

RCA-Enhanced Protein Detection Arrays

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Summary

There are many instances in which it is desirable to generate profiles of the relative abundance of a multiplicity of protein species. Examples include studies in embryonic development, immunobiology, drug responses, cancer biology, biomarkers, and so on. Microarray formats provide a convenient, high-throughput vehicle for generating such profiles, and the repertoire of proteins that can be measured is growing continuously as larger panels of specific antibodies become available. Here we describe methods for the use of antibody microarrays, whereby the detection of specifically bound antigens is enhanced by rolling circle amplification (RCA). RCA-enhanced protein detection on antibody microarrays provides a means for rapid protein profiling at high sensitivity. The set of RCA reagents remains unchanged for different microarray formats and compositions, and signal readout is performed using standard fluorescent dyes and scanners. The method is sensitive enough for the most challenging applications, such as the detection of low-abundance components of human serum.

Key Words: Microarrays; proteomics; rolling circle; antibody arrays; signal amplification; protein profiling.

1. Introduction

The feasibility and utility of antibody microarrays for the highly multiplexed analysis of proteins is now well established (*1–11*). Two major types of antibody microarray detection systems have emerged: (a) sandwich assays, in which unlabeled analytes are detected by a matched pair of antibodies specific for every protein target; and (b) label-based detection, in which the protein analyte is directly labeled by covalent attachment of tags, such as biotin or fluorophores (for example, Cy3 and Cy5), to enable detection after each targeted protein binds to the array. Sandwich assays can provide both high sensitivity and high specificity, and have been effectively demonstrated in the parallel measurements of low-abundance cytokines in culture supernatants and body fluids (*3,10*). Label-based

detection is an attractive complementary alternative to the sandwich assay, and its major advantage is ease in assay development, because only one antibody per target is required. Importantly, multicolor fluorescence detection is made possible when the targeted proteins are labeled. Because different samples may be labeled with different tags, a reference sample may be co-incubated with a test sample to provide internal normalization to account for concentration differences between spots. Although label-based detection is accurate and reproducible in the analysis of higher-abundance proteins, the detection sensitivity has not been sufficient to reliably detect lower-abundance proteins in biological samples using current methodology. Rolling-circle amplification (RCA) has been used for sensitivity enhancement in DNA quantitation (*12*), DNA mutation detection (*13,14*), and array-based sandwich immunoassays (*3,15*). RCA is well suited for multiplexed assays on solid surfaces, because the covalently attached amplified DNA cannot diffuse away. The isothermal process used in RCA takes place at moderate temperatures, and preserves the integrity of antibody–antigen complexes. RCA has been adapted to microarray-format immunoassays employing sandwich designs, as well as label-based detection. A schematic diagram illustrating alternative designs for antibody microarray assays utilizing RCA signal enhancement is shown in **Fig. 1**. In this chapter, we will focus on methods for the implementation of label-based assays, which are the most straightforward in implementation.

2. Materials

2.1. Microarray Preparation

1. Glass slides coated with a polyacrylamide hydrogel (hydrogel slides) (PerkinElmer Life Sciences, Boston, MA).
2. Antibodies, preferably of documented high specificity (*see Note 1*), are obtained from various commercial suppliers or from collaborators.
3. Aluminum foil tape #425–3 (R. S. Hughes Company, Sunnyvale, CA).

2.2. Protein and DNA Labeling

1. Amino-reactive fluorescent dyes, comprising *N*-hydroxysuccinimide (NHS) ester-linked Cy3 or Cy5 (Amersham Biosciences, Piscataway, NJ).
2. Amino-reactive haptens, NHS-digoxigenin, and NHS-biotin (Molecular Probes, Eugene, OR).
3. Chromatography spin columns with a molecular-weight cutoff of 6000 Da (Bio-spin P6) (Bio-Rad Laboratories, Hercules, CA).
4. Phosphoramidites coupled covalently to Cy3 or Cy5 dyes, for synthesis of labeled oligonucleotides (Glen Research, Sterling, VA).

2.3. RCA, Electrophoresis, and Microarray Washing Reagents

1. Phi 29 DNA polymerase (New England Biolabs, Beverly, MA).
2. Tango buffer (Fermentas, Hanover, NH).

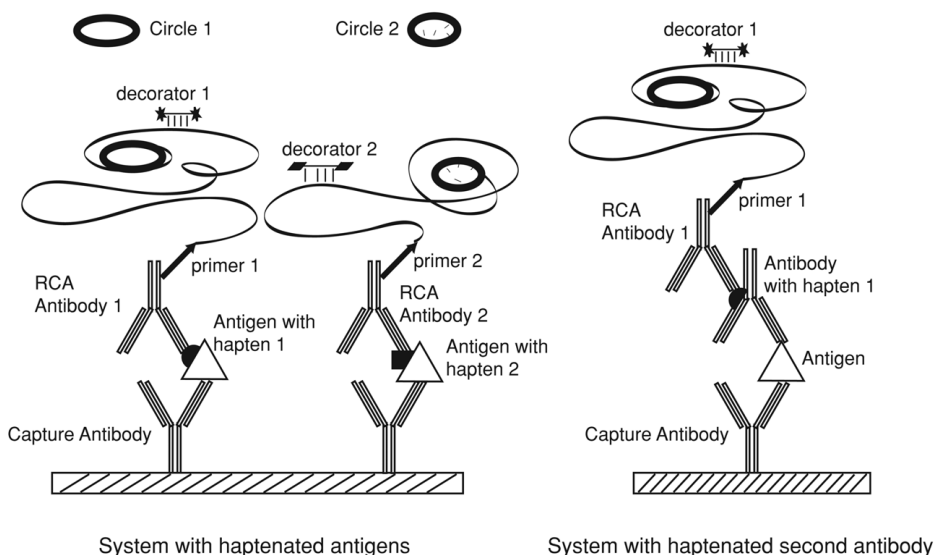


Fig. 1. Alternative designs for detection of antigens on the surface of antibody microarrays. In the label-based system employing haptenated antigens (diagram on the left) a capture antibody binds an antigen (labeled with hapten 1 or hapten 2). Preferred haptens are biotin and digoxigenin. The captured antigen is then detected by binding of an antibody-primer conjugate (rolling circle amplification [RCA] antibody 1 or 2), which generates amplified DNA after RCA, followed by fluorescent labeling of the amplified DNA with decorator 1 or decorator 2. A more elaborate assay system, based on a sandwich design, is depicted in the diagram on the right side. A capture antibody printed on a microarray binds an unlabeled antigen. The antigen is then bound by a haptenated second antibody (antibody with hapten 1) that recognizes a second epitope on the antigen. A third antibody, coupled to an oligonucleotide primer, binds to hapten 1 and functions as the signal generator. RCA is used to generate amplified DNA, followed by fluorescent labeling with decorator 1. Note that the sandwich system requires a matched pair of antibodies specific for every protein target, which insures extremely high specificity at the cost of added complexity. Because this system employs an unlabeled antigen, it is not straightforward to perform simultaneous two-color microarray experiments, and for this reason most investigators use a single-channel (single-color) detection system for sandwich assays. In this chapter, we describe methods for the label-based system based on haptenated antigens, which is simpler to implement and permits higher multiplexing.

3. TBE buffer: a 1X TBE solution contains 0.089 M Tris base, 0.089 M boric acid (pH 8.3), and 2 mM Na₂ ethylenediamine tetraacetic acid (EDTA).
4. PBST0.1 buffer: phosphate-buffered saline (PBS) with 0.1% Tween-20. Prepare by dissolving 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na₂HPO₄, and 0.24 g of KH₂PO₄

in 800 mL distilled H₂O. Adjust pH to 7.4 with HCl, add 1 mL of Tween-20, and adjust volume to 1 L with additional distilled H₂O. Sterilize by autoclaving.

5. 2X sodium saline citrate (SSC) or 1X SSC. 1X SSC is 0.15 M NaCl, 0.015 M Na citrate.

2.4. DNA Primers for RCA, To Be Conjugated to Antibodies

1. The oligonucleotide p1, thiol-5'-CACAGCTGAGGATAGGACsAsT serves as the first RCA primer. The symbol "s" is used to indicate the presence of a phosphorothioate linkage in the last two residues proximal to the 3'-end of the oligonucleotide, which serve to protect from degradation by 3'-5' exonuclease activity. The 5'-thiol group enables covalent coupling to antibodies. 5'-Thiol and phosphorothioate phosphoramidites for oligonucleotide synthesis are obtained from Glen Research, Sterling, VA.
2. The oligonucleotide p2, thiol-5'-TGTCTCAGTAGCTCGTCAsGsT serves as the second RCA primer. This primer (p2) was named primer 4.2 in publications from the Haab laboratory.

2.5. Preparation of DNA Circles

1. Circularization of oligonucleotide template for primer p1: The oligonucleotide c1, pGTCAGAACTCACCTGTTAGAACTGTGAAGATCGCTTATTATGTCC-TATCCTCAGCTGTGTAACAACATGAAGATTGTAG contains a 5'-phosphate (the terminal-phosphate phosphoramidite is obtained from Glen Research) to enable circularization with DNA ligase. The oligonucleotide g1, GTGAGTTCTGACCTACAATCTTCA-amino, which has a 3'-amino group to prevent priming by DNA polymerase (3'-amino phosphoramidite, Glen Research) serves as a guide for circularization. A 2 mL ligation reaction is prepared by mixing 200 μ L of oligonucleotide c1 (12 μ M), 200 μ L of oligonucleotide g1 (16 μ M) 200 μ L of 10X ligation buffer, and 1348 μ L of distilled water, followed by vortexing and heating to 65°C for 2 min. Then ligation is begun by adding 40 μ L of bovine serum albumin (5 mg/mL stock) and 12.5 μ L of T4 DNA ligase (400 U/mL), followed by incubation at 37°C for 2 h. DNA ligase is inactivated by incubation at 90°C for 2 min (*see Note 2*).
2. Circularization of oligonucleotide template for primer p2: The oligonucleotide c2, pCTGTGAGGTACTACCCTAATCGGACCTGTGAGGTACTACCCTAAT-TACTGACGAGCTACTGAGACATGTACAATCGGAC contains a 5'-phosphate (Glen Research) to enable circularization with DNA ligase. The oligonucleotide g2, AGTACCTCACAGGTCCGATTGTAC-amino, which has a 3'-amino group (Glen Research) serves as a guide for circularization. Circularization is performed as in **Subheading 2.5., step 1**.
3. Purification of circularized DNA. The reactions containing ligated circles are split into three microcentrifuge tubes (1.5-mL capacity) and deproteinized with an equal volume of a mixture of phenol:chloroform (1:1) by vigorous shaking (15 s) in a vortex mixer. The aqueous phase (upper layer) is recovered after centrifugation for 30 s at 7000g. The aqueous phase is washed with an equal volume of chloroform,

centrifuged, and washed with chloroform a second time. The aqueous layer is now brought up to 0.3 M salt by addition of one-tenth volume of 3 M Na acetate (pH 5.2). DNA is precipitated with 2.5 volumes of 100% ethanol. After addition of ethanol, the tubes are vortexed vigorously and then placed at -20°C for at least 1 h (or left in the freezer overnight). DNA is recovered by centrifugation for 15 min at 12,000 RPM in a benchtop microcentrifuge. The pellet is washed with 200 μL of 80% ethanol and again recovered by centrifugation. Finally, the pellet containing circular DNA is dissolved in 85 μL of 10 mM Tris-HCl (pH 7.7), 0.1 mM EDTA. The three aliquots of circular DNA are pooled, yielding a total volume of 255 μL . Confirmation of circularization is by electrophoresis in denaturing polyacrylamide gels. A denaturing 10% polyacrylamide gel is prepared in Tris-borate-EDTA buffer containing 8 M urea. One microliter of circularized DNA is mixed with 7 μL of gel loading buffer (0.5X TBE, 10 M urea), and denatured for 2 min in a heat block set to 92°C . As a control, a mixture of all DNAs and reagents used for ligation, minus DNA ligase, is prepared and mixed with gel load buffer, to serve as an indicator of the electrophoretic mobility of noncircular DNA. The gel is run for 25 min at 24 volts/cm. The gel is stained for 35 min with SYBR Green II (Molecular Probes, Eugene, OR), which is used at a 1/10,000 dilution, as recommended by the manufacturer, and then photographed with UV illumination. Successful generation of circular DNA will result in the presence of a band of considerably lower mobility than the linear form of the 80-base c1 or c2 oligonucleotide. In general, the circular molecules visible in the gel are present as a strong 80-mer, a fairly strong 160-mer, and a weaker 240-mer. The dimeric and trimeric circular concatemers work just as well as the monomeric circle as templates for RCA.

2.6. Antibody(Ab)-Primer Covalent Adducts

1. Preparation of covalent adducts is based on conjugation of thiol-labeled DNA oligonucleotides (sequences specified under **Subheading 2.4.**) to reactive amino groups in antibodies, using a heterobifunctional cross-linker, essentially as described by Schweitzer et al. (15). Anti-biotin antibody is obtained from Zymed (mouse monoclonal 03-3700, Zymed Laboratories, San Francisco, CA) and covalently conjugated to a 20-base oligonucleotide primer (p1) containing a 5'-thiol group, as follows. Desalted Ab (41 nmoles) is treated with a 10-fold molar excess of sulfo-GMBS (Pierce Chemicals, Dallas, TX) under nitrogen in the dark for 30 min at 37°C , followed by 30 min at room temperature. Unreacted sulfo-GMBS is removed by chromatography over a PD-10 column (Bio-Rad Laboratories, Hercules, CA) equilibrated with sodium phosphate (pH 7.5)/150 mM NaCl. The Ab is then concentrated in a Centricon YM-30 (Millipore Corp., Billerica, MA) at 4°C . The number of maleimides per Ab is determined by utilizing Ellman's reagent (Pierce) to measure sulfhydryls following titration of β -mercaptoethanol by the activated Ab. An amount equal to 28.1 nmol of sulfo-GMBS-activated Ab and 142 nmol of 5' thiol oligonucleotide is conjugated in a volume of 825 mL for 2 h at room temperature, followed by overnight at 4°C . Ab conjugated to oligonucleotide is purified by anion exchange chromatography on Q-Sepharose (Amersham Pharmacia) using a

salt gradient. Fractions containing conjugate are pooled and subjected to size exclusion chromatography on Superdex-200 (Amersham Biosciences-GE Healthcare, Piscataway, NJ) at 4°C to remove free oligonucleotide. A 10:1 ratio of oligonucleotide/Ab is typically used in conjugations, which results in a more reproducible production of conjugates, with three to five oligonucleotides per Ab molecule. Size exclusion chromatography is used to remove unconjugated oligonucleotide, ensuring a final preparation that is free of unconjugated Ab or DNA. This conjugation procedure yields a recovery of approx 50% of the starting Ab. Linear RCA reactions in solution (**13**) can be carried out to assess the ability of the Ab-DNA conjugate to serve as a primer. Standard RCA reactions contain 5 nM primer or Ab-DNA conjugate, 10 nM circular DNA template, and 0.4 U/μL of Phi29 DNA polymerase (New England Biolabs), 0.4 mM each dATP, dTTP, dGTP, and dCTP in 25 μL of Tango buffer (Fermentas). Additions are performed on ice and then shifted to 31°C. RCA products generated after 10, 20, and 30 min of incubation are assayed by electrophoresis for 2 h at 2.5 V/cm in a standard 0.7% agarose gel. The gel is stained for 35 min with Sybergreen II (Molecular Probes, Eugene, OR), which is used at a 1/10,000 dilution, as recommended by the manufacturer, and then photographed with UV illumination.

2. Anti-digoxigenin Ab covalently conjugated to a 20-base oligonucleotide primer (p2). Methods for generation of this adduct are identical to **Subheading 2.6. step 1**, except for the use of an anti-digoxigenin antibody (sheep anti-DIG IgG, Anawa Biomedical Services and Products, Switzerland) and a DNA oligonucleotide of different sequence.

2.7. Fluorescent “Decorator” Oligonucleotides

1. The oligonucleotide d1, cy3-ATGTCCTATCCTCAGCTGTG is used for fluorescent “decoration” of the amplified DNA generated by the RCA reaction on template c1.
2. The oligonucleotide d2, cy3-ACTGACGAGCTACTGAGACA is used for fluorescent “decoration” of the amplified DNA generated by the RCA reaction on template c2.

3. Methods

3.1. Printing of Antibody Microarrays

1. Antibody processing. The first step in producing antibody microarrays is the preparation of a set of antibodies. Antibodies work best in the microarray assay when they are highly purified. A high concentration of other proteins in the antibody solution usually results in a weakened or nonspecific signal, because binding sites on the microarray are occupied by the other proteins. Polyclonal antibodies collected from antisera should be antigen-affinity purified. Monoclonal antibodies that are provided in ascites fluid should be further purified using a kit such as the Bio-Rad Affi-gel Protein A MAPS kit. If bovine serum albumin (BSA) or gelatin has been added by the manufacturer, the IgG portion of the solution should be isolated

using Protein A purification. Glycerol is sometimes added to antibody solutions to prevent freezing at -20°C . Although glycerol will not interfere with the assay, the added viscosity may negatively affect the printing process. Glycerol concentrations above approx 20% should be avoided. To remove glycerol or to change the buffer of an antibody, we recommend the Bio-Rad Micro Bio-Spin P30 column (*see Note 3*). If the antibody is subsequently to be labeled, do not put the antibody in a Tris or amine-containing buffer, which will interfere with primary amine-based labeling reaction. The concentration of the antibody solutions should be adjusted to approx 500 $\mu\text{g/mL}$. Lower concentrations may also work well for some antibodies, and higher concentrations could yield higher signal intensities and may be desirable if consumption of antibody is not a concern.

2. Assembly of print plates. After the antibodies have been prepared at the proper purity and concentration, they are assembled into a “print plate”—a microtiter plate used in the robotic printing of the microarrays. Polypropylene microtiter plates are preferable to polystyrene because of lower protein adsorption. The plate should be rigid and precisely machined for optimal functioning with printing robots. The amount of antibody solution to load into each well of the print plate depends on the requirements of the printing robot—usually 10–15 μL is sufficient. If printing is sometimes inconsistent or variable between printing pins, it is desirable to fill multiple wells with the same antibody solution, so that different printing pins spot the same antibody and the data from replicate spots can be averaged. Store the 384-well print plates sealed in the refrigerator until ready to use. Aluminum foil tape provides a good seal (*see Materials*). Long-term evaporation-free storage is ensured by enclosing the covered plate in a sealed plastic bag (*see Note 4*). Prepare a spreadsheet containing the well identities for use in downstream data-processing applications.
3. Printing substrates. Various substrates for antibody microarrays have been demonstrated, such as poly-L-lysine-coated glass (*1*), aldehyde-coated glass (*2*), nitrocellulose (*4,16*), and a polyacrylamide-based hydrogel (*17,18*). Microscope slides with these various coatings can be purchased commercially. Because each application is unique in some aspects, the choice of which to use should be determined empirically by each user. We recommend simply preparing arrays on several different substrates and running the arrays in parallel, using the same sample on each array. Several criteria could be used to determine which surface type is best. The user should look at the signal-to-background ratio at each antibody spot, the reproducibility between replicate arrays, consistency in spot morphology, and the consistency in the background within each array.
4. Microarray printing hardware. Several varieties of commercial microarray printers are available, either contact printers or noncontact printers. Contact printers, commonly used for printing cDNA arrays, use metal pins to pick up antibody solutions from the print plate and deposit a small amount of the solutions (usually approx 1 nL) onto microarray substrates by contacting the surface. Noncontact printers deposit solutions without contacting the substrate, usually by ejection of a sub-nanoliter droplet from a quartz tip by a piezo-electric mechanism. Contact printers

can produce arrays faster than noncontact printers, especially if multiple printing pins are used simultaneously. The low production capacity of the noncontact printers can be a problem for some users, but the advantages of better spot reproducibility and morphology can be significant. Also, noncontact printers usually allow completely flexible array configurations, whereas the choices are usually more constrained using contact printers. The ability to print multiple replicate arrays on a single slide can be very useful, because the microarray substrates are used more efficiently and the throughput of the experiments can be higher. If multiple arrays per slide are printed, a method to separate the arrays is needed, so that different samples can be incubated on each array. A simple method is to draw a hydrophobic border around each array with a hydrophobic marker (*see Subheading 2.*). Another method for that purpose is the ProPlate (Grace Biolabs, Bend, OR), which can form up to 16 separate “wells” around 16 different microarrays on each slide.

5. Microarray printing. The details of the printing process will depend on the type of printing robot used, but we give some general notes here. Minimize the time that the print plates are unsealed and exposed in order to keep evaporation of the antibody solutions low. Evaporation may be minimized by cooling the print plate and maintaining an appropriate humidity in the printing environment. In a room-temperature environment, cooling the print plate to 5°C at a humidity of approx 43% will minimize both evaporation and condensation. The user should confirm that the print tips are sufficiently washed between loads, so that no measurable contamination between antibodies occurs. This test can be performed by printing a dye-labeled protein solution and a buffer solution in successive spots. If fluorescence is seen in the buffer spots, the pins need to be washed more stringently. Most microarrayers will allow the printing of replicate spots on each array, which are useful to obtain more precise data through averaging and to ensure the acquisition of data if a portion of the array is unusable. Three to six spots per array per antibody are usually sufficient. The handling of the arrays after printing depends on the surface used—see the manufacturer recommendations. Usually it will be advised to let the arrays incubate for some time to allow the antibodies to fully bind to the substrate. We recommend vacuum sealing and refrigerating the arrays for storage to minimize loss of antibody activity (*see Note 5*).

3.2. Sample Labeling With Haptens

1. Hapten-labeled protein samples are prepared by covalent labeling of an aliquot of sample with NHS-digoxigenin, and another aliquot with NHS-biotin. Each protein sample aliquot is diluted 1:15 with 50 mM carbonate buffer at pH 8.3, followed by addition of 1/20 volume of 6.7 mM NHS ester linked to digoxigenin or biotin, dissolved in dimethylsulfoxide (DMSO). After the reactions proceed for 1 h on ice, one-tenth volume of 1 M Tris-HCl (pH 8.0) is added to each tube to quench the reactions, and the solutions are allowed to sit for another 20 min. The unreacted dye is removed by passing each solution through a size-exclusion chromatography spin column (Bio-Spin P6). The digoxigenin-labeled samples are pooled, and equal

amounts of the pool are transferred to each of the biotin-labeled samples. Each hapten-labeled protein solution is supplemented with nonfat milk to a final concentration of 3%, Tween-20 to a final concentration of 0.1%, and 1X PBS to the final desired dilution. The optimal dilution of the samples may vary depending on the surface type and antibodies used, and should be determined empirically.

3.3. Microarray Immunoassay and RCA Signal Generation

The following reagents, described under **Subheading 2.**, are used: Anti-biotin antibody covalently conjugated to a 20-base oligonucleotide p1; anti-digoxigenin antibody covalently conjugated to a different 20-base oligonucleotide p2; 80-base circular DNA c1 with a portion complementary to primer 1; 80-base circular DNA c2 with a portion complementary to primer p2; Phi29 DNA polymerase; and decorator oligonucleotides d1 and d2.

1. Contacting of labeled protein sample with antibody microarrays: A 100- μ L sample of each hapten-labeled serum sample mix is incubated on a microarray with gentle rocking at room temperature for 1 h. The microarrays are rinsed briefly in PBST0.1 to remove the sample, washed three times for 3 min each in PBST0.1, and dried by centrifugation. (*Optional:* for indirect detection *without* RCA, microarrays are incubated under gentle rocking for 1 h at room temperature with primer 1-labeled anti-biotin and primer 2-labeled anti-digoxigenin antibodies, each prepared at 0.5 μ g/mL in 1X PBST0.1 with 1% BSA. A Cy3-labeled 20-bp oligonucleotide (d1) complementary to primer 1 and a Cy5-labeled 20-bp oligonucleotide (d2) complementary to primer 2 are prepared at 0.2 mM each in 2X SSC with 0.1% Tween-20 and 0.5 mg/mL herring sperm DNA. This solution is incubated on the microarrays for 1 h at 37°C. By using these labeled “decorator” oligos, d1 and d2, the antibodies are labeled by hybridization to the covalently bound primers.)
2. For RCA-enhanced detection, the microarrays are incubated for 1 h at room temperature with a solution containing 75 nM oligonucleotide c1, 75 nM oligonucleotide c2, 1.0 μ g/mL primer p1-conjugated antibiotin, and 1.0 μ g/mL primer p2-conjugated anti-digoxigenin in PBST0.1 with 1 mM EDTA and 5 mg/mL BSA. The microarrays are rinsed briefly in PBST0.1 and washed at room temperature with gentle rocking three times for 3 min each in PBST0.1. Phi29 DNA polymerase (Biolabs) in 100 μ L of 1X Tango buffer (Fermentas) solution with 0.1% Tween-20 and 1 mM dNTPs is incubated on the microarrays at 37°C for 30 min. The final concentration of Phi29 DNA polymerase should be 0.4 U/ μ L. After RCA, the microarrays are rinsed briefly in 2X SSC/0.1% Tween-20, washed three times for 3 min each at room temperature with gentle rocking in 2X SSC/0.1% Tween-20, and dried by centrifugation. A Cy3-labeled 20-bp decorator oligonucleotide (d1) complementary to the repeating DNA strand generated from primer p1 and a Cy5-labeled 20-bp decorator oligonucleotide (d2) complementary to the repeating DNA strand from primer p2 are prepared at 0.2 mM each in 2X SSC with 0.1% Tween-20 and 0.5 mg/mL herring sperm DNA. This solution is incubated on the microarrays for 1 h at 37°C, followed by washing for 2 min in 1X SSC.

3.4. Scanning of Microarrays

Several commercial microarray scanners are available, and each will have slightly different capabilities and functionality. Features such as adjustable resolutions, adjustable laser and photomultiplier settings, and autofocus abilities may vary between scanners. Some have the option to scan more than two channels of fluorescence, or are equipped with autoloaders for the convenient scanning of many slides (*see Note 6*). That capability is especially valuable for high-resolution scans of multiple slides.

1. Scanner settings. Most currently available scanners allow the user to adjust the laser and photomultiplier tube (PMT) settings, which is necessary because signal strengths can vary significantly between experiments, depending on the surface and sample types and other factors. The following guidelines should be adhered to. A significant saturation of signal at any of the spots should be avoided, because the values for those spots cannot be known if the signal is saturated (*see Note 7*). Therefore, the settings should be as high as possible without saturating any spots; usually, the average spots reach about 50% of the detector capacity. Most scanners offer a “quick scan” option at low resolution, to allow the user to rapidly determine the signal levels on the array and to set the optimal scanner settings. The laser power should be preferentially increased over the PMT gain. Higher laser power can improve signal-to-noise ratios (*19*), and laser outputs are more stable (less variable) at higher powers. The laser should be set as high as possible—up to approx 90–95% of maximum power. If the laser power is maximized and more signal is desired, increase the PMT setting. When scanning a group of slides that, together, compose an experiment set, it is usually desirable to use the same scanner settings for all slides. The user may then perform a quick scan on each slide in the set to verify that all the slides may be scanned at the chosen setting. The prescans should be limited to minimize photobleaching of the fluorophores on the arrays.
2. After the laser and PMT settings have been chosen, scan the slides at a resolution appropriate to the spot sizes. Ten to twenty pixels across the diameter of each spot are optimal. So if the spots have a diameter of 200 μM , use a resolution of no more than 20 μM . Fewer than 10 pixels across a spot can lead to imprecise averaging, and more than 20 pixels across does not further improve data quality and results in unnecessarily large image file sizes. Most scanners save the images in the TIFF format, with a separate image for each color channel that was scanned. An image of a scanned sub-array is shown in **Fig. 2**.

3.5. Data Analysis

Data analysis involves quantification of fluorescence intensity, spot flagging, averaging of replicate spots, normalization, and statistical analysis. The logical flow of these steps is outlined as follows, but a full treatment of the complex subject of microarray data analysis is beyond the scope of this chapter. The reader is referred to recent reviews (*21–23*) of the extensive literature on the subject.

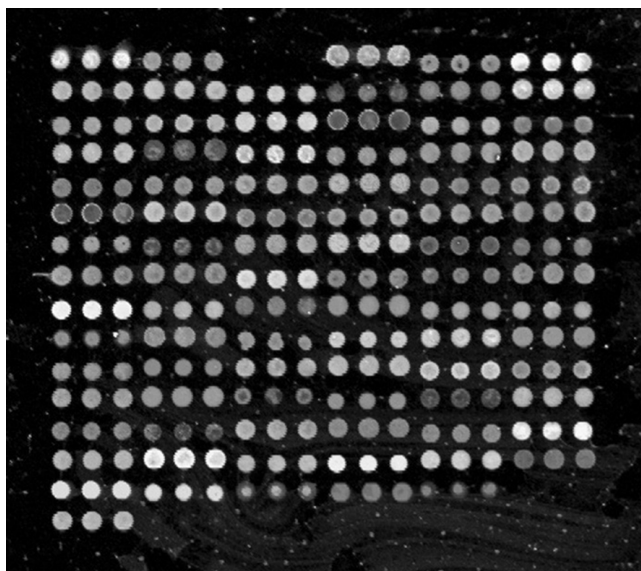


Fig. 2. Scanned image of an antibody sub-array. Antibody replication is in triplicate.

1. Spot localization and quantification of fluorescence intensity. The data analysis begins with quantification of the scanned images using a microarray gridding program, such as GenePix Pro from Axon Laboratories. This program will assign user-supplied identifications to each spot, locate the boundaries of each spot, and calculate various statistics for each spot, such as the average signal intensity of the pixels within a spot and outside of a spot, or the standard deviation between the pixels. The use of these numbers then depends on the type and purpose of the experiments. Two-color, label-based assays have significantly different primary data analysis methods (7,16) as compared with single-color sandwich assays (20).
2. Low-level data processing. The primary processing of the data involves spot flagging, averaging of replicate spots, and normalization. Spot flagging is the acceptance or rejection of spots based on signal intensities or other parameters. All arrays should be visually inspected, and spots with obvious defects should be flagged manually. A useful threshold for flagging spots by intensity is calculated by the formula $3 \cdot B \cdot CV_b$, where B is each spot's median local background, and CV_b is the average coefficient of variation (standard deviation divided by the average) of all the local backgrounds on the array. This threshold is similar to the standard deviation of the local background but minimizes the effects from spikes in the local backgrounds. For two-color data, the relevant parameter for subsequent analysis is the ratio of the fluorescence from the two color channels. For each spot, use the formula $(F1 - B1)/(F2 - B2)$, where $F1$ and $F2$ are the median intensities of the pixels inside the spot for color channels 1 and 2, respectively, and $B1$ and $B2$ are the

median intensities of the pixels locally outside the spot for the two color channels. GenePix Pro calculates this parameter automatically, and the user can simply extract the values of the nonflagged spots. The averaging of replicate spots helps to reduce variability caused by spot-to-spot differences. The “geometric mean” of the ratios should be calculated. That is, the logarithm of the ratios should be calculated (base 2 is useful) prior to averaging. The averages can then be anti-logged or left as logs, depending on the next analysis.

3. Normalization is typically applied to microarray data to account for possible systematic experimental variation in factors such as sample labeling efficiency, scanner readout efficiency, and microarray quality (24,25). Methods developed for two-color cDNA array data may be useful here, but the differences in antibody microarray experiments, such as a smaller and more selected set of targets and a different labeling method, may mean that the optimal normalization methods may be different. A simple “global normalization” method, which sets the median or mean ratio on each array to a fixed value, usually works quite well, but that method relies on the assumption that the average abundance of the measured proteins does not change between samples. A better method might be to select a subset of the spots within each array—spots that are expected not to change between samples—and to set the mean of those spots equal between arrays. If that method is used, the arrays should be designed to include many common, housekeeping, or structural proteins for use in the normalization.
4. Once the data are normalized, data from an experiment set can be brought together for statistical analyses. A variety of well-documented microarray analysis packages are available (microarrayworld.com/SoftwarePage.html), and the most appropriate tools will depend on the goals of the experiment. A common goal is to identify differences between samples, or to test the use of groups measurements for the classification of sample types. Often it is useful simply to visualize the expression profiles in a data set. Clustering is a useful tool for that, and a widely used program is freely available (rana.lbl.gov). Clustering allows the convenient visualization of patterns from many proteins over many samples and also the assessment of the degree of similarity between samples or proteins.

4. Notes

1. Antibodies should be of very high specificity, as determined by enzyme-linked immunosorbent assay (ELISA) or other methods. Specificity can be tested on the microarray format by performing competition assays, provided that the intended antigen is readily available. Serum is spiked with antigen, and labeling is performed as indicated. Increasing amounts of unlabeled antigen are added in a series of microarray experiments, to measure the reduction of the fluorescent signal as a function of the concentration of competing antigen.
2. Exonuclease digestion after generation of circular DNA was used in previously published protocols to destroy the remaining guide oligonucleotides after ligation. However, we have eliminated this step by using guide oligonucleotides with blocked 3' termini, which have no priming activity. An amino group located at the

3' end of the guide oligonucleotide can serve this purpose. The guide oligonucleotides remain hybridized to the circular oligos during purification, but are readily displaced by phi29 DNA polymerase during the RCA reaction.

3. The Biospin columns come prepacked with two types of buffers: SSC and Tris buffer. The packing buffer comes out of the column with the sample that was applied to it. That is, after a sample is run through the column, it will be in the buffer with which the column was packed. The packing buffer can be changed by running a different buffer through the column three times. The P30 column removes solution components smaller than 30 kD, and the P6 column removes components smaller than 6KD. Thus the P30 column is better for purification of antibodies, and the P6 column is better for the purification of complex mixtures in which low-molecular-weight species should be preserved.
4. Food storage sealers work well for this purpose. Some models have the option of applying vacuum, or simply sealing without vacuum. To completely prevent evaporation of fluid from the print plate, insert a moist piece of paper towel into the bag with the print plate before sealing; this will keep the humidity in the bag high.
5. We do not know of systematic studies on the shelf life of antibody microarrays. Our own experience has show that arrays stored up to 2 mo under vacuum in a refrigerator or cold-room show undiminished signal and performance properties.
6. A cautionary note when using autoloaders is that high levels of atmospheric ozone can be destructive to certain dyes, such as Cy5 (26). High levels of ozone can build up in closed compartments with electrical motors inside.
7. Saturation occurs when the upper limit of quantification of a signal has been exceeded. Most scanners will have some indication of pixels that have been saturated, such as white-colored pixels.

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