *Curr Eye Res* 35

- (12):1116–1127. <https://doi.org/10.3109/02713683.2010.510257>
80. Wu H, Hwang DK, Song X, Tao Y (2017) Association between aqueous cytokines and diabetic retinopathy stage. *J Ophthalmol* 2017:9402198. <https://doi.org/10.1155/2017/9402198>
 81. Dong N, Xu B, Chu LQ, Tang X (2015) Study of 27 aqueous humor cytokines in type 2 diabetic patients with or without macular edema. *PLoS One* 10(4):ARTN. e0125329. <https://doi.org/10.1371/journal.pone.0125329>
 82. Lim SW, Bandala-Sanchez E, Kolic M, Rogers SL, McAuley AK, Lim LL, Wickremasinghe SS (2018) The influence of intravitreal ranibizumab on inflammation-associated cytokine concentrations in eyes with diabetic macular edema. *Invest Ophthalmol Vis Sci* 59(13):5382–5390. <https://doi.org/10.1167/iops.17-23325>
 83. Roh MI, Kim HS, Song JH, Lim JB, Kwon OW (2009) Effect of intravitreal bevacizumab injection on aqueous humor cytokine levels in clinically significant macular edema. *Ophthalmology* 116(1):80–86. <https://doi.org/10.1016/j.ophtha.2008.09.036>
 84. Forooghian F, Kertes PJ, Eng KT, Agron E, Chew EY (2010) Alterations in the intraocular cytokine milieu after intravitreal bevacizumab. *Invest Ophthalmol Vis Sci* 51(5):2388–2392. <https://doi.org/10.1167/iops.09-4065>
 85. Yu SY, Nam DH, Lee DY (2018) Changes in aqueous concentrations of various cytokines after intravitreal bevacizumab and subtenon triamcinolone injection for diabetic macular edema. *Graefes Arch Clin Exp Ophthalmol* 256(1):39–47. <https://doi.org/10.1007/s00417-017-3819-2>
 86. Jeon S, Lee WK (2014) Effect of intravitreal triamcinolone in diabetic macular edema unresponsive to intravitreal bevacizumab. *Retina* 34(8):1606–1611. <https://doi.org/10.1097/iae.0000000000000109>
 87. Quigley HA (2011) Glaucoma. *Lancet* 377(9774):1367–1377. [https://doi.org/10.1016/S0140-6736\(10\)61423-7](https://doi.org/10.1016/S0140-6736(10)61423-7)
 88. Sun X, Dai Y, Chen Y, Yu DY, Cringle SJ, Chen J, Kong X, Wang X, Jiang C (2016) Primary angle closure glaucoma: what we know and what we don't know. *Prog Retin Eye Res*. <https://doi.org/10.1016/j.preteyeres.2016.12.003>
 89. Tamm ER (2009) The trabecular meshwork outflow pathways: structural and functional aspects. *Exp Eye Res* 88(4):648–655. <https://doi.org/10.1016/j.exer.2009.02.007>. S0014-4835(09)00045-1 [pii]
 90. Izzotti A, Longobardi M, Cartiglia C, Sacca SC (2010) Proteome alterations in primary open angle glaucoma aqueous humor. *J Proteome Res* 9(9):4831–4838. <https://doi.org/10.1021/pr1005372>
 91. Bagnis A, Izzotti A, Centofanti M, Sacca SC (2012) Aqueous humor oxidative stress proteomic levels in primary open angle glaucoma. *Exp Eye Res* 103:55–62. <https://doi.org/10.1016/j.exer.2012.07.011>
 92. Sacca SC, Centofanti M, Izzotti A (2012) New proteins as vascular biomarkers in primary open angle glaucomatous aqueous humor. *Invest Ophthalmol Vis Sci* 53(7):4242–4253. <https://doi.org/10.1167/iops.11-8902>
 93. Micera A, Quaranta L, Esposito G, Floriani I, Pocobelli A, Sacca SC, Riva I, Manni G, Oddone F (2016) Differential protein expression profiles in glaucomatous trabecular meshwork: an evaluation study on a small primary open angle glaucoma population. *Adv Ther* 33(2):252–267. <https://doi.org/10.1007/s12325-016-0285-x>
 94. Peng C, Wu Y, Ding X, Chen D, Zeng C, Xu L, Guo W (2020) Characteristic cytokine profiles of aqueous humor in glaucoma secondary to Sturge-Weber syndrome. *Front Immunol* 11:4. <https://doi.org/10.3389/fimmu.2020.00004>
 95. Kuchtey J, Rezaei KA, Jaru-Ampornpan P, Sternberg P Jr, Kuchtey RW (2010) Multiplex cytokine analysis reveals elevated concentration of interleukin-8 in glaucomatous aqueous humor. *Invest Ophthalmol Vis Sci* 51(12):6441–6447. <https://doi.org/10.1167/iops.10-5216>
 96. Gramlich OW, Beck S, von Thun Und Hohenstein-Blaul N, Boehm N, Ziegler A, Vetter JM, Pfeiffer N, Grus FH (2013) Enhanced insight into the autoimmune component of glaucoma: IgG autoantibody accumulation and pro-inflammatory conditions in human glaucomatous retina. *PLoS One* 8(2):e57557. <https://doi.org/10.1371/journal.pone.0057557>
 97. Weiskirchen R, Weiskirchen S, Tacke F (2019) Organ and tissue fibrosis: molecular signals, cellular mechanisms and translational implications. *Mol Asp Med* 65:2–15. <https://doi.org/10.1016/j.mam.2018.06.003>
 98. Gajda-Derylo B, Stahnke T, Struckmann S, Warsaw G, Birke K, Birke MT, Hohberger B, Rejdak R, Fuellen G, Junemann AG (2019) Comparison of cytokine/

- chemokine levels in aqueous humor of primary open-angle glaucoma patients with positive or negative outcome following trabeculectomy. *Biosci Rep* 39(5). <https://doi.org/10.1042/BSR20181894>
99. Ekins R, Chu F (1997) Immunoassay and other ligand assays: present status and future trends. *J Int Fed Clin Chem* 9(3):100–109
 100. Davuluri G, Espina V, Petricoin EF III, Ross M, Deng J, Liotta LA, Glaser BM (2009) Activated VEGF receptor shed into the vitreous in eyes with wet AMD: a new class of biomarkers in the vitreous with potential for predicting the treatment timing and monitoring response. *Arch Ophthalmol* 127(5):613–621. <https://doi.org/10.1001/archophthalmol.2009.88>
 101. Ecker SM, Pfahler SM, Hines JC, Lovelace AS, Glaser BM (2012) Sequential in-office vitreous aspirates demonstrate vitreous matrix metalloproteinase 9 levels correlate with the amount of subretinal fluid in eyes with wet age-related macular degeneration. *Mol Vis* 18:1658–1667
 102. Biasutto L, Chiechi A, Couch R, Liotta LA, Espina V (2013) Retinal pigment epithelium (RPE) exosomes contain signaling phosphoproteins affected by oxidative stress. *Exp Cell Res* 319(13):2113–2123. <https://doi.org/10.1016/j.yexcr.2013.05.005>
 103. Babel I, Barderas R, Diaz-Uriarte R, Moreno V, Suarez A, Fernandez-Acenero MJ, Salazar R, Capella G, Casal JI (2011) Identification of MST1/STK4 and SULF1 proteins as autoantibody targets for the diagnosis of colorectal cancer by using phage microarrays. *Mol Cell Proteomics* 10(3):M110.001784. <https://doi.org/10.1074/mcp.M110.001784>
 104. Chatterjee M, Ionan A, Draghici S, Tainsky MA (2006) Epitomics: global profiling of immune response to disease using protein microarrays. *OMICS* 10(4):499–506. <https://doi.org/10.1089/omi.2006.10.499>
 105. Chatterjee M, Mohapatra S, Ionan A, Bawa G, Ali-Fehmi R, Wang X, Nowak J, Ye B, Nahhas FA, Lu K, Witkin SS, Fishman D, Munkarah A, Morris R, Levin NK, Shirley NN, Tromp G, Abrams J, Draghici S, Tainsky MA (2006) Diagnostic markers of ovarian cancer by high-throughput antigen cloning and detection on arrays. *Cancer Res* 66(2):1181–1190. <https://doi.org/10.1158/0008-5472.CAN-04-2962>
 106. Wang X, Yu J, Sreekumar A, Varambally S, Shen R, Giacherio D, Mehra R, Montie JE, Pienta KJ, Sanda MG, Kantoff PW, Rubin MA, Wei JT, Ghosh D, Chinnaiyan AM (2005) Autoantibody signatures in prostate cancer. *N Engl J Med* 353(12):1224–1235. <https://doi.org/10.1056/NEJMoa051931>
 107. Nagele E, Han M, Demarshall C, Belinka B, Nagele R (2011) Diagnosis of Alzheimer's disease based on disease-specific autoantibody profiles in human sera. *PLoS One* 6(8):e23112. <https://doi.org/10.1371/journal.pone.0023112>
 108. Nagele EP, Han M, Acharya NK, DeMarshall C, Kosciuk MC, Nagele RG (2013) Natural IgG autoantibodies are abundant and ubiquitous in human sera, and their number is influenced by age, gender, and disease. *PLoS One* 8(4):e60726. <https://doi.org/10.1371/journal.pone.0060726>
 109. DeMarshall C, Sarkar A, Nagele EP, Goldwasser E, Godsey G, Acharya NK, Nagele RG (2015) Utility of autoantibodies as biomarkers for diagnosis and staging of neurodegenerative diseases. *Int Rev Neurobiol* 122:1–51. <https://doi.org/10.1016/bs.irn.2015.05.005>
 110. Han M, Nagele E, DeMarshall C, Acharya N, Nagele R (2012) Diagnosis of Parkinson's disease based on disease-specific autoantibody profiles in human sera. *PLoS One* 7(2):e32383. <https://doi.org/10.1371/journal.pone.0032383>
 111. Barderas R, Babel I, Diaz-Uriarte R, Moreno V, Suarez A, Bonilla F, Villar-Vazquez R, Capella G, Casal JI (2012) An optimized predictor panel for colorectal cancer diagnosis based on the combination of tumor-associated antigens obtained from protein and phage microarrays. *J Proteome* 75(15):4647–4655. <https://doi.org/10.1016/j.jprot.2012.03.004>
 112. Barderas R, Villar-Vazquez R, Fernandez-Acenero MJ, Babel I, Pelaez-Garcia A, Torres S, Casal JI (2013) Sporadic colonic cancer murine models demonstrate the value of autoantibody detection for preclinical cancer diagnosis. *Sci Rep* 3:2938. <https://doi.org/10.1038/srep02938>
 113. Babel I, Barderas R, Diaz-Uriarte R, Martinez-Torrecuadrada JL, Sanchez-Carbayo M, Casal JI (2009) Identification of tumor-associated autoantigens for the diagnosis of colorectal cancer in serum using high density protein microarrays. *Mol Cell Proteomics* 8(10):2382–2395. <https://doi.org/10.1074/mcp.M800596-MCP200>
 114. Fasolo J, Im H, Snyder MP (2015) Probing high-density functional protein microarrays to detect protein-protein interactions. *J Vis Exp* 102:e51872. <https://doi.org/10.3791/51872>

115. Cox E, Uzoma I, Guzzo C, Jeong JS, Matunis M, Blackshaw S, Zhu H (2015) Identification of SUMO E3 ligase-specific substrates using the HuProt human proteome microarray. *Methods Mol Biol* 1295:455–463. https://doi.org/10.1007/978-1-4939-2550-6_32
116. Ayoglu B, Haggmark A, Khademi M, Olsson T, Uhlen M, Schwenk JM, Nilsson P (2013) Autoantibody profiling in multiple sclerosis using arrays of human protein fragments. *Mol Cell Proteomics* 12(9):2657–2672. <https://doi.org/10.1074/mcp.M112.026757>
117. Uhlen M, Fagerberg L, Hallstrom BM, Lindskog C, Oksvold P, Mardinoglu A, Sivertsson A, Kampf C, Sjostedt E, Asplund A, Olsson I, Edlund K, Lundberg E, Navani S, Szigarto CA, Odeberg J, Djureinovic D, Takanen JO, Hober S, Alm T, Edqvist PH, Berling H, Tegel H, Mulder J, Rockberg J, Nilsson P, Schwenk JM, Hamsten M, von Feilitzen K, Forsberg M, Persson L, Johansson F, Zwaalen M, von Heijne G, Nielsen J, Ponten F (2015) Proteomics. Tissue-based map of the human proteome. *Science* 347(6220):1260419. <https://doi.org/10.1126/science.1260419>
118. Uhlen M, Oksvold P, Fagerberg L, Lundberg E, Jonasson K, Forsberg M, Zwaalen M, Kampf C, Wester K, Hober S, Wernerus H, Bjorling L, Ponten F (2010) Towards a knowledge-based Human Protein Atlas. *Nat Biotechnol* 28(12):1248–1250. <https://doi.org/10.1038/nbt1210-1248>
119. Frederick PA, Kleinman ME (2014) The immune system and AMD. *Curr Ophthalmol Rep* 2(1):14–19. <https://doi.org/10.1007/s40135-013-0037-x>
120. Morohoshi K, Goodwin AM, Ohbayashi M, Ono SJ (2009) Autoimmunity in retinal degeneration: autoimmune retinopathy and age-related macular degeneration. *J Autoimmun* 33(3–4):247–254. <https://doi.org/10.1016/j.jaut.2009.09.003>
121. Patel N, Ohbayashi M, Nugent AK, Ramchand K, Toda M, Chau KY, Bunce C, Webster A, Bird AC, Ono SJ, Chong V (2005) Circulating anti-retinal antibodies as immune markers in age-related macular degeneration. *Immunology* 115(3):422–430. <https://doi.org/10.1111/j.1365-2567.2005.02173.x>
122. Morohoshi K, Patel N, Ohbayashi M, Chong V, Grossniklaus HE, Bird AC, Ono SJ (2012) Serum autoantibody biomarkers for age-related macular degeneration and possible regulators of neovascularization. *Exp Mol Pathol* 92(1):64–73. <https://doi.org/10.1016/j.yexmp.2011.09.017>
123. Boehm N, Wolters D, Thiel U, Lossbrand U, Wiegel N, Pfeiffer N, Grus FH (2012) New insights into autoantibody profiles from immune privileged sites in the eye: a glaucoma study. *Brain Behav Immun* 26(1):96–102. <https://doi.org/10.1016/j.bbi.2011.07.241>
124. Dervan EW, Chen H, Ho SL, Brummel N, Schmid J, Toomey D, Haralambova M, Gould E, Wallace DM, Prehn JH, O'Brien CJ, Murphy D (2010) Protein microarray profiling of serum autoantibodies in pseudoexfoliation glaucoma. *Invest Ophthalmol Vis Sci* 51(6):2968–2975. <https://doi.org/10.1167/iovs.09-4898>
125. Gramlich OW, Bell K, von Thun Und Hohenstein-Blaul N, Wilding C, Beck S, Pfeiffer N, Grus FH (2013) Autoimmune biomarkers in glaucoma patients. *Curr Opin Pharmacol* 13(1):90–97. <https://doi.org/10.1016/j.coph.2012.09.005>
126. Kim JM, Kyung H, Azarbod P, Lee JM, Caprioli J (2014) Disc haemorrhage is associated with the fast component, but not the slow component, of visual field decay rate in glaucoma. *Br J Ophthalmol* 98(11):1555–1559. <https://doi.org/10.1136/bjophthalmol-2013-304584>
127. Lorenz K, Beck S, Keilani MM, Wasielica-Poslednik J, Pfeiffer N, Grus FH (2016) Longitudinal analysis of serum autoantibody-reactivities in patients with primary open angle glaucoma and optic disc hemorrhage. *PLoS One* 11(12):e0166813. <https://doi.org/10.1371/journal.pone.0166813>
128. Lorenz K, Beck S, Keilani MM, Wasielica-Poslednik J, Pfeiffer N, Grus FH (2016) Course of serum autoantibodies in patients after acute angle closure glaucoma attack. *Clin Exp Ophthalmol*. <https://doi.org/10.1111/ceo.12864>
129. Gramlich OW, Teister J, Neumann M, Tao X, Beck S, von Pein HD, Pfeiffer N, Grus FH (2016) Immune response after intermittent minimally invasive intraocular pressure elevations in an experimental animal model of glaucoma. *J Neuroinflammation* 13(1):82. <https://doi.org/10.1186/s12974-016-0542-6>

Part VI

Statistical Methods for Analyzing Protein Microarray



Chapter 18

Statistical Methods for Analysis of Protein Microarray Data Using R

Yunro Chung

Abstract

This chapter aims to provide statistical methods for analyzing protein microarray data. It uses a publicly available protein array dataset and emphasizes practical applications in statistics using R, a statistical software. A wide range of statistical methods will be demonstrated, including descriptive statistics, hypothesis testing, false discovery rate, receiver operating characteristic curve, correlation, visualization, and power analysis. The R code used to perform the statistical analyses will be provided.

Key words Biostatistics, Correlation, False discovery rate, Mann-Whitney *U* test, Nonparametric test, Sample size calculation, Significance level, *t*-Test, *p*-Value, *q*-Value

1 Introduction

Statistics is a field that deals with the collection, summarization, analysis, and interpretation of data [1]. It has been widely applied to a variety of academic disciplines and research areas. In basic science or translational research, various statistical methods and techniques are frequently used to analyze and interpret data. For example, first, scientists develop a hypothesis that addresses their research questions. Then, they conduct laboratory experiments to examine the samples that they collected. Finally, they analyze their data to test their hypothesis. During this research process, statistics plays a crucial role particularly for the early stage of study design and the later stage of data analysis. In research journals or scientific articles, biological or clinical findings are reported along with statistical analysis results and include several statistical terminologies such as significance level or *p*-value. Hence, it is useful to understand the basic concepts of statistics and learn their applications.

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There are a wide range of statistical techniques and tools available. Traditional statistical methods focused on simple research questions. In the late 1990s and 2000s, DNA microarray technologies emerged as key tools for genomic expression analysis. It enabled us to quantify hundreds of thousands of genes together, generating a high-dimensional microarray dataset where the number of genes is far larger than the sample size. Since then, several statistical methods have been developed to tailor to microarray data analysis. Most of these methods are directly applicable in analyzing protein microarray datasets because DNA and protein microarray experiments generate datasets with similar structures.

In this chapter, we explain basic statistical concepts and demonstrate how to use R to analyze nucleic acid programmable protein array (NAPPA) datasets. NAPPA is a core technology at the Biodesign Institute at Arizona State University and has successfully discovered protein biomarkers for several diseases [2]. We will provide R code to analyze the sample NAPPA dataset. The code will include topics such as descriptive statistics, graphics, t -tests, Wilcoxon rank-sum tests, adjusted p -values, evaluation of biomarkers, and correlation. Note that while we use the NAPPA dataset as an example, the methods we introduce are general and can be applied to other protein microarray datasets.

2 Materials

1. NAPPA datasets: The dataset we will analyze is named `nappa.csv` and is provided in the supplementary materials. The data includes a total of 90 samples, with 45 basal-like breast cancer (BLBC) samples and 45 healthy control samples, and 100 selected proteins. In the `nappa.csv` file, each column represents a sample and each row represents a protein. The first and second rows are sample ID and BLBC status ($y = 1$ for BLBC or $y = 0$ control). The 3rd to 102nd rows are (normalized) intensity values for the 100 selected proteins named P1, P2, ..., P100. The full dataset is publicly available and can be downloaded from the Protein Microarray Database (<http://www.proteinmicroarray.cn/>) [3].
2. R statistical software: We will use R version 4.0.2 which is an open-source software and can be downloaded for free from the R Project website (<http://www.r-project.org/>) [4].
3. The R file named `nappa.R` includes all of the R codes in this chapter and can be found in the supplementary materials.

3 Methods

3.1 Exploratory Data Analysis

Since each protein in the BLBC study is measured on a continuous scale, we can use the mean and standard deviation to summarize their characteristics. Alternatively, the median and interquartile range (IQR) can be used when outliers or extreme values exist, where the IQR is $Q3 - Q1$, and $Q1$, $Q2$ (or median), and $Q3$ are the 25th, 50th, and 75th percentile of the data. Mean and median measure the central tendency of the data. Standard deviation and IQR measure the variability of the data. For the protein P1, for example, we see that the mean $\pm$ standard deviation is 2.68 ± 0.85 and 2.18 ± 0.79 and the median $\pm$ IQR is 2.77 ± 0.85 and 2.32 ± 0.97 for the BLBC and control groups.

A box plot is commonly used to visualize data by representing the five-number summary: maximum, $Q3$, median, $Q2$, and minimum. The maximum and minimum values are often replaced with $Q3 + 1.5 \times \text{IQR}$ and $Q1 - 1.5 \times \text{IQR}$, where any value above or below the threshold value could be regarded as potential outliers (Fig. 1).

Several other summary measures or figures would be applicable to summarize the data. These types of analyses are called exploratory data analyses [5], which is generally the first step of data analysis.

3.2 Differential Analysis of a Single Protein

In the subsection above, we computed the summary statistics for protein P1 for the BLBC and control groups. The next step would be to decide whether we can claim that the response of P1 is significantly different between the BLBC and control groups.

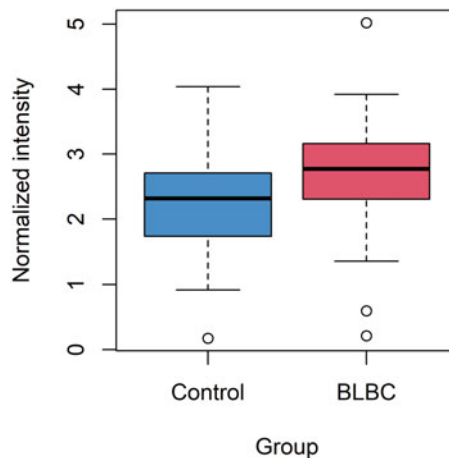


Fig. 1 Box plot of normalized intensities for the protein P1

Because we will draw a conclusion based on the sample, the decision should be affected by how we collect our samples. Statistical hypothesis testing allows us to consider sampling variability and make a decision using probability theory.

Statistical hypothesis testing includes multiple steps. First, we develop the null and alternative hypotheses. In general, the null hypothesis states no difference, no effect, or no relationship. The alternative hypothesis states that the null hypothesis is not true. Researchers usually want to reject the null hypothesis and conclude the new finding stated in the alternative hypothesis. The null and alternative hypotheses are often denoted as H_0 and H_1 , respectively. For example, for the BLBC study, H_0 states that there is no difference in P1's mean intensity between the BLBC and healthy groups, and H_1 states that there is a difference between them. Second, we set a type I error rate, denoted as α , which is the probability of rejecting the H_0 when it is actually true. The type I error rate is commonly referred to as the significance level. Third, we collect our samples and calculate the p -value, which is the probability of getting an extreme result assuming that H_0 is true. Lastly, we compare the p -value to α . If the p -value is smaller than α , we reject H_0 and conclude that the mean difference of intensity is significantly different between the two groups. Otherwise, we fail to reject H_0 and conclude that we have no sufficient evidence to support H_1 .

Using statistical hypothesis testing, we can control the type I error when we make a decision. In most cases, α is set to 0.05, which implies that 5 out of 100 null hypotheses are erroneously rejected when they are true, given many repeated experiments. A smaller or larger α such as 0.01 or 0.1 can be used depending on the research question. It is not recommended to compute the p -value first and then find α to get significant results. The significance level α should be determined before analyzing the data. It is important to note that statistical significance does not imply clinical or scientific significance.

For the BLBC study, we can use either a t -test or Wilcoxon rank-sum test to compare the mean intensity between the BLBC and control groups. Specifically, we use a t -test if the data follows a normal distribution. Otherwise, we use the Wilcoxon rank-sum test, also known as the Mann-Whitney U or Wilcoxon-Mann-Whitney test. Note that the normal distribution is a bell-shaped curve, symmetric about the mean, and frequently used in many statistical methods. To check the normality assumption, one of the popular approaches is to produce a histogram with a normal curve and normal quantile-quantile (Q - Q) plot. We can then visually check the normality assumption if the histogram approximately follows the normal curve (Fig. 2) and the Q - Q plot is approximately a straight line (Fig. 3). Here, we only show these plots for the BLBC group. The same type of plots can be produced for the control group.

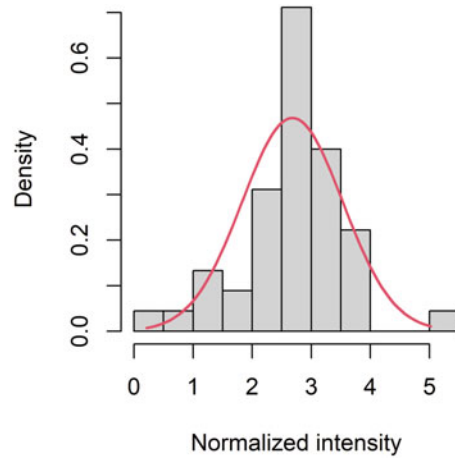


Fig. 2 Histogram for the protein P1 in the BLBC group

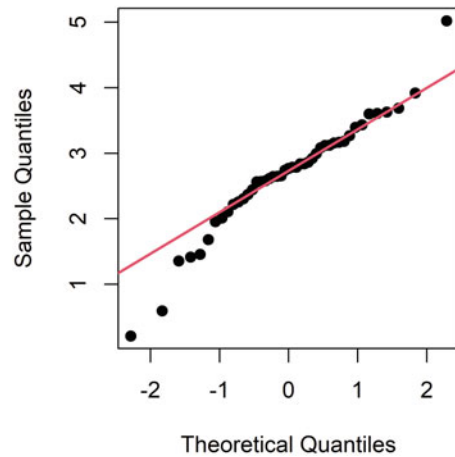


Fig. 3 Normal quantile-quantile plot for the protein P1 in the BLBC group

We can also use the Shapiro-Wilk test to test the normality assumption. We see that p -values are 0.95 and 0.98 for the BLBC and control groups, which are greater than 0.05 and do not support non-normal distributions. We can thus use the t -test.

To apply the t -test, we first need to check whether the variance between the two groups is equal or not. Unless there is a specific reason, we perform Barlett's test to check for equal or unequal variance using the following R code. The p -value is 0.21, which is greater than 0.05 and does not support an unequal variance. Thus, we apply the t -test assuming equal variance. The p -value is 0.005, which is smaller than 0.05. Therefore, we reject H_0 and conclude that P1's mean difference of intensity is significantly different between the two groups.

The output from the t -test also shows 95% confidence interval (CI). Specifically, P1's mean difference of intensity between the two groups is $-0.50 (=2.18-2.68)$ and its 95% CI is $(-0.84, -0.15)$, where -0.84 is called the lower confidence limit and -0.15 is called the upper confidence limit. It implies that if we repeated the experiment 100 times and computed 95% CIs for each experiment, 95 out of 100 CIs would cover the true mean difference and 5 would not.

Suppose that the data is not normally distributed or the sample size is too small to check the normality assumption. In this case, the Wilcoxon rank-sum test can be used to compare the two groups. The p -value is 0.002, less than 0.05, so we reject the null hypothesis.

In summary, check the normality assumption. If it holds, additionally verify the equal variance assumption and use the t -test assuming equal or unequal variance. Otherwise, use the Wilcoxon rank-sum test, which does not require any distributional assumption and is often called a nonparametric or distribution-free test.

3.3 Differential Analysis of Multiple Proteins

Since the NAPPA file contains 100 proteins, we apply statistical hypothesis testing to each of the proteins. For example, 49 proteins were identified as significant, with p -values less than 0.05, if we applied the t -test to each protein. A major problem with this approach is the inflated type I error rate. In other words, since we set the type I error rate at 0.05 for testing each protein, the probability of observing at least one significant protein due to chance, called the family-wise error rate (FWER), is $1 - (1 - 0.05)^{100} = 0.99$. This implies that with the 100 proteins being tested, we have a 99% chance of identifying at least one significant protein even if all of the proteins are not actually significant. To control the inflated type I error rate, several statistical methods have been proteins.

The Bonferroni correction [6] controls the FWER by setting the significance cutoff at $\alpha/100$. Suppose we identify two proteins with p -values less than $0.05/100 = 0.0005$. Using the Bonferroni correction, we would control the FWER at $1 - (1 - 0.0005)^{100} = 0.0488$ which is approximately 0.05. Equivalently, instead of comparing the p -value to the adjusted type I error, we can adjust the p -value and compare the adjusted p -value to the FWER of 0.05 directly. However, the Bonferroni correction is known to be conservative, which could miss some significant proteins.

A more popular approach is to control the false-positive rate (FDR), which is the expected proportion of discoveries that are false-positive proteins (incorrect rejections of the null hypothesis) among all significant proteins (rejections of the null hypothesis). The Benjamini and Hochberg correction [7] sorts p -values from smallest to largest and sets the significance cutoff at α times $k/100$

for the k th smallest p -value. For the BLBC study, we identified 35 significant proteins by controlling the FDR at 0.05.

Storey [8, 9] suggested controlling the positive FDR by omitting the probability of rejecting the null hypothesis that is greater than 0 in the FDR. This probability is usually close to 1, especially for protein microarray studies. Thus, controlling the FDR or positive FDR would yield the same if not similar results in practice. The positive FDR-adjusted p -value is called the q -value, which can be computed using the q -value R package [10].

3.4 Analysis of Protein Biomarkers

The BLBC study was designed to identify protein biomarkers for screening or early diagnosis. Sensitivity and specificity are some of the more popular measures to evaluate the performance of biomarkers. Specifically, we set an arbitrary threshold value and classify samples whose intensities are greater than the threshold value as marker positives. Otherwise, those whose intensities are less than or equal to the threshold value are classified as marker negatives. Four possible outcomes can occur: true positive (TP), true negative (TN), false positive (FP), and false negative (FN). TP and TN are correct decisions, where TP is the number of samples who are marker positive and have BLBC, and TN is the number of samples who are marker negative and do not have BLBC (control). FP and FN are incorrect decisions, where FP is the number of samples who are marker positive but do not have BLBC, and FN is the number of samples who are marker negative but have BLBC. Sensitivity and specificity are defined as the proportion of marker positives among the BLBC samples and marker negatives among the control samples. Thus, it is of interest to discover biomarkers with high sensitivity and high specificity.

To better explain these terminologies, we produced a dot plot for the protein P1 with an arbitrary threshold value of 4 (Fig. 4).

The TP count is 15, FN is 30, FP is 5, and TN is 40. Thus, sensitivity and specificity are computed as $15/45 = 0.33$ and $40/45 = 0.89$, respectively. Clearly these values depend on which threshold value we used. For example, if a higher threshold value were chosen, we would observe more marker positives, which increase sensitivity but decrease specificity. For a lower threshold value, we would observe more marker negatives, which decrease sensitivity but increase specificity. In other words, it is not possible to find a threshold value to increase both sensitivity and specificity. To avoid threshold selection problems, the receiver operating characteristic (ROC) curve is used by plotting sensitivity versus one minus specificity for all possible threshold values (Fig. 5).

We can evaluate the classification performance of P1 using the ROC curve. A popular evaluation measure is the area under the ROC curve (AUC). AUC values range from 0 to 1, where an AUC value close to 1 indicates better performance, whereas an AUC value close to 0.5 or below indicates a lower performance.

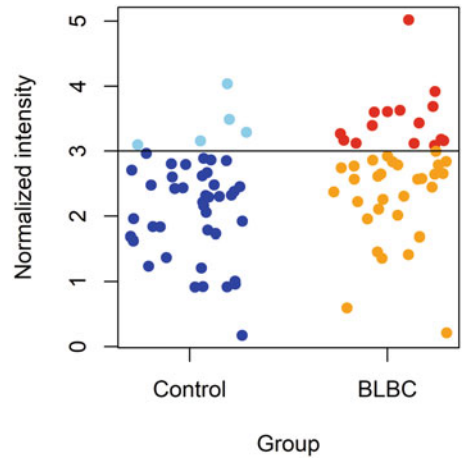


Fig. 4 Dot plot with a threshold value of three, where the red points are true positives, the orange points are false negatives, the sky points are false positives, and the blue points are true negatives

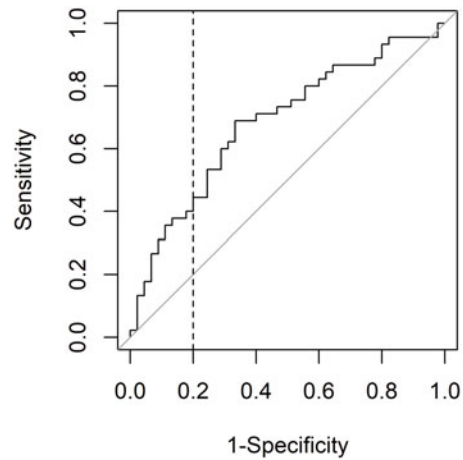


Fig. 5 Receiver operating characteristic curve, where the gray line is the 45° degree straight line, and the dashed line is a vertical line at the specificity of 0.8

Theoretically, an ineffective biomarker has an ROC curve on the 45° straight line yielding an AUC value of 0.5. Two other measures commonly used are also based on the ROC curve. One is partial AUC which is the AUC in a specific specificity range. The other is sensitivity at a fixed specificity. The range of specificity can be determined clinically. In general, a higher specificity is preferred; however, no data will be available if the specificity is set too high. Suppose that we are interested in a sensitivity of 0.8. Using the pROC R package [11], the AUC is 0.68 (95% CI: 0.57, 0.79), the partial AUC between 1 and 0.8 specificity is 0.053 (95% CI: 0.025,

0.093), and the sensitivity at a specificity of 0.8 is 0.44 (95% CI: 0.27, 0.71) for P1. We can compare these evaluation measures to an ineffective biomarker. Recall that an ineffective biomarker produces a 45° straight line on the ROC curve, yielding an AUC value of 0.5, partial AUC of $0.2^2 = 0.004$, and sensitivity at specificity = 0.8 of 0.2. We can compute these evaluation measures for the other proteins as well, where the P38 protein has the maximum AUC value of 0.73.

3.5 Protein Correlation Analysis

It is often of interest regarding how proteins are correlated with each other. Pearson's correlation coefficient measures a linear relationship between two proteins. A linear relationship indicates that the two proteins will produce a straight line if we create a two-dimensional scatterplot. Alternatively, spearman's correlation coefficient can be used to capture a nonlinear relationship. Correlation coefficients range from -1 to 1 . Correlation coefficients close to 1 , -1 , and 0 imply that two proteins are more positively, more negatively, and less correlated, respectively. The correlation plot shows the correlation coefficients among the first ten proteins P1, P2, ..., P10, which are all positively correlated (Fig. 6). We interpret the correlation in a pairwise fashion. For example, correlation coefficients between P1 and P2, P1 and P3, and P2 and P3 are 0.58, 0.78, and 0.61, respectively.

3.6 Power Analysis

In statistical hypothetical testing (explained in Subheading 3.3), because either H_0 or H_1 is true, two errors can occur when we make a decision. Type I error occurs when we reject H_0 when it is actually true. Type II error occurs when we do not reject H_0 when it is actually false. One minus type II error is called the statistical power. Theoretically, it is not possible to decrease type I error and increase

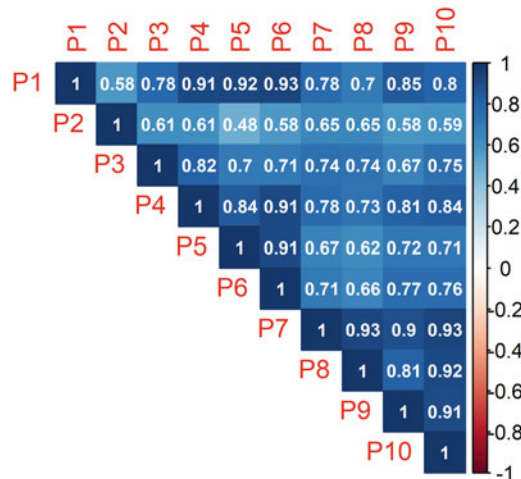


Fig. 6 Pairwise correlations between the first ten proteins

statistical power together. Alternatively, a general strategy is to fix type I error and collect sufficient data to achieve adequate statistical power. With regard to multiple testing, we fix the FDR instead of type I error.

A basic approach to statistical power analysis is to set an adequate statistical power. We then conduct a small pilot study to compute the effect size and calculate how many more samples are needed to achieve adequate power. Literature or historical data can also be used to compute the effect size. In other words, the effect size is an input parameter to calculate the power analysis. Calculating the effect size is often unclear because it depends on the experimental design, the aim of the study, and the statistical hypothesis. When applying multiple t -tests with FDR correction for NAPPA or similar protein arrays, Liu and Hwang [12] computed power based on the (a) effect size, which is the mean difference between the two groups divided by the common standard deviation of intensities; (b) proportion of nonsignificant proteins; (c) FDR; and (d) sample size. Using the `ssize.fdr` R package [13], we compute powers under various scenarios. When (c) is 0.2, 0.4, 0.7, and 0.9, at least 11, 16, 23, and 29 per group are required to achieve at least more than 0.08 power at the FDR threshold of 0.05, given that the effect size is one (Fig. 7).

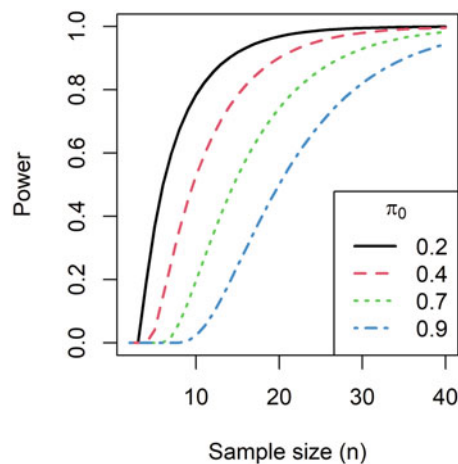


Fig. 7 Plot of power versus sample size for the t -test, where π_0 is a proportion of nonsignificant proteins

References

1. Elston RC, Johnson WD (1987) Essentials of biostatistics. F.A. Davis Company, Philadelphia, PA
2. Díez P, González-González M, Lourido L, Dégano RM, Ibarrola N, Casado-Vela J, LaBaer J, Fuentes M (2015) NAPPA as a real new method for protein microarray generation. *Microarrays* 4(2):214–227
3. Protein Microarray Database (2020) PMDA156-PMDA160. <http://www.proteinmicroarray.cn/>. Accessed 8 Sept 2020
4. R core team (2014) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. <https://www.r-project.org/>. Accessed 8 Sept 2020
5. Tukey JW (1977) Exploratory data analysis, vol 2. Addison-Wesley, Boston, pp 131–160
6. Bonferroni C (1936) Teoria statistica delle classi e calcolo delle probabilita. *Pubblicazioni del R Istituto Superiore di Scienze Economiche e Commerciali di Firenze* 8:3–62
7. Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Stat Soc B Methodol* 57(1):289–300
8. Storey JD (2002) A direct approach to false discovery rates. *J R Stat Soc B Stat Methodol* 64(3):479–498
9. Storey JD (2003) The positive false discovery rate: a Bayesian interpretation and the q-value. *Ann Stat* 31(6):2013–2035
10. Storey JD, Bass AJ, Dabney A, Robinson D (2020) q-value: Q-value estimation for false discovery rate control. R package version 2.20.0. <http://github.com/jdstorey/qvalue>. Accessed 8 Sept 2020
11. Robin X, Turck N, Hainard A, Tiberti N, Lisacek F, Sanchez JC, Müller M (2011) pROC: an open-source package for R and S+ to analyze and compare ROC curves. *BMC Bioinformatics* 12(1):1–8
12. Liu P, Hwang JG (2007) Quick calculation for sample size while controlling false discovery rate with application to microarray analysis. *Bioinformatics* 23(6):739–746
13. Orr M, Liu P (2009) Sample size estimation while controlling false discovery rate for microarray experiments using the size. *fdr package. R Journal* 1(1):47–53

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