

# On-Chip Protein Synthesis for Making Microarrays

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## Summary

Protein microarrays are a miniaturized format for displaying in close spatial density hundreds or thousands of purified proteins that provide a powerful platform for the high-throughput assay of protein function. The traditional method of producing them requires the high-throughput production and printing of proteins, a laborious method that raises concerns about the stability of the proteins and the shelf life of the arrays. A novel method of producing protein microarrays, called *nucleic acid programmable protein array* (NAPPA), overcomes these limitations by synthesizing proteins *in situ*. NAPPA entails spotting plasmid DNA encoding the relevant proteins, which are then simultaneously transcribed and translated by a cell-free system. The expressed proteins are captured and oriented at the site of expression by a capture reagent that targets a fusion protein on either the N- or C-terminus of the protein. Using a mammalian extract, NAPPA expresses and captures 1000-fold more protein per feature than conventional protein-printing arrays. Moreover, this approach minimizes concerns about protein stability and integrity, because proteins are produced just in time for assaying. NAPPA has already proven to be a robust tool for protein functional assays.

**Key Words:** Protein microarrays; functional proteomics; protein expression; protein purification; microarray surface chemistry.

## 1. Introduction

The recent development of functional protein microarrays has stirred excitement in the proteomics community (1–7). The power of this approach is that, by spotting many proteins on a single array surface, many biochemical activities can be studied simultaneously. These activities include identifying interacting proteins, examining the selectivity of drug binding, finding substrates for active enzymes, and looking for unintended drug interactions. Typically, the array is probed with a labeled query molecule to identify interactions with proteins on

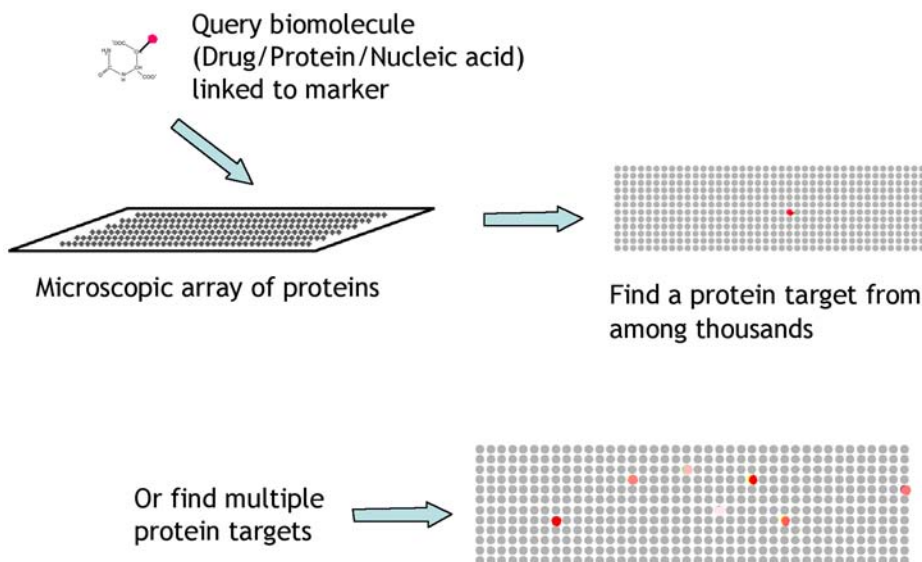


Fig. 1: Rapid screening of target protein arrays. A labeled query molecule can be used to screen an array of target proteins to identify potential interactor(s).

the array (Fig. 1). For example, a labeled candidate kinase inhibitor might be used to screen an array of kinases to determine which kinase(s) the inhibitor binds directly. In order to build protein microarrays, one needs the content to spot on the array and an appropriate binding chemistry to capture the protein. These components must be optimized to produce and present proteins of good integrity and stability. The goal is to preserve the functionality of the protein in order to minimize false-negatives. Here we will address the issues pertaining to building functional protein microarrays.

### 1.1. Issues for Protein Array Production

1. *Availability of array content.* Assembling the proteins for printing on the array remains a major challenge for most researchers, because recombinant expression of proteins in the numbers anticipated for protein microarrays relies on the availability of large collections of cDNAs in protein expression-ready formats. It also requires methods to produce and purify the proteins. Although several collections of cDNAs are available, the methods and robotic equipment required for the high-throughput (HT) expression and purification of thousands of proteins remain outside the realm of most laboratories (8–10).
2. *Protein integrity.* Ensuring that properly folded proteins can be produced and captured remains a challenge. Proteins are more likely to fold naturally if heterologous

systems can be avoided and if proteins are synthesized in a milieu as close to their natural setting as possible. For example, mammalian proteins are more likely to fold naturally in mammalian (or at least animal) cells. Yet, many expression systems are too cumbersome and expensive to allow thousands of proteins to be easily processed. Bacterial cells, which are readily adapted to HT protein expression, can be counted on to produce only 50% of mammalian proteins ([10](#)).

3. *Protein stability.* Proteins are notoriously fragile, raising concerns about the stability of the isolated proteins before and after they have been arrayed on a glass slide. We expect some proteins to remain relatively stable, with good shelf life, whereas others display greater lability and are unable to withstand prolonged array conditions. Moreover, it is difficult to determine which of the proteins remain active at the time of the assay. In general, to ensure proper assay conditions and minimize false-negatives, it is best to use the array soon after the protein is synthesized.
4. *Microarray surface chemistry.* Several surface chemistries have been developed and validated for microarray platforms, particularly for DNA microarrays. The chemistry for binding DNA is simple compared with the chemical demands necessary to immobilize functional protein. DNA molecules, which are all negatively charged, bind to surfaces based on charge alone, enabling positively charged arrays such as a polylysine-coated slides to bind all DNA. In contrast, proteins display a staggering range of hydrophobicity and charge, making it a challenge to find a single method that provides good binding for most, let alone all, proteins. Factors to consider include:
  - a. Generality of binding: ability to bind all proteins that will be spotted on the array.
  - b. Binding capacity: maximum amount of protein captured per feature.
  - c. Efficiency of capture: fraction of spotted protein that is captured on the array.
  - d. Orientation: specific vs random orientation. Proteins can be immobilized either in an orientation-specific manner (e.g., by binding via either an N-terminus or a C-terminus tag) or in random orientations (e.g., by chemical attachment). Random rather than specific orientations may allow many areas of the protein to be exposed, increasing accessibility to the protein. Although this may increase the likelihood of an interaction, there have been no significant differences observed between these approaches ([11](#)); it may be necessary to evaluate this on an experiment by experiment basis.
  - e. Distance from surface: some attachment methods allow for a spacer (e.g., a large polypeptide tag) that separates the protein from the array surface; other methods (e.g., chemical attachment) bring the proteins in direct contact with the array surface. Increasing the distance between the protein and the array surface might alleviate some of the steric hindrance caused by the surface and potentially increase accessibility to the protein.
  - f. Native or denatured protein: surface chemistry can be formulated to contain hydrophobic or hydrophilic residues. Given that many proteins have a hydrophilic exterior and a hydrophobic interior, the choice of the surface chemistry could support the binding of nondenatured or denatured protein ([12](#)).

Early demonstration of the feasibility of printing proteins on a microscopic surface has been promising. To demonstrate that spotted proteins maintain their functional integrity upon immobilization, well characterized and specific interactions among proteins, lipids, and small molecules, as well as enzyme-substrate screens, were recapitulated with proteins on the arrays ([1,3,6,7,11,13–20](#)). Even in light of these achievements, the widespread use of this technology has remained limited, largely as a result of the labor-intensive protein production, the quality of proteins expressed in heterologous systems, and the stability of the proteins during storage. To address these persistent concerns, we developed a self-assembling protein microarray method.

### **1.2. Nucleic Acid Programmable Protein Array**

To circumvent the need to express, purify, and spot the protein, this approach prints the plasmids bearing the genes on the array and the proteins are synthesized *in situ*. The genes are configured such that each expressed protein contains a polypeptide tag used to capture the protein to the array surface. The proteins are expressed using a cell-free transcription/translation extract, which can be selected to match the source of the genes (e.g., rabbit reticulocyte lysate for mammalian genes), thus enabling the proteins to be expressed in a more native milieu. The use of appropriate cell-free extracts helps to encourage natural folding and, at least in the case of reticulocyte lysate, is highly successful at expressing most proteins. In addition, some natural posttranslational modifications occur in these extracts and/or can be induced by using supplemented lysates ([21,22](#)).

Arranging the genes so that each has an appropriate capture tag is facilitated by using vectors with recombinational cloning sites. Coding regions inserted in recombinational cloning systems, such as the Invitrogen Gateway system or Clontech Creator system, can be readily moved into expression vectors that append the appropriate tag(s) to the coding regions. The transfer reactions themselves are simple, highly efficient, error-free, and automatable. The assembly of large collections of genes in these systems is currently in progress ([10,23–27](#)).

A significant advantage of the nucleic acid programmable protein array (NAPPA) approach is that it eliminates concerns about protein stability. Proteins on the array are not produced until the array is ready for use in experiments; that is, they are made just in time. Prior to activation with the cell-free transcription/translation extract, the arrays are stable and can be stored dry on the bench for months.

Using this approach in a recent study, 30 human DNA replication proteins were expressed and captured on NAPPA microarrays ([28](#)). The yield of captured protein was 400–2700 pg/feature, which was 1000-fold more than con-

ventional protein-spotting arrays, 10–950 fg/feature (11). Arrays were used to determine protein-protein interactions (recapitulating 85% of the previously known interactions), to map protein interaction domains by using partial-length proteins, and to assemble multiprotein complexes.

## 2. Materials

### 2.1. Equipment

1. Arrayer with solid pins, humidity control.
2. Microarray scanner.
3. Programmable chilling incubator.
4. SpeedVac.
5. Centrifuge: Sorvall RC12, Eppendorf 5417C, IEC Centra GP8.
6. Ultraviolet (UV) light, UVP UVLMS-38, set at 365 nm.

### 2.2. Preparation of the Slides

1. Glass slides (VWR 48311-702).
  2. Solution of 2% aminosilane (Pierce 80370) in acetone. Make up 300 mL just before use.
  3. Stainless steel 30-slide rack (Wheaton), handle removed.
  4. Glass staining box (Wheaton).
  5. Lock & Lock 1.5 cup boxes (Heritage Mint Ltd., ZHPL810).
  6. Prepare a 50 mM dimethyl suberimidate-2 HCl (DMS) stock solution: 1 g of DMS linker (Pierce 20700) in 40 mL dimethylsulfoxide (DMSO). Store at  $-20^{\circ}\text{C}$ .
  7. To coat slides with linker only (used if NAPPA strategy is to spot avidin/streptavidin along with plasmid DNA and anti-glutathione *S*-transferase [GST] antibody): 2 mM DMS in phosphate-buffered saline (PBS), pH 9.5 (*see Note 1*).
- Or
8. To coat slides with avidin/streptavidin (used if NAPPA strategy is to spot only plasmid DNA and anti-GST antibody): 2 mM DMS, plus avidin (Cortex CE0101) at 1 mg/mL or streptavidin (Cortex CE0301) at 3.5 mg/mL, in PBS, pH 9.5. For material in either **step 7** or **8**, make fresh at the time of coating, otherwise the DMS linker may hydrolyze over time (*see Note 1*).
  9. Cover slips (VWR 48393-081).
  10. Bioassay dishes with dividers (Genetix x6027).

### 2.3. DNA Preparation

1. The plasmid DNA is prepared in 300-mL cultures usually grown in Terrific Broth media. The DNA preparation is derived from Sambrook et al. (29) and is summarized below.
2. Solution 1 (GTE): 50 mM glucose, 25 mM Tris-HCl (pH 8.0), 10 mM ethylenediamine tetraacetic acid (EDTA), pH 8.0, and 0.1 mg/mL RNase. Store at  $4^{\circ}\text{C}$ .
3. Solution 2: 0.2 N NaOH with 1% sodium dodecyl sulfate (SDS).
4. Solution 3: 3 M KOAC; add glacial acetic acid until pH is 5.5.

5. 250-mL conical Corning centrifuge bottle.
6. Glass fiber 0.7- $\mu$ m filter plate, long drip (Innovative Microplate F20060).
7. 96-well deep-well block (Marsh AB-0661).

## **2.4. Preparation of Samples and Arraying**

1. Plasmid DNA (prepared in **Subheading 2.3.**).
2. Microcon YM-100 (100 kDa) tube (Millipore), or DNA binding plate: 100 kDa 96-well filter plate (Millipore plasmid plate).
3. BrightStar Psoralen-biotin kit (Ambion 1480). Just before use, prepare psoralen-biotin: dissolve the contents (4.17 ng) of the kit in 50  $\mu$ L DMF (also in kit).

Or

4. EZ-Link Psoralen-PEO-Biotin (Pierce 29986). Prepare stock solution of 5 mg/mL in water and store at  $-20^{\circ}\text{C}$ .
5. UV-transparent 96-well plate (Corning 3635).
6. Sephadex G50 (Sigma-Aldrich).
7. 1.2- $\mu$ m glass fiber filter plate, long drip (Innovative Microplate F20021).
8. Collection plate, round bottom (Corning 3795).
9. 384-well plate for arraying (Genetix x7020).
10. Polyclonal anti-GST antibody (Amersham Biosciences 27457701).
11. Purified GST protein (Sigma G5663). Prepare stock solution of 0.03 mg/mL in PBS.
12. Whole mouse immunoglobulin (Ig)G antibody (Pierce 31204). Prepare stock solution of 0.5 mg/mL in PBS.
13. Bis(sulfosuccinimidyl) suberate (BS3) linker (Pierce 21580).
14. Bioassay dish dividers to be used as slide racks (Genetix x6027) and deeper bioassay dishes (e.g., Corning 431111 or 431272; do not use “low profile” dishes).

## **2.5. Expression of Proteins**

1. HybriWell gaskets (Grace HBW75).
2. Cell-free expression system (Rabbit reticulocyte lysate) (Promega L4610).
3. RNaseOUT (Invitrogen 10777–019).
4. SuperBlock blocking solution in TBS (Pierce 37535).
5. Milk blocking solution: 5% milk in PBS with 0.2% Tween-20 (Sigma).

## **2.6. Detection and Analysis**

1. Primary AB solution: mouse anti-GST (Cell Signaling 2624) 1:200 in SuperBlock (Pierce 37535). Store at  $4^{\circ}\text{C}$ .
2. Primary AB solution: mouse anti-HA (Cocalico) 1:1000 in SuperBlock. Store at  $4^{\circ}\text{C}$ .
3. Secondary AB solution: horseradish peroxidase (HRP)-conjugated anti-mouse (Amersham NA931) 1:200 in SuperBlock. Store at  $4^{\circ}\text{C}$ .
4. Tyramide Signal Amplification (TSA) stock solution: use TSA reagent (PerkinElmer SAT704B001EA). Prepare per kit directions. Keep this solution at  $4^{\circ}\text{C}$ .
5. Milk blocking solution: 5% milk in PBS with 0.2% Tween-20 (Sigma).

6. Cover slips (VWR 48393–081).
7. PicoGreen (Molecular Probes P11495) stock solution: to the 100  $\mu\text{L}$ /vial that comes in kit, add 200  $\mu\text{L}$  TE buffer. Before use, do a 1:600 dilution in SuperBlock.

### 3. Methods

NAPPA chemistry relies on efficient immobilization of plasmid DNA onto a solid surface without compromise to integrity, and on rapid capture of the expressed target proteins. In order to immobilize the plasmid, we use a psoralen-biotin bis-functional linker that derivatizes the plasmid DNA (**Fig. 2**). Under long-wave UV (365 nm), psoralen intercalates into the DNA, creating a biotinylated plasmid. The reaction is fairly robust over a wide range of pH and salt concentrations. The biotinylated plasmid is tethered to the array surface by high-affinity binding to either avidin or streptavidin. In addition to the plasmids, target protein capture molecules are also immobilized on the slide. Currently, our plasmids are programmed to express target proteins with a C-terminal GST fusion protein; therefore, a polyclonal anti-GST antibody is bound to the array as the capture molecule to immobilize the expressed target proteins (**Fig. 3**). The presence of the C-terminal fusion tag can later be confirmed by incubating the slides with an antibody that recognizes a different epitope on the tag than the antibody used for capture. The presence of the C-terminal tag indicates that the full-length protein was expressed.

In order to make this chemistry robust and reproducible, we have used high-affinity capture reagents that are well characterized and stable throughout arraying and storage. Moreover, the schemes outlined previously can be altered by the user to accommodate different immobilization chemistries for the plasmid DNA and/or target proteins.

#### 3.1. Preparation of the Slides

1. Prepare 300 mL of aminosilane coating solution (2% aminosilane reagent in acetone).
2. Put slides in metal rack (30-slide Wheaton rack).
3. Treat glass slides in the aminosilane coating solution, approx 1–15 min in glass staining box on shaker. Rinse with acetone in rack using wash bottle. Briefly rinse with Milli-Q water. Spin dry in SpeedVac or dry using 0.2- $\mu\text{m}$  filtered air cans or use house air with  $2 \times 0.25 \mu\text{m}$  filters (*see Note 2*). It is important to use clean air to dry slides in order to prevent contaminating debris from binding to the surface.
4. Store at room temperature in metal rack in Lock & Lock box.
5. Just before use, prepare linker solution according to **Subheading 2.2. step 7** or **step 8**, depending on the array strategy.
6. Set slides on divider in bioassay dish, with water in the bottom of the tray. Treat each slide with 150–200  $\mu\text{L}$  linker solution and cover slip (*see Note 3*). Incubate for 2–4 h at room temperature or overnight in cold-room.

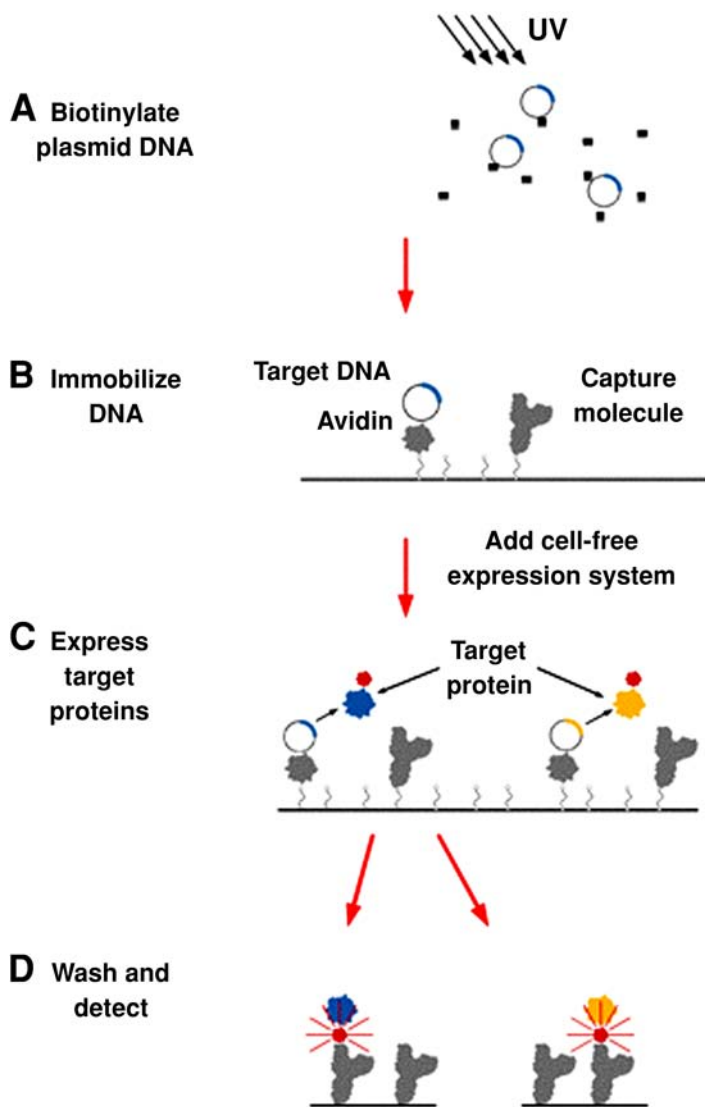


Fig. 2. Nucleic acid programmable protein array (NAPPAs) chemistry. **(A)** Derivatization of plasmid DNA. Plasmid DNA is mixed with psoralen-biotin, and cross-linked using ultraviolet light. **(B)** Sample preparation. The DNA is mixed with avidin/streptavidin, crosslinker, and the anti-glutathione *S*-transferase (GST) capture antibody, and this mix is arrayed on the aminosilane-coated glass slides. **(C)** Protein expression. After blocking, cell-free expression mix is applied to the slide, and during a temperature-programmed incubation the proteins are produced and bind to the capture antibody. **(D)** Detection. The slide is washed, and the proteins are detected by detecting the GST tag (using a monoclonal anti-GST antibody, an horseradish peroxidase (HRP)-labeled anti-mouse antibody, and Cy3-tyramide [TSA] HRP substrate).

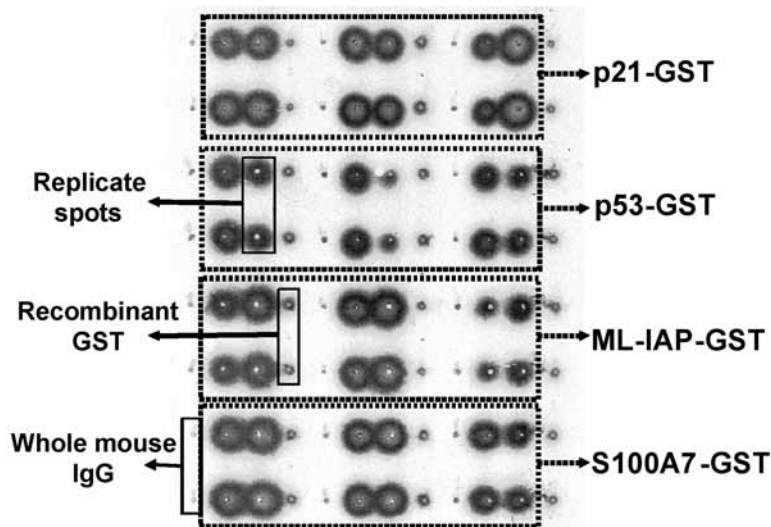


Fig. 3. Protein expression on nucleic acid programmable protein array (NAPPA) arrays. Plasmids encoding for four control proteins (p21, p53, ML-IAP, and S100A7) were biotinylated and immobilized via streptavidin onto the array surface. Arrays are activated by adding rabbit reticulocyte lysate, and the expressed proteins are detected using  $\alpha$ -glutathione *S*-transferase (GST) antibody. Protein expression and its immediate replicate using the same pin from the same well are indicated on the left. The neighboring spots of the same gene were arrayed from different wells with different pins. Two registration spots are also included—purified recombinant GST protein and whole mouse immunoglobulin G.

7. Wash with Milli-Q water.
8. Put slides in metal rack. Spin dry in SpeedVac.
9. Store at room temperature in metal rack in Lock & Lock box.

### 3.2. DNA Preparation

1. Grow 300-mL culture: in a 2-L culture flask, make a 300-mL culture of TB with 10% KPI. Add 300  $\mu$ L 100 mg/mL ampicillin stock solution. Add 0.5  $\mu$ L glycerol stock. Put it on a shaker for 16–24 h at 37°C, 300 rpm.
2. Pellet in 450-mL centrifuge bottle: spin 15 min at 5300g (Sorval RC12).
3. Add 30 mL of solution 1 and resuspend.
4. Add 60 mL of solution 2 and swirl, no more than 5 min.
5. Add 45 mL of solution 3 and shake briefly.
6. Spin 15 min at 5300g (Sorval RC12).
7. Pass through cheesecloth into 250-mL conical Corning centrifuge bottles.
8. Add 75 mL of isopropanol and shake.
9. Spin at 5300g 15 min (Sorval RC12).

10. Pour off supernatant.
11. Dissolve pellet in 2 mL of Tris-EDTA buffer (pH 8.0) and transfer to a 2-mL microfuge tube. Plasmid DNA yield from this preparation is approx 0.5–1.5  $\mu\text{g}/\mu\text{L}$ .
12. Add 200–250  $\mu\text{L}$  to each well of the long drip glass fiber 0.7- $\mu\text{m}$  filter plate (F20060). Stack on top of a deep-well block.
13. Spin 20 min at 890g (IEC Centra GP8).
14. Store the filtrate in the deep-well block at  $-20^{\circ}\text{C}$ , or in individual microfuge tubes.

### 3.3. Preparation of Samples and Arraying

1. Either spin 200  $\mu\text{L}$  of DNA (0.5–1.5  $\mu\text{g}/\mu\text{L}$ ) in a Microcon 100-kDa tube at 1000g for 20 min, or spin 200  $\mu\text{L}$  of DNA in a 100-kDa 96-well filter plate, stacked on top of a discard plate, for 20 min at 890g (IEC Centra GP8).
2. Resuspend in 100  $\mu\text{L}$  water. DNA concentration should be 1–2  $\mu\text{g}/\mu\text{L}$ . The goal is to achieve 100  $\mu\text{L}$  of roughly 1  $\mu\text{g}/\mu\text{L}$  of plasmid DNA. This is because the following UV exposure conditions for biotinylation of the plasmid have been optimized for a 100- $\mu\text{L}$  volume. Increasing or decreasing the volume is feasible, but the height of the liquid in the well may affect the UV dose. This may require a re-optimization of UV time and biotin dose to achieve efficient intercalation of the psoralen.
3. Just before use, prepare the BrightStar psoralen-biotin (*see Subheading 2.4., step 3*): dissolve the contents (4.17 ng) of the kit in 50  $\mu\text{L}$  DMF (also in kit); or for EZ-Link Psoralen-PEO-Biotin (*see Subheading 2.4., step 4*) prepare a 0.25 mg/mL solution in water.
4. Add the resuspended DNA into a UV plate for UV cross-linking. Add 1.3  $\mu\text{L}$  of BrightStar psoralen-biotin or 2  $\mu\text{L}$  of 0.25 mg/mL EZ-Link Psoralen-PEO-Biotin solution per 100  $\mu\text{L}$  DNA.
5. Cross-link for 20 min for BrightStar psoralen-biotin or for 30 min for EZ-Link Psoralen-PEO-Biotin with 365 nm UV, with the plate right up to the light; plate on ice; entire setup covered with foil. (The light covers five columns of the plate, so use only five columns of wells.) Note: 30 min with this setup corresponds to 8000  $\text{mJ}/\text{cm}^2$ .
6. Prepare Sephadex slurry, 25–50 mg/mL in water. Add 200  $\mu\text{L}$  of slurry to a 1.2- $\mu\text{m}$  glass fiber filter plate. Spin briefly at 890g (IEC Centra GP8) for 1 min into a discard plate. Add 100  $\mu\text{L}$  of water to the filter plate for the Sephadex to swell. Add 100  $\mu\text{L}$  of DNA and spin briefly again into the collection plate. Add 100  $\mu\text{L}$  water to the filter plate and spin briefly into the collection plate again.
7. Add eluate (approx 250  $\mu\text{L}$ ) to either a Microcon 100-kDa tube or a 100-kDa 96-well filter plate stacked on top of a discard plate. For the Microcon tube, spin at 1000g for 20 min (Eppendorf 5417C). For the filter plate, spin for 20 min at 890g (IEC Centra GP8).
8. Resuspend in 50  $\mu\text{L}$  water (2  $\mu\text{g}/\mu\text{L}$  plasmid DNA). Check that  $\text{OD}_{260}$  at 1:300 dilution is approx 0.6 (the absorbance reading is applicable only with the above mentioned method of DNA preparation; different DNA preparation methods yield different purity with different absorbance). Note: the desired final plasmid DNA concentration depends on the level of expression for the particular gene of interest.

Final plasmid DNA concentration may vary from 0.5  $\mu\text{g}/\mu\text{L}$  for genes with good expression to 3  $\mu\text{g}/\mu\text{L}$  for genes with poor expression.

9. Prepare spotting mix in arraying plate: 10  $\mu\text{L}$  DNA + 1.5  $\mu\text{L}$  of master mix.  
Master mix: *For linker-only slides*: GST polyclonal AB (0.5 mg/mL) + BS3 crosslinker (2 mM) + avidin (1 mg/mL) or streptavidin (3.5 mg/mL). *For avidin/streptavidin-coated slides*: GST polyclonal AB (0.5 mg/mL) + BS3 crosslinker (2 mM).
10. GST registration spots: 0.03 mg/mL in water or PBS.
11. Mouse IgG registration spots (whole mouse IgG antibody): 0.5 mg/mL in water or PBS.
12. Spin down plate, 1 min at 210g (IEC Centra GP8).
13. Array, using humidity control at 40–60%.
14. Store spotted slides in cold-room with water in the bottom of the tray, at least overnight. The bioassay dish divider should be placed in a deeper bioassay dish, so that the slides can be placed face-up on the rack without hitting the cover. Water in the bottom of the tray maintains high humidity.
15. Store slides the next day at room temperature. Storage conditions have been tested at room temperature to  $-80^{\circ}\text{C}$  in the dark for up to 2 mo without loss in expression and capture.

### 3.4. Expression of Proteins

1. Block slides for approx 1 h at room temperature or  $4^{\circ}\text{C}$  overnight in the cold-room with SuperBlock or milk. Use approx 30 mL in a pipet box for four slides. The slides need to be shaken during this initial step to wash away unbound NAPPA reagents (plasmid, avidin/streptavidin, capture antibody).
2. Quickly rinse with milli-Q water. Dry with filtered compressed air. Do not let slides stand to dry, as the watermarks will increase background.
3. Prepare in-vitro transcription/translation (IVT) mix. For one slide, 100  $\mu\text{L}$  is needed (*see Note 4*):
  - a. 4  $\mu\text{L}$  TNT buffer.
  - b. 2  $\mu\text{L}$  T7 polymerase.
  - c. 1  $\mu\text{L}$  of  $-\text{Met}$ .
  - d. 1  $\mu\text{L}$  of  $-\text{Leu}$  or  $-\text{Cys}$ .
  - e. 2  $\mu\text{L}$  of RNaseOUT.
  - f. 40  $\mu\text{L}$  of diethylpyrocarbonate (DEPC)-treated water.
4. Apply a HybriWell gasket to each slide. Use the wooden stick to rub the areas where the adhesive is, to make sure it is well stuck all around.
5. Add IVT mix from the nonspecimen end. Pipet the mix in slowly; it may bead up temporarily at the inlet end. Gently massage the HybriWell to get the IVT mix to spread out and cover all of the area of the array. Apply the small, round port seals to both ports.
6. Incubate for 1.5 h at  $30^{\circ}\text{C}$  for protein expression (30 is key; 28 or 32 gives reduced yield), followed by 30 min at  $15^{\circ}\text{C}$  for the query protein to bind to the immobilized protein.

7. Remove the HybriWell; wash with milk three times, 3 min each, in pipet box on a shaker. Use approx 30 mL per wash.
8. Block with SuperBlock or milk overnight at 4°C or room temperature for 1 h.

### 3.5. Detection and Analysis

1. Apply primary AB (mouse anti-GST or mouse anti-HA) by adding 150  $\mu$ L to the nonspecimen end of the slide, then apply a cover slip. Incubate for 1 h at room temperature; wash with milk (three times, approx 5 min). Drain.
2. Apply secondary AB (anti-mouse HRP) by adding 150  $\mu$ L to the nonspecimen end of the slide, then apply a coverslip. Incubate for 1 h at room temperature; wash with PBS (three times, approx 5 min). Then do a quick rinse with Milli-Q water. Drain.
3. Before applying TSA solution, make sure that the slides are not too wet, but do not let them fully dry. (If they are too wet, it will dilute the TSA.) Apply TSA mix and place cover slip. Incubate for 10 min at room temperature. Rinse in Milli-Q water; dry with filtered compressed air.
4. Scan in microarray scanner, using settings for Cy3.

As a quality check, select a couple of slides per arraying batch, and detect the arrayed DNA:

5. Block with SuperBlock 1 h.
6. For a single slide: apply 150  $\mu$ L PicoGreen mix and apply cover slip. Let sit for 5 min at room temperature. For four slides, add 20 mL in a box and shake for 5 min.
7. Wash with PBS (three times, approx 5 min). Then do a quick rinse with Milli-Q water.
8. Dry with filtered compressed air.
9. Scan, using Cy3 settings.

## 4. Notes

1. Part of the slide preparation process involves coating the slide with an activated *N*-hydroxysuccinimide (NHS) ester cross-linker (DMS). In our experience, this has not affected protein binding of the arrayed sample, but it significantly reduces the background.
2. We have used both streptavidin and avidin to immobilize the DNA onto the array surface. We have also coated the slides with avidin or streptavidin instead of adding it to the array mixture. Avidin in the array mixture tends to precipitate and affects arraying of the sample, whereas streptavidin does not precipitate and hence is a better choice for adding to the array mixture. Coating the slides with either streptavidin or avidin is feasible; however, we do observe better binding when the proteins are in the array mixture.
3. A key step in processing microarray slides is to *never* let them air dry. Any attempt to dry them slowly will leave watermarks, which will result in high background. As suggested in the protocol, use a clean air source to quickly dry the slides. It is also important to rinse the slides in clean, filtered water before drying, especially if the

arrays have been incubating in salt or protein solutions. Drying arrays directly from salt/protein solutions can also generate high background.

4. Good expression of the NAPPA arrays depends on correctly preparing the rabbit reticulocyte lysate. It is advisable to test a small sample of your prepared lysate for expression using the positive control provided in the kit.

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