

## Epitope Mapping Using Peptide Microarray in Autoantibody Profiling

Sebastian Henkel, Robert Wellhausen, Dirk Woitalla, Katrin Marcus, and Caroline May

### Abstract

The use of peptide microarrays for epitope mapping of autoantibodies greatly facilitates the early diagnosis of allergic, cytotoxin-associated diseases and especially inflammatory diseases. A common approach to create the microarrays utilizes nitrocellulose-coated glass slides for peptide probe binding, which is based on surface adsorption. Advantages of this method include excellent peptide binding capacity and long-term stability. To ensure equal accessibility to all antibodies on the peptide microarray during epitope mapping, all probes are immobilized in a random manner, thus avoiding concentration-dependent effects on signal intensity.

In this chapter, we provide a step-by-step protocol on how to construct the peptide microarrays and perform epitope mapping of autoantibodies using them. Finally we present a comparative approach for the evaluation of the data.

**Key words** Peptide microarray, Autoantibody profiling, Epitope mapping, Nitrocellulose-coated glass slides

---

### 1 Introduction

Peptide and protein microarrays are effective time-saving methods for the simultaneous analysis of greater than 20,000 different biomolecules in a nearly automated manner. This high-throughput method has been used for the identification of new drugs and biomarkers [1]. Numerous applications are available and they can be used to address many scientific questions. Typical applications include enzymatic activity determination and pathway mapping. Furthermore, one of the more prominent applications is immune response profiling, particularly in the field of autoimmune disease diagnostics, to evaluate biomarker efficacy as well as in vaccine design [2, 3]. A particular application is epitope mapping which is able to identify the polyclonal antibody repertoire elicited by

vaccination [4], produced due to the immunogenicity of cytotoxins [5] and of allergens [6], and/or produced in neuronal diseases [3] and in inflammation [7, 8].

A key mechanism of the demonstrated microarray approach is the antibody–antigen reaction that is similar to the one used in Western blot analysis and that is based on the unique recognition of an antigen (i.e., a peptide) by its specific antibody. We used a non-contact micro-dispensing instrument to spot the peptide solutions onto a nitrocellulose-coated slide. A major advantage of peptide microarrays in comparison to the commonly used Western blot analysis is the direct immobilization of the peptides to the nitrocellulose surface [9]. In contrast to interaction studies wherein a controlled orientation of the peptides is preferred [10], in immune response profiling, a randomly oriented environment of peptides is preferred to facilitate equal access to functional domains for the antibodies [9]. All possible immunogenic epitopes and various structures are represented by a uniform distribution of peptides on the nitrocellulose surface [11]. We describe the application of antibody-containing human serum to a nitrocellulose-coated peptide microarray for the identification of immunogenic peptides. The major advantages of this approach include the relatively high load of peptide probes in each spot and the long-term stability of the probes [12].

We describe the major steps for the successful epitope mapping of a polyclonal antibody using the amino acid sequence of a corresponding immunogenic protein that has been divided into peptides with a length of 21 residues and an overlap of 16 residues. The peptides were synthesized and subsequently lyophilized for storage. The subsequent steps consist of: (a) sample preparation with buffer handling, in which the peptides are dissolved; (b) the spotting process, which includes the transfer of peptides onto the nitrocellulose membrane; (c) the incubation of the microarrays with human serum, and (d) data acquisition (Fig. 1).

The epitope mapping method described in this chapter includes general recommendations from our experience [3, 13] and notes for quality assurance. In addition, we show the dynamic range of detecting the test epitope (Fig. 4). It should be noted that this protocol varies between proteins and peptides and must be optimized for each application and biomolecule.

---

## 2 Materials

### 2.1 Blood Sampling

1. Serum separator tubes (9 mL).
2. Safety syringe.
3. Safety holder to connect the syringe to the tube.
4. Needle and blade waste container.

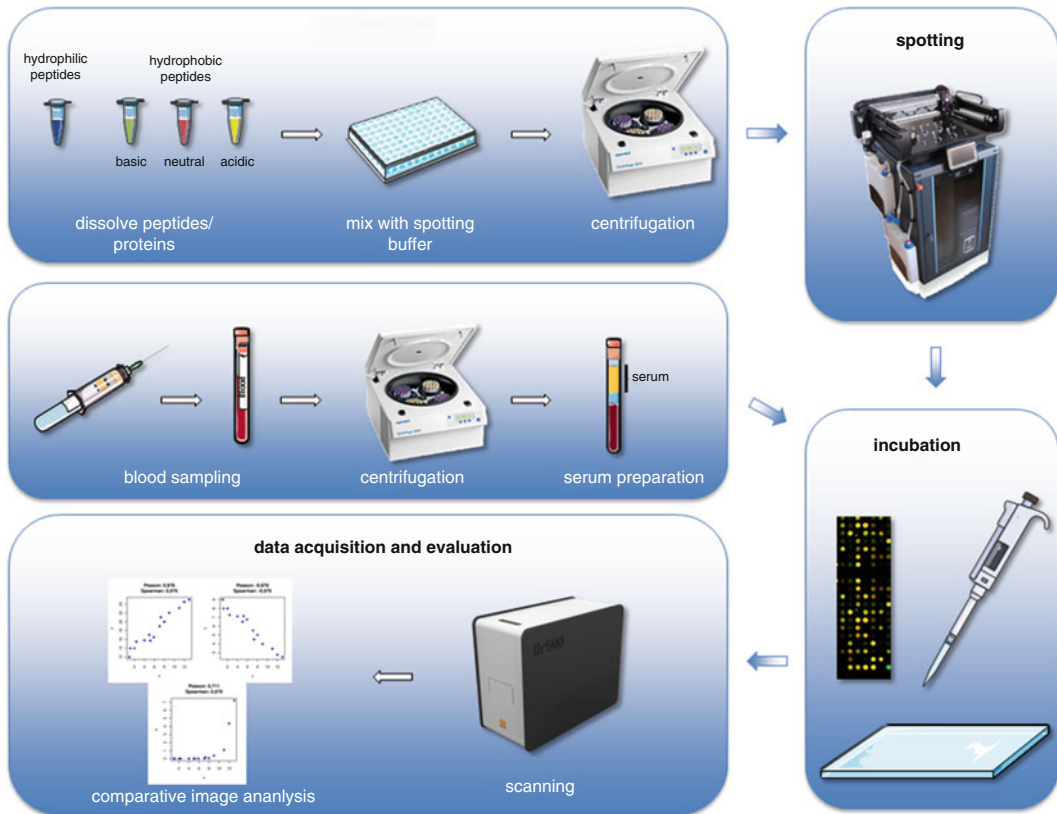


**Fig. 1** Overview of the epitope mapping workflow using nitrocellulose-coated microarray slides and a non-contact micro-dispensing instrument. (1) The peptides and proteins are dissolved based on their physico-chemical characteristics. (2) Simultaneously, the collection of the desired sera for a comparative study should be completed. (3) After spotting, the microarrays are ready for incubation with the sera and subsequent incubation with a fluorescently labeled secondary antibody. (4) The final step consists of data acquisition to detect and measure the fluorescence signals resulting from antibodies produced during the immune response and data evaluation to compare the different human subject groups (i.e., diseased vs. control)

## 2.2 Serum Preparation

1. 1 mL cryogenic tubes.

## 2.3 Peptide and Protein Preparation

1. 0.1 M (w/v) ammonium bicarbonate.
2. >99.0 % (v/v) trifluoroacetic acid.
3. PBS (phosphate-buffered saline, 10×), pH=7.4; without calcium chloride and without magnesium chloride.
4. >99.0 % (v/v) *N,N*-dimethylformamide.
5. 100 % (w/v) glycerol.
6. 100 % (v/v) Triton X-100.

**2.4 Control Samples**

1. Control spot antibody (Alexa Fluor 555 Goat Anti Rabbit IgG, Life Technologies, Darmstadt).
2. Positive control antigen (HA-epitope extended with neutral amino acids and present as the following amino acid frequency SNCYPYDVPDYASLRSLVASS, Peps4Life, Heidelberg).

**2.5 Peptide Array Preparation**

1. Non-contact micro-dispensing instrument (instrumentONE; M2-Automation, Berlin).
2. Piezo-element-driven micro-dispensing nozzle (instrumentONE; M2-Automation, Berlin).
3. PATH® plus Microarray Ultrathin Nonporous Nitrocellulose Slides (Grace Bio-Labs, Bend, Oregon).
4. Glass slides coated with water-sensitive paper.

**2.6 Peptide Array Processing**

1. Circular Microtiter Plate (MTP) shaker.
2. Table centrifuge.
3. Vacuum pump.
4. Fluorescence scanner.
5. Four-cell tray with lid.
6. Forceps.
7. 500 mL vacuum filtration unit containing a filter disk with a pore size of 0.45  $\mu\text{m}$  (Filtropur V50 500 ml, 0.45  $\mu\text{m}$ , Sarstedt, Nümbrecht).
8. Polyacetal rack.
9. Polyacetal trough.
10. Secondary antibody (Alexa Fluor® 647 Goat Anti-Human IgG, Life Technologies, Darmstadt).
11. Synthetic block 10 $\times$  (from Invitrogen™).
12. Blocking buffer (50 mM HEPES, pH = 7.5; 25 % (w/v) glycerol; 0.08 % (w/v) Triton X-100; 200 mM NaCl; and 20 mM reduced glutathione).
13. 1 M (w/v) dithiothreitol.
14. Tween 20 (50 % (w/v) solution).

**2.7 General**

1. Laminar flow hood, HEPA filtered and UV sterilized.
2. Safety workbench.
3. Centrifuge, swinging bucket rotor, adjustable acceleration and braking, cooling option.
4. Containment for biowaste.
5. pH meter.
6. Water squirt bottle.

7. 0.1–2.5, 0.5–10, 2–20, 20–200, 100–1000, and 500–5000  $\mu\text{L}$  micropipettors.
8. 2.5  $\mu\text{L}$ , 10  $\mu\text{L}$ , 20  $\mu\text{L}$ , 200  $\mu\text{L}$ , 1000  $\mu\text{L}$ , and 5 mL sterile micropipette tips.
9. Sterile 15 and 50 mL conical centrifuge tubes.
10. Stand for centrifuge tubes.
11. 0.5 mL microtubes.
12. 384-well microtiter plates.
13. Ultrapure deionized water.
14.  $\geq 96\%$  isopropyl alcohol (technical grade).
15. Disinfectant.
16. Ice box.
17. 4  $^{\circ}\text{C}$  refrigerator,  $-20^{\circ}\text{C}$  freezer,  $-80^{\circ}\text{C}$  freezer.

---

### 3 Methods

#### 3.1 Blood Sampling

1. Perform blood withdrawal according to local legislative regulations by authorized medical personal only.
2. Assemble  $4 \times 9$  mL serum separator tubes with safety syringes using appropriate adapters (*see Note 1*).
3. Fill each tube with blood obtained by venipuncture of a peripheral vein (*see Note 2*).
4. Slowly invert tubes three times to ensure homogenous mixing of blood and uniform coagulation (*see Note 3*).

#### 3.2 Serum Preparation

1. Centrifuge the serum tubes at  $3000 \times g$  for 10 min at room temperature.
2. Label the cryo tubes with a unique serial identification number.
3. Prepare 500- $\mu\text{L}$  serum aliquots in cryo tubes using micropipettes under a safety workbench and freeze them immediately.
4. Aliquots are stored at  $-80^{\circ}\text{C}$ . Avoid freeze–thaw cycles (*see Note 4*).

#### 3.3 Peptide and Protein Preparation

Samples are prepared from lyophilized synthetic peptides using a total quantity of 300  $\mu\text{g}$ . The peptide sequences should partially overlap to cover all possible sequences and ensure that potential epitopes are not separated. We used peptides consisting of 21 residues with an overlap of 16 residues.

1. Subdivide all lyophilized peptides into four groups according to their solubility and isoelectric characteristics (*see Note 5*).
2. The first group includes all water-soluble peptides, regardless of isoelectric properties (WS-group). Pipette 360  $\mu\text{L}$  of water

into a microtube with 300 µg of peptide, and pipette an additional 40 µL of PBS (10×) into the tube (*see Note 6*).

3. The second group includes all insoluble peptides with an acidic pH value. Pipette 40 µL of PBS (10×) into a microtube with 300 µg of peptide. If the peptide does not dissolve, add 5 µL of 0.1 M ammonium bicarbonate followed by water giving a total volume of 400 µL (*see Note 6*).
4. The third group includes all insoluble peptides with basic pH values. Use 5 µL of 100 % TFA to dissolve these peptides, and add 355 µL of water and 40 µL of PBS (10×) to each microtube (*see Note 6*).
5. The fourth group includes all insoluble peptides with neutral pH values. Dissolve these hydrophobic peptides with 5 µL of 100 % DMF (*see Note 7*). Pipette an additional 355 µL of water and 40 µL of PBS (10×) into each microtube (*see Note 6*).
6. Prepare 5 mL of peptide spotting buffer, which is a mixture of 3.75 mL of water, 1.238 mL of glycerol and 12 µL of Triton X-100 (*see Note 8*).
7. Equimolar concentrations of 0.75 µg/µL should be used for all peptides. Subsequently, add 50 µL of spotting buffer into each microtube.
8. Pipette 25 µL of each peptide into each well in a 384-well microtiter plate. Prepare a table that includes the information for the peptide present in each well (*see Note 9*). This information can be subsequently uploaded for analysis.
9. Centrifuge the microtiter plate at 1000×*g* for 10 s, and seal the plates with an airtight seal until subsequent analysis.
10. Store the microtiter plates at 4 °C if they will be analyzed the following day. Otherwise, the plates can be stored at −20 °C for approximately 3 months.

### 3.4 Control Samples

For quality assurance, control samples should be included within the peptide array.

1. Negative control samples should consist of all of the different solutions representing the different buffers used for peptide dissolution. Perform the various preparation steps (*see Subheading 3.3*) without the peptides, and mix the solutions with the spotting buffer. If there is no contamination, the spots should not exhibit a fluorescence signal on the final peptide array after all incubation steps.
2. Use the HA-epitope as a positive control sample. Dissolve this antigen and immobilize it on the slide in a similar fashion as the peptides (*see Note 10*).

3. Immobilize the control spot antibody, which is labeled with a fluorescent probe that is monitored at a different wavelength than the secondary antibody for incubation (*see* Subheading 3.6, **step 12**), in a similar fashion as the peptides (*see* Subheading 3.3). The spots obtained from this sample serve as guide dots to ensure correct array alignment during the entire assay process.

### 3.5 Peptide Array Preparation

The peptide arrays are prepared using a piezoelectric non-contact micro-dispensing instrument that is able to dispense liquid droplets with a volume of 20–100 pL (*see* **Note 11**).

1. Clean the entire system and rinse the hoses with isopropyl alcohol. The entire process should be performed under low-dust and high-purity conditions (*see* **Note 12**).
2. Place the microtiter plate with the samples (peptides) in the MTP holder of the circular MTP shaker. Shake at 150 rpm for 5 min.
3. Adjust the micro-dispensing parameters according to the physicochemical characteristics of the peptide solutions in contrast to water with the software provided by the manufacturer. A longer pulse duration ( $\mu\text{s}$ ) and higher pulse amplitude (voltage) may be necessary for solvents, which are more viscous than water (*see* **Note 13**).
4. A washing step, which is performed after each new biomolecule has been spotted, is established via the software. Dispense 100  $\mu\text{L}$  of solvent and 200 droplets, and wash the nozzle for 3 s at the wash station. The external wash consists of a 6-s wash at the wash station and a 4-s pause interval. Individualized parameters for washing are also possible (*see* **Note 14**).
5. Pre-dispense 150 droplets to discard any liquid that gets into the nozzles due to the washing fountain.
6. Use a nonporous nitrocellulose film-coated glass slide with low fluorescence for improved detection at a high signal-to-noise ratio (*see* **Note 15**). Place the slides in the slide holder. The first and last slides are coated with water-sensitive paper. These slides are important for a quick quality assurance. After the spotting step, an indicator for the complete spotting process is the visualization of these slides. However, spotting of a loading control of all spotted peptides labeled with an antibody or fluorescent dye is strongly recommended.
7. On the software, load the content table and create a grid by enabling various fields with blocks, which are indicated by guide dots, to structure the microarray. This step facilitates dot orientation after scanning. Furthermore, choose a randomized setting of triplicates for each peptide (*see* **Note 16**).

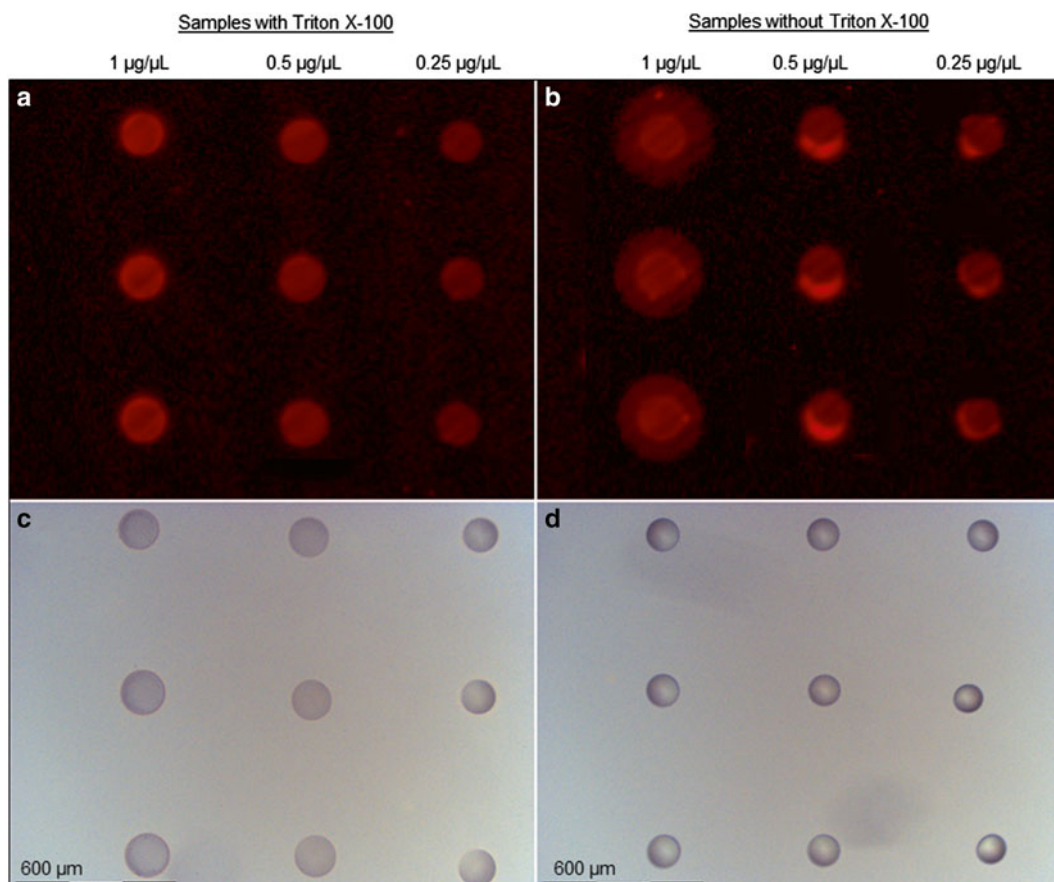


**Fig. 2** Comparison of a water-soluble peptide with 0.08 % Triton X-100 (**a** and **c**) and a hydrophobic peptide in buffer without Triton X-100 (**b** and **d**). The peptides are spotted in triplicate at concentrations of 1, 0.5, and 0.25  $\mu\text{g}/\mu\text{L}$ . Part(**a** and **b**) show the fluorescence signals detected with a high sensitivity interline charge-coupled scanner at a resolution of  $10\text{ }\mu\text{m}^2/\text{pixel}$  with a dynamic range larger than 12-bit and an exposure time of 10,000 ms. Part(**c** and **d**) show the spots prior to incubation using a microscope with fivefold magnification. In (**a**), homogenous signal intensity from the spots due to the presence of Triton X-100 in the spotting buffer is shown. Enhanced adsorption of the spots onto the nitrocellulose membrane is also apparent in (**c**) in comparison with (**d**). The light microscope image without Triton X-100 (**d**) shows raised spots because of higher surface tension compared with the samples containing Triton X-100 (**c**). The spots in (**b**) exhibit a halo-shaped structure and an inhomogeneous fluorescence signal; this is often observed in samples without detergent and derives from high surface tension with subsequent irregular drying from the inside to the outside [10]. Within the spot, immobilization and accumulation of the biomolecules is favored at the outer rim [2]

8. Adjust the spacing distance between the spots to 600  $\mu\text{m}$  (see **Note 17**). A typical image of these spots dispensed on a slide is shown in Fig. 2.
9. When the settings are complete, begin a test run by using water or a cheap test peptide prior to using the samples to produce the arrays. If there are any errors in the automated workflow, the wastage of the samples is prevented (see **Note 18**).

10. If necessary, optimize the parameters and produce the peptide arrays under standardized conditions (low-dust environment, room temperature and 40–50 % humidity).
11. If some samples could not be spotted, they can be re-spotted as long as the array slide has not been moved.
12. Dry the spotted microarray slides for 48 h at room temperature under the dust-free workbench and do not move them. Then, the arrays can be stored at  $-20^{\circ}\text{C}$  for 3 months.

### 3.6 Peptide Array Processing

The following peptide array processing procedures have been modified from the instructions from Invitrogen™ for ProtoArray® Human Protein Arrays and the modified procedures have been previously tested in various applications for immune profiling [3, 14–16]. First, prepare the blocking buffer and washing buffer solutions. The step-by-step instructions provide an example of how to incubate four peptide arrays.

1. All reagents and vessels should be cooled to  $4^{\circ}\text{C}$  prior to use.
2. Prepare 50 mL of blocking buffer working solution by mixing 5 mL of synthetic block solution (10×) and 45 mL of blocking buffer (*see Note 19*) and 0.1 mL of 1 M DTT.
3. Prepare washing buffer as a mixture of 60 mL of PBS (10×), 3 mL of Tween 20 (10×), 60 mL of synthetic block (10×), and deionized water to a total volume of 600 mL.
4. Filter the washing buffer using the vacuum filtration unit together with a pump to remove lint by vacuum suction.
5. Thaw the self-made peptide arrays at  $4^{\circ}\text{C}$  for 15 min, then at room temperature for an additional 15 min. During this time, cool the required volume of deionized water at  $4^{\circ}\text{C}$  (*see Note 20*).
6. Scan the arrays at the wavelength at which the control sample spots fluoresce prior to incubation with antibodies (*see Note 21*).
7. Place the arrays in the tray (*see Note 22*), and pipette 5 mL of the prepared blocking buffer into each tray chamber.
8. Incubate the arrays with the blocking buffer at  $4^{\circ}\text{C}$  for 60 min with horizontal rotation using the circular MTP shaker (150 rpm). During this time, dilute the human serum with washing buffer at a ratio of 1:250 (*see Note 23*).
9. Remove the blocking buffer carefully and rapidly using a vacuum pump. Pipette 5 mL of washing buffer into the chamber, and incubate the array for 5 min at  $4^{\circ}\text{C}$  with horizontal rotation (*see Note 24*).
10. Remove the washing buffer and add the diluted serum to each chamber. Incubate the arrays for 90 min at  $4^{\circ}\text{C}$  with horizontal rotation.

11. Remove the sera and pipette 5 mL of washing buffer into each chamber. Incubate the arrays for 5 min at room temperature, and repeat this washing step for a total of five times.
12. Dilute the secondary antibody at a ratio of 1:2000 with washing buffer, and pipette 5 mL into each chamber. Incubate the arrays with these antibody for 90 min at 4 °C with horizontal rotation.
13. Remove the antibody and repeat the five washing steps (*see* Subheading 3.6, step 7).
14. Perform the last washing step with water. The microarrays will be sequentially sorted into a prepared rack that is located in a watertight box (*see* Note 25).
15. Place the racks into dry boxes without lids and centrifuge at  $3000\times g$  for 2 min.
16. After centrifugation, take images of the microarrays, and digitalize the different images directly for optimal comparability of the results using a fluorescence scanner (*see* Note 26).
17. After scanning, the arrays may be stored for a longer time under a modified atmosphere (argon) in sealed opaque pouches at 4 °C.
18. Import the data into R (a free software environment for statistical computing) *via* Bioconductor using the Limma package, and choose cyclic local regression (cyclic loess) as the normalization method to preprocess the arrays (*see* Note 27). Subsequent selection and sorting of peptide candidates can be performed based on the “minimum M-Statistic” *p*-value (“M-score” [13, 17]).

---

## 4 Notes

1. The appropriate blood sampling system/adapters should be chosen by authorized and experienced medical personal.
2. Coagulation times may differ according to the instruction manual for the serum tubes.
3. Ensure that the blood is coagulated and non-hemolytic.
4. Although human serum can be frozen for years without a significant loss in antibody activity [18], for a comprehensive analysis, the subsequent incubation of serum on the peptide microarray should be performed over a short period of time under standardized conditions (particularly, temperature and humidity).
5. If the information is not available for the peptides, use a free peptide property calculator (e.g., Innovagen) to obtain biophysical properties and an estimation of the water solubility.

If possible, use water to dissolve the peptides. In most cases, peptides are readily soluble in water; however, experimental verification may be required.

6. We recommend the use of low bind tubes (e.g., Eppendorf LoBind Tubes), to prevent loss of peptide by sample-to-surface binding. If the quantity of the peptides is not known, perform an analysis using UPLC. Adjust all concentrations to 0.75  $\mu\text{g}/\mu\text{L}$ .
7. Use glass as the reaction container, and do not store DMF-containing liquids in polymeric containers. DMF is a polar organic solvent that dissolves polyacrylic fibers, vinyl resins and polyacetylene. DMF also causes first degree burns upon short exposure and it is toxic to the liver [19].
8. Triton X-100 is required to reduce the surface tension of water to prevent annular (or donut-shaped) inhomogeneous fluorescent spots (*see* Fig. 2). Glycerol is primarily used to increase physical adsorption, and hence, to increase the binding capacity [20].
9. The template of the table with the peptide information depends on the software used.
10. There are various options in the selection of positive controls. The specified option is only a technical control to verify that the incubation was properly performed and that all solutions and antibodies function well. Different peptides with physico-chemical properties that are similar to those of the sample peptides (particularly in their affinity for the antibody) may be used as positive controls. However, this control approach is difficult to implement for an immune response profile because of the magnitude of interindividual differences. Furthermore, there are also intra-individual fluctuations in antibody titer [21]. Therefore, this positive control approach is more suitable for peptide–protein and protein–protein interactions.
11. The dispensed volume usually varies between different nozzles obtained from the manufacturer. Therefore, always make an individualized adaption of dispensing parameters according to the data sheet, which the nozzle included.
12. Dust or other particles that are visible after scanning may lead to false-positive results.
13. The micro-dispensing parameters must be adjusted according to the viscosity of the spotting buffer and samples. All samples should be handled using identical global parameters once the settings have been tuned. If the characteristics of the peptides substantially differ, individual parameter settings are also possible. Figure 3 shows the perfect trajectory of a drop (stable size, linear and vertical trajectory without satellite droplets). Note that these parameters must always be evaluated in two mutually orthogonal planes, to ensure a three-dimensional assessment of the mentioned drop morphology and trajectory.

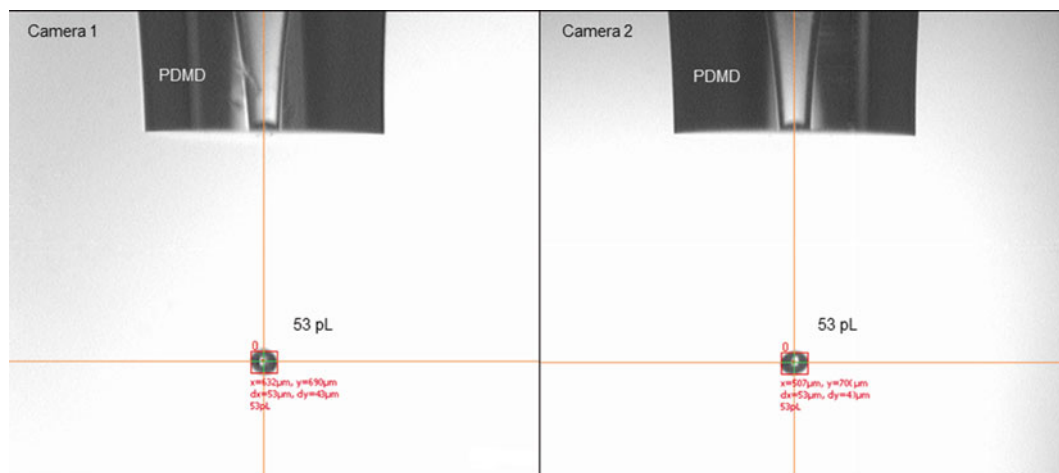


**Fig. 3** Sample trajectory of a 53-pL droplet during micro-dispensing recorded with two orthogonally oriented cameras. The droplet was formed using the following settings of a piezo-element-driven micro-dispensing (PDMD) nozzle: pulse duration = 10  $\mu$ s, voltage = 87 V, frequency = 200 Hz. The droplet volume may vary and depends on both the nozzle and the physicochemical properties of the solutions (peptides and buffer)

14. The wash should consist of a complete wash (i.e., a wash after the sample is spotted and an external wash conducted to prevent cross-contamination). Individualized parameters are necessary if you use peptides with specific physicochemical properties and particularly viscous buffers so that the nozzle does not get clogged.
15. Background noise is reduced when the slide surface is coated with ultrathin, nonporous dark nitrocellulose (Fig. 4) [22]. We recommend the use of PATH plus Microarray slides from Grace Bio-Labs.
16. The randomized settings are necessary to avoid cross-reaction and mixing of the various peptide samples.
17. When more droplets are dispensed, the distance should be carefully adapted to avoid bleeding and cross-reactivity. For instance, for the deposition of five droplets with a volume of approximately 80 pL, the center-to-center spacing is 600  $\mu$ m.
18. A test run should be performed to verify that the micro-dispensing parameters apply to all of the samples, so that the wash is effective, so that the dispensing nozzle does not get clogged, and so that possible exceptions are spotted. In this occasion, possible exceptions are peptides with special or different physicochemical properties in contrast to the remaining peptides. Adjust the micro-dispensing parameters and save the settings for these peptides in your software for an automated production of microarrays.

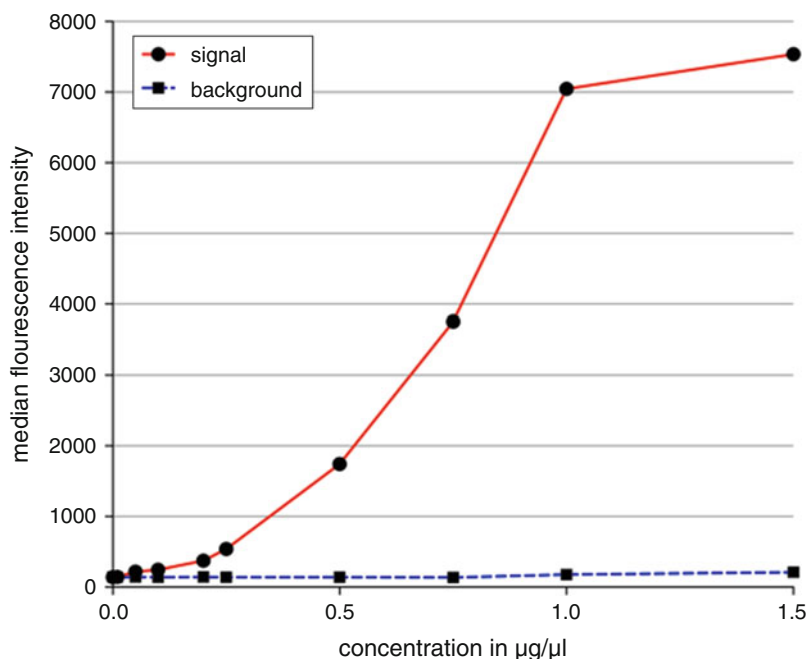


**Fig. 4** Dynamic range of the test sample (modified HA-epitope). We probed five separate arrays with the modified HA-epitope. The fluorescence signal is obtained from the secondary antibody (Alexa Fluor® 647 Goat Anti-Human IgG) measured by a fluorescence scanner with a 635 nm wavelength laser diode. Graphs displaying median fluorescence signal of a dilution series of the modified HA-epitope ranging from 0.01 to 1.5  $\mu\text{g}/\mu\text{l}$  and the corresponding background signal, in triplicates

19. The specified buffer has a low fluorescent background and it also protects the biomolecules on the surface of the array [20].
20. During the entire experiment, all materials and solutions should be cooled to 4 °C to ensure a standardized environment and to avoid rearrangements of the molecules (peptides) at high temperatures. Additionally, tighter binding of human IgG antibodies and peptides is observed at 4 °C [23, 24]. After the solutions have been prepared, the following guidelines for handling microarrays should be observed: (a) Use gloves in handling the microarray slide, and it should only be touched on the edge or on unspotted locations (never touch the spotted area!). (b) The microarray surface should only be pointed upwards. The only exception is during the scanning process, wherein the microarray orientation depends on the manufacturer.
21. The quality control step detects the guide dots and checks for impurities on the array. Prior to scanning, the scanner should be started 30 min to warm up.
22. After this step, the entire experiment will be performed under a clean bench. Solutions should not be directly pipetted onto the slide. Add solutions next to the array inside the chamber using only a 5 mL micropipette.

23. A total of 5 mL of diluted serum is required for each chamber of the tray. Therefore, mix 20  $\mu$ L of the four different sera with 5 mL of washing buffer in four different 15 mL tubes.
24. The surface of the array must be wet throughout the procedure. The microarray should only be dried at the end of the incubation prior to scanning as described in the protocol (a short immersion in deionized water and subsequent centrifugation). This drying step ensures low background from solvents and artificial residues, which can increase the background signal.
25. Prevent drying of the spotted surface before and during microarray sorting by working quickly and maintaining the humidity at approximately 50 %.
26. Choose excitation wavelengths and emission filters according to the control sample spots and the secondary antibodies for detection. Scanner settings are as follows: detection: high sensitivity interline charge-coupled device; resolution: 10  $\mu$ m<sup>2</sup>/pixel; dynamic range: larger than 12-bit; and exposure time: 10,000 ms.
27. Limma [25] is one of the few R [26] packages that provides full non-Affymetrix single-channel microarray support including generic data objects, background correction, log transformation and three different normalization approaches (i.e., variance stabilization, quantile and cyclic loess normalization). This method is adopted by Jiang et al. [27] and described by May et al. [3] for highly immune-reactive IgG antibody selection of biomarkers in amyotrophic lateral sclerosis patients.

---

## Acknowledgments

This work was supported by P.U.R.E. (Protein Unit for Research in Europe), which is a project of the federal state of Nordrhein-Westfalen, Germany, and by the Ministerium für Innovation, Wissenschaft und Forschung des Landes Nordrhein-Westfalen and WTZ Brasilien, which is a project of the German Ministry of Education and Research (BMBF), Germany (01DN14023).

## References

1. Abel L, Kutschki S, Turewicz M, Eisenacher M, Stoutjesdijk J, Meyer HE, Voitalla D, May C (2014) Autoimmune profiling with protein microarrays in clinical applications. *Biochim Biophys Acta* 1844(5):977–987
2. Wellhausen R, Seitz H (2012) Facing current quantification challenges in protein microarrays. *J Biomed Biotechnol* 2012:831347
3. May C, Nordhoff E, Casjens S, Turewicz M, Eisenacher M, Gold R, Brüning T, Pesch B, Stephan C, Voitalla D, Penke B, Janáky T, Virók D, Siklós L, Engelhardt JI, Meyer HE (2014) Highly immunoreactive IgG antibodies directed against a set of twenty human proteins in the sera of patients with amyotrophic lateral sclerosis identified by protein array. *PLoS One* 9(2), e89596

4. Cohen O, Mechaly A, Sabo T, Alcalay R, Aloni-Grinstein R, Seliger N, Kronman C, Mazor O (2014) Characterization and epitope mapping of the polyclonal antibody repertoire elicited by ricin-holotoxin based vaccination. *Clin Vaccine Immunol* 21(11):1534–1540
5. Akada J, Okuda M, Hiramoto N, Kitagawa T, Zhang X, Kamei S, Ito A, Nakamura M, Uchida T, Hiwatani T, Fukuda Y, Nakazawa T, Kuramitsu Y, Nakamura K (2014) Proteomic characterization of *Helicobacter pylori* CagA antigen recognized by child serum antibodies and its epitope mapping by peptide array. *PLoS One* 9(8):e104611
6. Lin J, Bardina L, Shreffler WG, Andrae 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
7. Ng JK, Malotka J, Kawakami N, Derfuss T, Khademi M, Olsson T, Linington C, Odaka M, Tackenberg B, Prüss H, Schwab JM, Harms L, Harms H, Sommer C, Rasband MN, Eshed-Eisenbach Y, Peles E, Hohlfeld R, Yuki N, Dornmair K, Meinl E (2012) Neurofascin as a target for autoantibodies in peripheral neuropathies. *Neurology* 79(23):2241–2248
8. Prüss H, Hölte M, Maier N, Gomez A, Buchert R, Harms L, Ahnert-Hilger G, Schmitz D, Terborg C, Kopp U, Klingbeil C, Probst C, Kohler S, Schwab JM, Stoecker W, Dalmau J, Wandinger KP (2012) IgA NMDA receptor antibodies are markers of synaptic immunity in slow cognitive impairment. *Neurology* 78(22):1743–1753
9. Price JV, Haddon DJ, Kemmer D, Delepine G, Mandelbaum G, Jarrell JA, Gupta R, Balboni I, Chakravarty EF, Sokolove J, Shum AK, Anderson MS, Cheng MH, Robinson WH, Browne SK, Holland SM, Baechler EC, Utz PJ (2013) Protein microarray analysis reveals BAFF-binding autoantibodies in systemic lupus erythematosus. *J Clin Invest* 123(12):5135–5145
10. Guo A, Zhu X-Y (2007) The critical role of surface chemistry in protein microarrays. In: Predki PF (ed) *Functional protein microarrays in drug discovery*. Drug discovery series, vol 8. CRC, Boca Raton, pp 53–72
11. Cretich M, Damin F, Pirri G, Chiari M (2006) Protein and peptide arrays: recent trends and new directions. *Biomol Eng* 23(2–3):77–88
12. Tonkinson JL, Stillman BA (2002) Nitrocellulose: a tried and true polymer finds utility as a post-genomic substrate. *Front Biosci* 7:c1–12
13. Turewicz M, May C, Ahrens M, Woitalla D, Gold R, Casjens S, Pesch B, Brüning T, Meyer HE, Nordhoff E, Böckmann M, Stephan C, Eisenacher M (2013) Improving the default data analysis workflow for large autoimmune biomarker discovery studies with ProtoArrays. *Proteomics* 13(14):2083–2087
14. Schweitzer B, Meng L, Mattoon D, Rai AJ (2010) Immune response biomarker profiling application on ProtoArray protein microarrays. *Methods Mol Biol* 641:243–252
15. Le Roux S, Devys A, Girard C, Harb J, Hourmant M (2010) Biomarkers for the diagnosis of the stable kidney transplant and chronic transplant injury using the ProtoArray® technology. *Transplant Proc* 42(9):3475–3481
16. Nguyen MC, Tu GH, Koprivnikar KE, Gonzalez-Edick M, Jooss KU, Harding TC (2010) Antibody responses to galectin-8, TARP and TRAP1 in prostate cancer patients treated with a GM-CSF-secreting cellular immunotherapy. *Cancer Immunol Immunother* 59(9):1313–1323
17. Sboner A, Karpikov A, Chen G, Smith M, Mattoon D, Dawn M, Freeman-Cook L, Schweitzer B, Gerstein MB (2009) Robust-linear-model normalization to reduce technical variability in functional protein microarrays. *J Proteome Res* 8(12):5451–5464
18. Linke RP (2012) Immunologische Techniken. In: Lottspeich F, Engels JW (eds) *Bioanalytik*, 3rd edn. Spektrum Akademischer Verlag, München, pp 77–124
19. Redlich CA, Beckett WS, Sparer J, Barwick KW, Riely CA, Miller H, Sigal SL, Shalat SL, Cullen MR (1988) Liver disease associated with occupational exposure to the solvent dimethylformamide. *Ann Intern Med* 108(5):680–686
20. Luttmann W, Bratke K, Küpper M, Myrtek D (2014) *Der experimentator: immunologie*. Spektrum Akademischer Verlag, Heidelberg
21. Isacson J, Trollfors B, Hedvall G, Taranger J, Zackrisson G (1995) Response and decline of serum IgG antibodies to pertussis toxin, filamentous hemagglutinin and pertactin in children with pertussis. *Scand J Infect Dis* 27(3):273–277
22. Balboni I, Limb C, Tenenbaum JD, Utz PJ (2008) Evaluation of microarray surfaces and arraying parameters for autoantibody profiling. *Proteomics* 8(17):3443–3449
23. Shopes B (1995) Temperature-dependent binding of IgG1 to a human high affinity Fc receptor. *Mol Immunol* 32(5):375–378

24. Dorrington KJ (1976) Properties of the Fc receptor on macrophages. *Immunol Commun* 5(4):263–280
25. Smyth GK (2005) Limma: linear models for microarray data. In: Gentleman R, Carey V, Dudoit S, Irizarry R, Huber W (eds) *Bioinformatics and computational biology solutions using R and bioconductor*. Springer, New York, pp 397–420
26. Rdc T (2011) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria
27. Jiang H, Deng Y, Chen HS, Tao L, Sha Q, Chen J, Tsai CJ, Zhang S (2004) Joint analysis of two microarray gene-expression data sets to select lung adenocarcinoma marker genes. *BMC Bioinformatics* 5:81