Chapter 19

Antibody Colocalization Microarray for Cross-Reactivity-Free Multiplexed Protein Analysis

Véronique Laforte, Pik-Shan Lo, Huiyan Li, and David Juncker

Abstract

Measuring many proteins at once is of great importance to the idea of personalized medicine, in order to get a snapshot of a person's health status. We describe the antibody colocalization microarray (ACM), a variant of antibody microarrays which avoids reagent-induced cross-reactivity by printing individual detection antibodies atop their corresponding capture antibodies. We discuss experimental parameters that are critical for the success of ACM experiments, namely, the printing positional accuracy needed for the two printing rounds and the need for protecting dried spots during the second printing round. Using small sample volumes (less than 30 μL) and small quantities of reagents, up to 108 different targets can be measured in hundreds of samples with great specificity and sensitivity.

Key words Microarray, Antibody, Sandwich immunoassay, Multiplexed, Fluorescence

1 Introduction

Immunoassays are currently used in the clinic to quantify specific proteins in the blood and plasma of patients to give clues about their health status. Proteomics, the measurement of tens or hundreds of proteins in a single sample, has the potential to empower diagnostics and patient monitoring by providing a more complete snapshot picture of the health status of a person using very little sample. To achieve this multiplexed measurement goal in the future, technologies that measure multiple proteins simultaneously with high sensitivity, precision, and reproducibility are required.

Sandwich immunoassays consist in capturing a target in a sample to the surface using a surface-bound capture antibody, followed by the binding of a detection antibody which recognizes the same target (e.g., a protein) but at a different epitope as the capture antibody. Sandwich immunoassays offer high sensitivity due to the high affinity of antibodies to their target and high specificity, thanks to the double recognition of different epitopes on that target. Antibody microarrays can measure multiple targets at once using the same

David W. Greening and Richard J. Simpson (eds.), *Serum/Plasma Proteomics: Methods and Protocols*, Methods in Molecular Biology, vol. 1619, DOI 10.1007/978-1-4939-7057-5_19,

amount of sample as a classical ELISA and minimal amounts of costly antibodies. However, when multiple detection antibodies are mixed, specificity is often lost due to cross-reactivity between reagents [1] which can be mitigated by extensive selection, optimization of the reagents, and limiting the number of targets measured simultaneously. Cross-reactivity leads to significant false-positive signals, which can mask significant binding or conversely give the appearance of target binding when none occurred [2].

The antibody colocalization microarray (ACM) was developed to avoid cross-reactivity in multiplexed measurements by physically separating individual detection antibody solutions and printing them directly atop their corresponding capture antibodies. Because detection antibodies are not mixed, the same high level of specificity as ELISA is reached with the ACM. Microscope slides are printed with capture antibodies using a microarray printer with silicon quill pins that are fabricated in-house [3]. After blocking and incubating samples on the microarray slides, slides are dried and moved back to the microarray printer where detection antibodies are then spotted over their respective capture antibodies with great positional accuracy. Microarray slides are incubated with a reporter molecule (streptavidin conjugated to Alexa Fluor 647 (AF647)) and scanned with a fluorescence scanner (*see* Fig. 1). Capture and detection antibodies are printed in different low-evaporation buffers which are suited for each step. These low-evaporation printing buffers allow for long printings of several hours without changing the composition of the printed solutions, allowing for better printing reproducibility [4].

Many other methods have been devised to circumvent cross-reactivity in multiplexed measurements. Similar to the ACM, a system was developed with two spotting rounds and an aqueous two-phase system to separate individual detection antibodies. The caveat of this system is the size of spots which limits the density of targets that can be measured in one sample [5]. Proximity extension assays (PEA) [6] and proximity ligation assays (PLA) [7, 8] make use of matched antibody or aptamer pairs conjugated to corresponding short DNA fragments and real-time PCR to quantify the amount of antigen bound. These methods have been shown to accurately measure up to 96 targets in as little as 1 μL samples; however, it requires the labeling of each individual antibody and the use of a separate microfluidic platform. The Simple Plex [9] is a simple polymer chip that uses microfluidics to separate the flow of individual detection antibodies over separate capture areas. The method can very quickly detect proteins in samples; however, the multiplexing capabilities are currently limited to detecting four targets in a same sample. Microarray chips that use force-based discrimination only leave tightly bound antibody on the surface, while cross-reacting antibodies that are expected to be weakly bound are removed [10]. This method was shown for eight

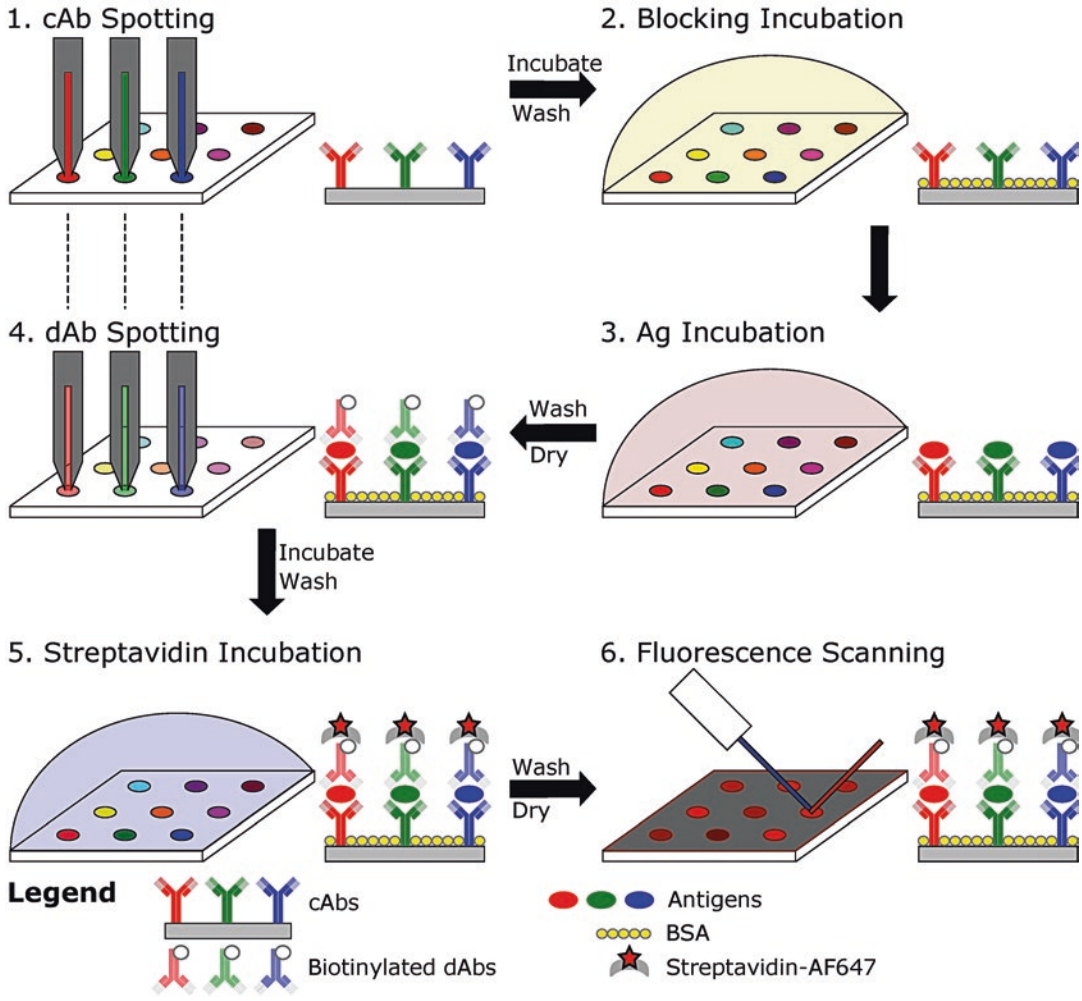

Fig. 1 Schematic of the antibody colocalization microarray. Capture antibodies are printed onto functionalized glass microarray slides (*1*) using silicon quill pins and incubated for 24 h. Microarray slides are washed and blocked (*2*), followed by an overnight incubation of diluted samples and antigen standard overnight at 4 °C (*3*). Microarray slides are then washed and dried before printing cognate biotinylated detection antibodies at the exact same location as previously printed capture antibodies (*4*). Microarray slides are incubated for 16–24 h before washing, followed by the incubation of fluorescent streptavidin (*5*). After washing and drying, microarray slides are scanned with a fluorescent scanner (*6*)

targets. In short, while PEA and PLA have good performance and multiplexing capabilities, these assays are very complex. On the other hand, Simple Plex and the aqueous two-phase system platform are simpler but have limited multiplexing capabilities. In comparison, ACM is simple and allows for more than one hundred targets to be measured.

Several aspects of the procedure described in this chapter are critical to the success of ACM experiments. These are printing positional accuracy, spots protection with trehalose during detection

antibody printing, duration of streptavidin incubation, features of the working environment, and experimental design. Each of these aspects contributes to the high accuracy, sensitivity, and reproducibility of the ACM platform, and they are described below.

Because detection antibodies are printed directly atop their corresponding capture antibodies, the microarray printer used, regardless of whether it is a contact printer or inkjet, should have excellent positional accuracy (10 μm) when a microarray slide is removed and handled between two spotting rounds on the same slide. In order to reach this level of performance, we found it important to avoid re-initializing the printer between printing rounds, as well as establishing a method for calibrating the printer head position. A microarray slide deck that is equipped with spring-loaded slots was used for accurate positioning of slides. Slides were pushed against a corner and the two adjoining sides. Precise and consistent alignment is also dependent on good manual dexterity, and slides were positioned at the same location on the deck.

Since spots containing the capture antibodies and targets are dried before printing the detection antibodies, it is important to coat the microarray slides with a protectant to prevent the degradation of antibodies and proteins at the surface [11]. Trehalose [12] was dried on the surface, forming a protective coating, without the presence of salts or buffer, which denature proteins because of the high salt concentration at the dry state. Detection antibodies are printed with a detection buffer containing glycerol and bovine serum albumin (BSA) that help protect the proteins at the surface during the following incubation. Printing many microarray slides (>10) takes several hours with our setup with four silicon pins used in parallel. In the absence of a trehalose coating just before detection antibody printing, we observed a slow degradation of capture antibodies and bound antigens at the surface that reduced assay reproducibility.

Following sample incubation which is done for ~18 h to allow the capture antibody to antigen binding to reach equilibrium, incubation times for the following steps are crucial. Because the detection antibody spots have very small volumes and high viscosity, which decreases the off-rate of the antibody-antigen complex, the quantity of bound antigen to the capture antibodies is not significantly decreased in spite of the long incubation time. However, during the following washes and streptavidin-AF647 incubation, a trade-off must be found between minimizing incubation times to limit off-rate unbinding and providing sufficient time for the streptavidin to bind in order to give a strong signal. We found that 20–30 min of streptavidin incubation is sufficient in our experiments. After the final washing and drying, all microarray slides were scanned at once unless they are kept in the dark and in vacuum. Fluorophores used are sensitive to ozone below levels that can normally be detected, and keeping them in the presence of

air or light for several hours leads to significant degradation to affect reproducibility and sensitivity.

A dedicated room was used for carrying out the procedures described below with lights turned off, ozone removal, and HEPA-filtered air. Ambient light and the presence of even small amounts (10 ppb) of ozone in the air during incubations, washes, and scanning can lead to fluorophore photobleaching and gradual degradation during scanning that can affect reproducibility of the assay. Alexa Fluor dyes are less sensitive to the effect of light than Cy dyes [13]; however, they are more sensitive to ozone [14]. Their use is still warranted by the fact that AF dyes have higher signals and decreased quenching compared to Cy5 and Cy3 [15, 16]. Their absorption and emission spectra also do not change when they are conjugated to antibodies [17]. Dust particles that are bigger than a spot size (~100 μm) can lead to one or several missing spots and, hence, missing data points in an experiment. Moreover, many dust particles are autofluorescent. The use of cellulose-based cotton from lab coats or paper to blot liquids in the work environment should be avoided to prevent contamination by dust particles. Microfiber cloths were used, along with clean-room quality lab coats. Generally following guidelines for a dust-free room (such as a clean room) is helpful to obtain high-quality defect-free microarrays.

If a microarray printer is not readily available, pairs of microarray slides containing preprinted, mirrored capture antibodies and detection antibodies, respectively, can be purchased from Parallex Bioassays (http://www.parallexbio.com, Montreal, Canada) along with a snap-chip device. The ACM assay can then be conveniently performed by using the snap chip to precisely transfer the detection antibodies to the spots with the corresponding capture antibody spots following sample incubation [18, 19]. The snap-chip procedure (*see* Fig. 2) corresponds to the steps described below involving sample incubation, washing, streptavidin-AF647 incubation, and scanning. Steps that are specific to the ACM are the printing steps and the protection of microarray slides with trehalose. This is not necessary in the snap-chip procedure because all detection antibodies are applied at once in parallel. Overall, an experiment using the snap chip is shorter in time than with home-printed microarray slides.

Experimental design is important to the success of ACM experiments (*see* Fig. 3). Two complete standard curves containing a mixture of known quantities of recombinant antigen that are serially diluted (1:2–1:4) with a minimum of seven points (but ideally 15 points to obtain an accurate curve fit) and a blank are included in the layout [20, 21]. Samples are measured at two different dilutions (e.g., 1:3 and 1:50) to allow the quantification of low- and high-abundance proteins. The well position of all samples is randomized to avoid measurement bias. Several blanks and normal replicate samples, for example, from a pooled normal serum or

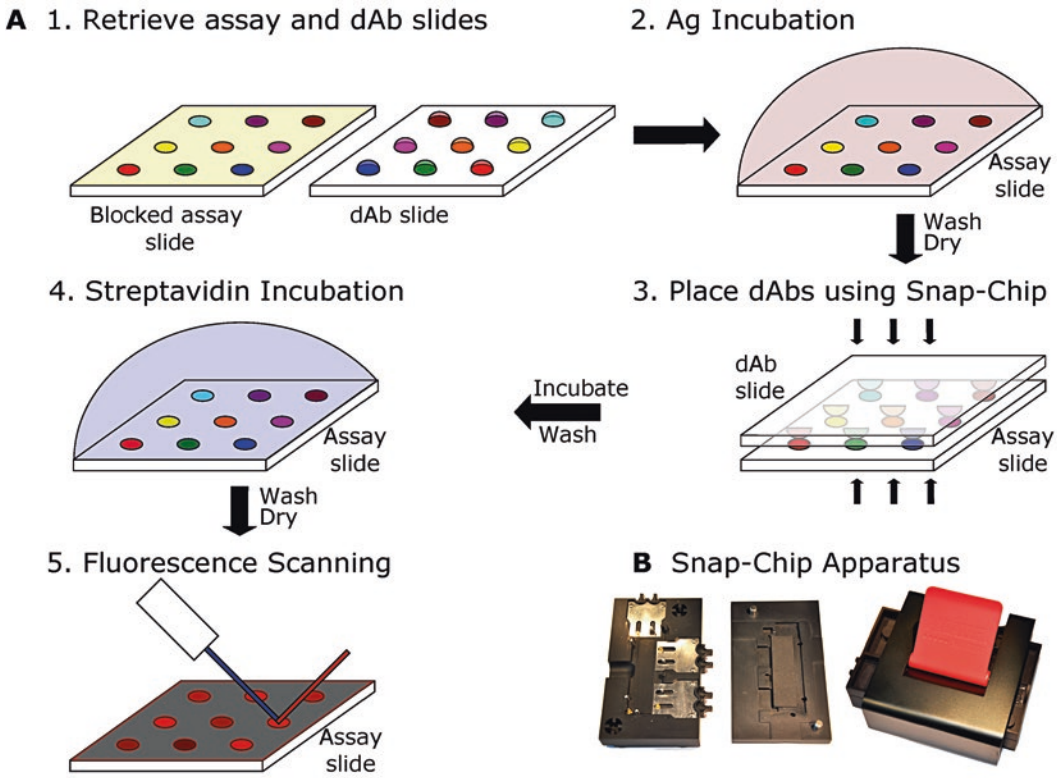

Fig. 2 Schematic of the ACM using a snap-chip apparatus. (**a**) The same number of assay (*capture*) and detection slides are pre-spotted with antibodies (*1*), and the assay slides are pre-rinsed, blocked, and dried prior to shipping. Assay slides are retrieved from storage by the user and incubated with diluted samples and antigen standards overnight at 4 °C (*2*). Assay slides are then washed and dried. After being retrieved from storage, detection slides are brought into contact with their respective assay slides using the snap-chip apparatus (*3*) which aligns the assay and detection slides and ensures incubation of each spot with a detection antibody solution for 1 h. Slides are then separated, and the assay slides are incubated with fluorescent streptavidin (*4*), washed, dried, and scanned (*5*) using a fluorescent scanner. (**b**) The snap-chip apparatus mechanically brings an assay slide and a detection slide in contact with precise force and alignment over the whole surface (*pictures from Parallex Bioassays*)

plasma sample, are measured at regular position intervals (e.g., once per microarray slide) in order to properly measure the limit of detection (LOD) and the reproducibility (coefficient of variation %CV) for each target measured. All values of samples and replicates are quantified by interpolating the log-transformed raw fluorescence value in a log-log curve fit using the standard curve values (without the blank).

The ACM has been used to measure up to 50 targets in 55 samples [1, 22]. Recently, the measurement of up to 108 targets with triplicate replicate spots per target and per sample and for upward of 300 samples with two dilutions per sample has been analyzed. The ACM has been used to measure human serum, plasma with different anticoagulants (EDTA, heparin, CTAD, and

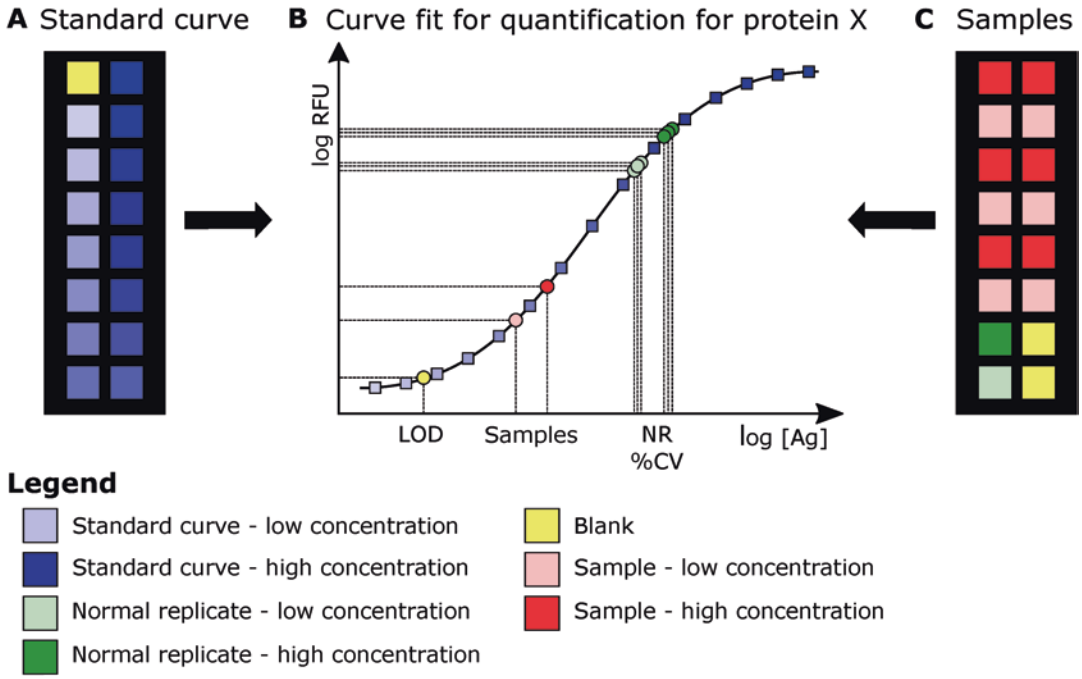

Fig. 3 Experimental design for ACM experiments. A slide containing 16 identical subarrays are incubated with (**a**) a serial dilution of recombinant antigen mixtures (*varying shades of blue*) and a blank (*yellow*). This standard curve is plotted on a log-log graph (**b**) and fitted with a four-parameter logistical curve. Other slides (**c**) are incubated with samples at different dilutions (*red*), blanks (*yellow*), and normal replicates (*pooled samples from healthy individuals*) at different dilutions (*green*). The concentration of each protein is derived by interpolating the values with the binding curves derived in (**a**). The limit of detection (LOD) is calculated as the mean + three times the standard deviation of blanks, whereas the assay coefficient of variation (%CV) is calculated from the interpolation of each normal replicate where the standard deviation is divided by the mean for each target

citrate), as well as other human fluids such as cerebrospinal fluid and urine. The sensitivity of assays using the ACM is comparable to that of ELISA, with LODs ranging from 0.1 to 300 pg/mL depending on the antibody pair used. Reproducibility of assays also varies depending on the antibody pair and can be as good as 10% variability over a large (>200 samples) experiment (unpublished results). Because of long duration of printing for large experiments, reproducibility is better when printing up to six to eight microarray slides with a 16-well gasket (*see* Fig. 2) which leads to printing rounds that are less than 3 h. Reproducibility can be further improved by normalizing the data [23].

2 Materials

2.1 Buffers and Materials

All buffers are prepared using ultrapure water which has a resistance of at least 18 MΩ·cm at 25 °C. All reagents are analytical grade and stored at room temperature unless otherwise indicated.

Follow local waste disposal regulations and MSDS recommendations for chemicals.

1. Wash buffer: 1× phosphate-buffered saline (PBS) containing 0.1% Tween 20. Mix 100 mL of 10× PBS stock to 900 mL of ultrapure water. Add 1 mL of Tween 20 using a viscous liquid pipette (*see* **Note 1**). 1× PBS can be prepared by other methods as long as it is free of small particles (*see* **Note 2**). This buffer can be stored in a squeeze bottle at 4 °C for 1 year.
2. Dilution buffer: 1× PBS containing 0.05% Tween 20. Mix 100 mL of 10× PBS stock to 900 mL of ultrapure water. Add 0.5 mL of Tween 20 using a viscous liquid pipette (*see* **Note 1**). This buffer can be stored at 4 °C for 1 year.
3. Blocking buffer: 3% protease-free bovine serum albumin (BSA) kept at 4 °C, 1× PBS, and 0.05% Tween 20. Mix 0.3 g BSA in 10 mL of dilution buffer. This solution is made fresh when needed.
4. Capture antibody printing buffer: make a 2.9 M betaine, 35.7% 2,3-butanediol in 1× PBS by mixing 1.6987 g of betaine to 1.785 mL 2,3-butanediol using the viscous liquid pipette (*see* **Note 1**), and 1.97 mL of 1× PBS. Dilute this concentrated printing buffer to the required concentration by mixing with 1× PBS before adding to individual capture antibodies. The final concentration for printing is 2 M betaine and 25% 2,3-butanediol in 1× PBS when antibodies have been added (*see* **Note 3**). This solution can be kept at room temperature for up to a week. Do not store this buffer at 4 °C (*see* **Note 4**).
5. Detection antibody printing buffer: make a concentrated BSA-T20 solution by mixing 0.15 g of protease-free BSA kept at 4 °C, 150 µL of wash buffer, and 4.85 mL of 1× PBS. When preparing detection antibodies, mix the appropriate volume of this concentrated BSA-T20 (3% BSA, 0.003% Tween 20, 1× PBS) to pure glycerol using the viscous liquid pipette (*see* **Note 1**) and 1× PBS so that the final concentration of the additives is 1% BSA, 0.001% Tween 20, and 45% glycerol solution in 1× PBS after adding the detection antibody stock solution (*see* **Note 3**). However, before adding the individual detection antibodies, filter the solution containing BSA, Tween 20, and glycerol with a 0.45 µm sterile filter, a syringe, and a hypodermic needle (*see* **Note 5**). Dispose of the hypodermic needle in a sharps container according to local waste management regulations. This solution is prepared fresh daily.
6. Slide rinsing solution: make 50 mL or more of 5% trehalose solution in water by mixing 2.5 g of trehalose to 50 mL of ultrapure water. This solution is kept at 4 °C in a squeeze bottle.

7. Microarray slides: Xenobind slides (Xenopore Corp.) are standard size glass microarray slides with a proprietary reactive aldehyde surface. PolyAn's 2D aldehyde microarray slides also work well with this protocol, as it has the right surface-binding capacity and chemistry (*see* **Note 6**). Slides should be clean and free of dust or visible smudges. Their surface coating should be homogeneous and can be verified by scanning slides at a high gain before using. If slides are not clean, an in-house cleaning and quality control step can be performed (*see* **Note 7**) but should be validated for each slide type.

2.2 Equipment

The ACM protocol requires a number of specialized equipment. Below is the list of equipment and their characteristics or performance parameters that are critical to the success of ACM assays.

1. Microarray printing: printing is done using a contact microarray printer using custom-made silicon quill pins with high liquid capacity [3]. The printer has a spot positioning accuracy of 10 μm or less when microarray slides are taken out of the slide deck and replaced in the same position after further processing. The capture and detection printing buffers are compatible with silicon quill pins. Quill pins are treated once with the flame from an ordinary kitchen torch which forms a plasma that makes the pin channels hydrophilic. During normal operation, pins are washed with a soap solution, followed by distilled water, and then dried using a vacuum pump or absorbent paper. Neither the source plate nor the slide deck is cooled. An inkjet-type microarray printer can be used if it meets minimum performance requirements (*see* **Note 8**). The printing chamber is kept free from dust by filtering the incoming air to the humidifier and minimizing manipulations with hands inside the chamber. Gloves and dust-free (clean room) lab coats are worn at all times in the room where the printer is located, and hair is tied or covered. A HEPA filter and dust-minimizing practices are also recommended for this room. If using a 1536-well plate to dispense liquids, make sure that the plate fits tightly in its enclosure and is well aligned. To facilitate loading the plates, they can be treated with a (gas) plasma. We found that 10 s at 100 W (PlasmaEtch PE-50) worked well for 1536-well plate for detection antibody solutions. The 1536-well plate for capture antibody solutions containing additives is not plasma-treated (*see* **Note 9**).
2. Rotary shaker: a type of flat rotary shaker that has a large surface area and small radius of rotation is used in order to maximize mixing within the 7 mm^2 wells. Moreover, it is compatible with temperatures down to 4 °C. In order to improve the adhesion between gaskets and the rotary shaker, a large, flat layer of polydimethylsiloxane (PDMS) is placed on top of the surface (*see* **Note 10**).

3. Fluorescent scanner: a microarray scanner which scans microarray slides in less than 15 min per slide at a resolution of 5 μm or less is recommended. At least one of the laser and filter combination should be compatible with the AF647 dye.

2.3 Antibody Pairs and Antigens

Assays should be performed with high-quality reagents that have undergone rigorous quality control. Only antibody pairs that have been validated for use as a pair in ELISA or on a microarray are used, and binding curves established using full-length proteins whenever available. All reagents are verified for binding against the species of the samples to be measured.

1. Capture antibodies: these antibodies are typically monoclonal antibodies, purified and unlabeled. If the stock concentration is not at least 0.25 mg/mL, a concentration and quantification step is required prior to aliquoting in working volumes and storing at the appropriate temperature as specified by the manufacturer. Capture antibodies should be free of carrier proteins (e.g., BSA) and contain less than 5% glycerol (*see* **Note 11**).
2. Detection antibodies: these antibodies can be monoclonal or polyclonal antibodies and are purified and labeled with biotin. Other labels are possible (*see* **Note 12**) but should be compatible with the labels of all other assays within a microarray slide's subarray. Their concentration is at least 0.1 mg/mL and they are aliquoted in working volumes and stored according to the manufacturer's instructions. If the concentration is too low, a concentration step, for example, using spin columns, may be used before aliquoting and storing.
3. Antigens: antigens are typically recombinant and purified. Antigens that are obtained from animals or humans and purified can lead to significant cross-reactivity with other targets within the subarray (*see* **Note 13**). Antigen stock solutions should have as high concentration as possible for better storage stability and can be stored in the presence of carrier protein such as BSA in order to improve their shelf life.

2.4 Other Materials and Equipment

1. Microarray gaskets: the microarray gaskets used are Grace Bio-Labs ProPlate® Multi-Array Slide System, more specifically the gaskets with metal clips and 16 wells (2 × 8) that allow us to incubate 16 different samples per microarray slide. Printing of microarray slides is designed to print 16 identical subarrays that correspond to the 16 wells of the gasket. Different gasket layouts can be used in order to have more or less targets and samples on each slide as long as the microarray printing layout is adjusted.

2. Compressed nitrogen stream: many fluorescent dyes are sensitive to heat, oxygen, ozone, and light. When drying microarray slides, it is very important to use a stream of compressed gas that is free of oxygen in order to avoid degradation of the AF647 reporter dye.
3. Square 24.5 cm^2 petri dishes (optional, *see* **Note 10**).
4. Solvent- and water-resistant permanent marker (*see* **Note 14**).
5. Microfiber cloth.
6. 1536-well plate, not plasma-treated (*see* **Note 9**).
7. 1536-well plate, plasma-treated (*see* **Note 9**).
8. Pure ethanol, for washing.
9. 8-channel multichannel pipette.
10. Dust-free, ozone-free sealed room with HEPA filter.
11. 4 °C storage, ideally a cold room.

3 Methods

3.1 Sample Storage

Samples of serum, plasma (EDTA, heparin, citrate, or CTAD), or other biological fluids such as urine, cell culture supernatant, saliva, or cerebrospinal fluid can be collected using standard procedures. Serum samples are allowed to coagulate for 30–60 min before centrifugation. All samples are centrifuged for 10 min at 1500 × *g* at room temperature to remove cells and other particles. The supernatant can be aliquoted in working volumes and kept at −80 °C for short periods of time (less than 1 year). For longer storage, samples are kept in the dry phase of a liquid nitrogen storage system.

3.2 Capture Antibody Printing

1. Mark clean slides with a small black line at the top right corner to identify the printed side, using a permanent marker that does not dissolve in water nor ethanol (*see* **Note 14**). In addition, identify each individual slide in the bottom corners using the same marker. Take care to always handle microarray slides by the sides, never touching the top or bottom with your hands or gloves.
2. Prepare the pin printer for printing by turning on the humidity to 65% at room temperature and cleaning the pins according to the manufacturer's instructions. Make sure all wash buffer containers are full and waste recipients are empty. The humidity in the printing chamber should be stable before capture antibody solutions are placed in the printer. Verify printer alignment (*see* **Note 15**).
3. Prepare capture antibody solutions. Capture antibodies are printed at a concentration of 0.1 mg/mL in the capture antibody printing buffer, and the final concentration of additives is

exactly 2 M betaine and 25% 2,3-butanediol. For example, mix 2 μL of an antibody that has a stock concentration of 1.0 mg/mL to 8 μL of a capture antibody printing solution that contains 2.5 M betaine and 31.3% of 2,3-butanediol. Any significant increase or decrease of the concentration of additives can prevent reproducible printing across all slides due to evaporation or swelling of solutions while printing. If loading a 1536-well plate, preparing 10 μL is sufficient for each individual antibody. If fluorescence signal is too low for all spots for a specific antibody solution, or if spots have streaks in their near vicinity, slightly increase or decrease the concentration accordingly until no streaking is seen and sufficient fluorescence is obtained for all spots (*see* **Note 16**).

4. Load 8 μL of each capture antibody solution into the 1536-well plate using pipette tips that are long and thin (e.g., as is used for loading polyacrylamide gels). Place the bottom of the tip at one of the bottom corner of a well before dispensing the solution slowly; this will ensure that no bubble is formed at the bottom of the well. The source well plate used for the capture antibody solutions is not plasma-treated (*see* **Note 9**).
5. Quickly remove dust from microarray slides using a stream of compressed nitrogen before loading them, as well as the 1536-well plate, into the printer when the relative humidity has reached 65% in the printing chamber. Set up the printing program to print at least three technical replicate spots of each solution per subarray, and 16 identical subarrays on each microarray slide, in locations that fit exactly the 16-well gaskets used. The spacing between spots is at least 200 μm to prevent spots from merging during printing or the subsequent incubation. Make sure that the microarray slides are secured in their position in a reproducible manner (*see* **Note 17**).
6. Print all microarray slides and incubate them for 24 h after printing is finished to allow capture antibodies to fully bind to the surface. Printed spots should be visible on the microarray slides, immediately after printing and also after the 24 h incubation.

3.3 Slide Blocking

1. Clean and assemble gaskets to make them free of dust or chemical residues (*see* **Note 18**).
2. Apply incubated microarray slides onto the gaskets. Mark the top right corner of the slide on the gasket using a small tape, along with the microarray slide number.
3. Wash slides with wash buffer in a squeeze bottle (*see* **Note 19**) by filling the gasket wells halfway with wash buffer, dumping the wash buffer, repeating twice, followed by filling the gasket wells halfway with wash buffer, and leaving on the rotary shaker for 5 min at room temperature and 450 rotations per min (rpm).

4. An entire wash cycle consists in repeating the above **step 3** times in total.
5. After microarray slides are washed, load 80 µL of blocking buffer into each well using an 8-tip multichannel pipette, and incubate on the rotary shaker for 3 h at room temperature, 450 rpm.

3.4 Antigens and Sample Incubation

1. Prepare the antigen standard curve. Antigens are added to dilution buffer as a mixture of low volumes of stock for each antigen before diluting. If the antigen stock concentration is too high to measure at least 1 µL of antigen stock solution into the mixture, then pre-dilute with dilution buffer as necessary. The final mixture containing all antigens to be assayed is diluted 1:2.5 15 times by mixing 66.6 µL of the previous concentration to 100 µL of dilution buffer. Each antigen starting concentration (i.e., its individual concentration in the original mixture) is picked such that 15 serial dilutions cover the entire s-shape of the assay standard curve (*see* Fig. 2). Increase or decrease the starting concentration of an individual antigen in the mixture as needed to shift the resulting s-shaped curve.
2. Retrieve samples from storage and let them thaw for at least 10 min at room temperature or longer at 4 °C. Mix individual samples by pipetting up and down before diluting them. Prepare the samples by diluting them at 1:3 and 1:50 in dilution buffer. For example, mix 31.8 µL of pure serum or plasma to 63.6 µL of dilution buffer to make the 1:3 dilution. Mix 5.4 µL of the 1:3 dilution to 84.6 µL of dilution buffer to make the 1:50 dilution for a sample. Use multiples of these quantities for replicate samples.
3. Dump the blocking buffer from the microarray slides. Knock the remaining liquid from the gaskets by hitting a dust-free surface. Use a dry microfiber cloth (*see* **Note 20**) to wipe the top of the gasket to prevent well-to-well contamination (*see* **Note 21**). Do not leave the microarray slide to dry. Immediately load the samples for one microarray slide before dumping the blocking buffer for the next microarray slide.
4. Place all the microarray slides in a square 24.5 cm^2 petri dish with PDMS at the bottom (*see* **Note 10**). Close the petri dish with its lid, and seal it with two full layers of paraffin film.
5. Incubate overnight (minimum 16 h) at 4 °C on the rotary shaker, at 450 rpm.

3.5 Detection Antibody Printing

1. Prepare the pin printer for printing by turning on the humidity to 65% at room temperature and cleaning the pins according to the manufacturer's instructions. Make sure all wash buffer containers are full and waste recipients are empty. The humidity in

the printing chamber should be stable before both the detection antibody solutions and the microarray slides are placed in the printer. Verify alignment of the printer head (*see* **Notes 15** and **17**).

2. Prepare detection antibody solutions. Detection antibodies are printed at a concentration of 0.01 mg/mL in the detection antibody printing buffer, and the final concentration of additives should be exactly 45% glycerol, 1% BSA, and 0.001% Tween 20. For example, mix 1 μL of an antibody that has a stock concentration of 0.2 mg/mL to 19 μL of a detection antibody solution that contains 47.4% glycerol, 1.053% BSA, and 0.001053% Tween 20. Because the detection antibody solutions are prone to making bubbles when mixing the components by pipetting up and down, make 20 μL even though only 8 μL is loaded onto a 1536-well plate.
3. Load 8 μL of each detection antibody solution into the 1536-well plate using the long and thin pipette tips. Place the bottom of the tip at one of the bottom corner of a well before dispensing the solution; this will ensure that no bubble is formed at the bottom of the well. The source well plate used for the detection antibody solutions is plasma-treated for proper loading without bubbles (*see* **Note 9**).
4. Incubate the slides at room temperature for 30 min before removing the paraffin film from the large square petri dish. This allows the slides to be at room temperature for further processing.
5. Wash microarray slides twice with washing buffer by performing two times Subheading 3.3, **step 3**.
6. Rinse microarray slides rapidly in their gasket three times with PBS without Tween 20. Dump the PBS from the gaskets. Add 80 μL of slide rinsing solution to the gaskets and incubate 5 min at room temperature on the rotary shaker at 450 rpm.
7. Dump the slide rinsing solution from the gaskets. Remove the gasket from the microarray slide and rinse the top side of the slide with more slide rinsing solution with a squeeze bottle.
8. Immediately dry the slide under a forceful, perpendicular stream of compressed nitrogen. This step is done to ensure that a small consistent film of trehalose is left on the surface to protect the complexed capture antibodies and antigens spots during the detection printing step (*see* **Note 22**). Wash and dry a single microarray slide at a time.
9. Print all slides in the same order that capture antibody solutions were printed. Detection antibodies are printed directly atop their corresponding capture antibody solutions. Incubate the slides for 24 h after printing is finished to allow detection antibodies to fully bind to the targets. Printed spots should be

visible on the microarray slides, immediately after printing and also after the incubation.

10. Clean and assemble gaskets (*see* **Note 18**).

3.6 Streptavidin Incubation

1. Apply incubated microarray slides onto clean gaskets. Mark the top right corner of the slide on the gasket using a small tape, along with the microarray slide number.
2. Wash microarray slides by performing Subheading 3.3, **step 4**.
3. Prepare a solution of 0.5 μg/mL of streptavidin-AF647 in blocking buffer and apply 80 μL in each well using a multichannel pipette. To apply to several slides, apply in the same order that washes will be performed, waiting 15–20 s in between each slide (*see* **Note 23**).
4. Incubate for 30 min at room temperature on the rotary shaker at 450 rpm. The microarray slides are kept in the dark at this point because of the presence of a fluorescence marker.
5. Wash microarray slides again by performing Subheading 3.3, **step 4**.
6. Remove the gasket and rinse both sides of the microarray slide with distilled water from a squeeze bottle or a gentle flow from a distilled water tap.
7. Immediately dry the microarray slide under a forceful stream of compressed nitrogen that is parallel to the small axis of the microarray slide, in order to remove all droplets of water. Rinse and dry a single microarray slide at a time.

3.7 Fluorescence Scanning

1. If using a fluorescence scanner that scans through the back of the slide, polish the back of all microarray slides using a dry microfiber cloth (*see* **Note 20**). Remove the microfiber dust particles using a stream of compressed nitrogen.
2. Turn on the scanner and allow enough time for the lasers to warm up. Refer to the manufacturer's instructions.
3. Set the photomultiplier gain to an appropriate number. This gain should be the highest that leads to no saturated pixel (*see* **Note 24**).
4. Scan all the microarray slides as quickly as possible after the experiment is done, and in as little time as possible (*see* **Note 25**).
5. Save all images as TIFF images. If compressing images, make sure that the compression algorithm is loss-less (*see* **Note 26**).

3.8 Data Extraction and Analysis

1. Verify that none of the microarray slide pictures have saturated pixels (*see* **Note 24**).
2. Align grids onto the TIFF images to extract all technical replicate spots on all subarrays and on all microarray slides. Grid spot size should be at least half of the size of the actual spots.

For spots of approximately 100 μm in size, we use a grid spot size of 60 μm diameter.

3. Extract the data by outputting the raw fluorescent intensity. There should be no negative value in this data.
4. Log-transform the data by calculating the log10 of each individual outputted value.
5. Perform outlier removal using Peirce's criterion or Grubbs' test (*see* **Note 27**). Calculate the mean and standard deviation of technical replicates to obtain a log-transformed raw fluorescence intensity value for a specific assay in a given sample, sample replicate, blank, or standard curve dilution. Note that the value of the concentration of antigen should also be log-10 transformed.
6. Perform a four-parameter logistical curve fit on each individual assay's standard curve using the values obtained in Subheading 3.8, **step 5**. It is important to use the standard deviations obtained for the standard curve dilutions to obtain a more accurate curve fit.
7. In order to calculate the limit of detection for a curve fit, calculate the mean and standard deviation for all blank values (obtained in Subheading 3.8 **step 5**) for a given assay within the experiment. Interpolate the value calculated by taking the mean + three times the standard deviation into the curve fit for the given assay. The concentration obtained is the log10 value of the lowest quantity of antigen that can be quantified using the assay.
8. In order to calculate the reproducibility for a given assay, interpolate all individual values from the replicate sample. Divide the standard deviation of all the quantities obtained by the mean of all those quantities. The value obtained is the coefficient of variation (%CV) for this assay.
9. Interpolate the values obtained in Subheading 3.8 **step 5** for all samples in the curve fit for a given assay to obtain the quantities measured in that sample. If the values obtained at dilution 1:3 cannot be quantified because they are above the maximum value from the standard curve, quantify the samples in the 1:50 dilution. Multiply the quantities obtained by the dilution in order to infer the concentration in the original sample (*see* **Note 28**).

4 Notes

1. The volume and concentration of viscous liquids to be measured are critical for this application. Therefore, in order to measure viscous liquids such as glycerol, Tween 20, or 2,3-butanediol, we recommend using a viscous liquid pipette which uses a piston to displace the viscous liquid rather than air.

2. The 1× PBS solution used throughout the protocol should be free of small particles that are often autofluorescent and can easily bind to the microarray surface or sometimes lead to missing spots if particles clog the silicon quill pins. For this reason we recommend buying a 10× PBS stock solution that has been prefiltered by the manufacturer.
3. Antibodies are normally supplied as liquid in a PBS buffer base, or freeze-dried, in which case they are reconstituted in PBS. The presence of up to 5% of glycerol or other cryopreservative chemicals did not affect our experiments. Antibodies supplied in a different buffer than PBS might also be used but should be individually tested. For calculations, we made the assumption that antibody stock solutions are the equivalent of 1× PBS.
4. At a concentration of 2 M betaine and 25% 2,3-butanediol, the capture printing buffer can safely be kept at 4 °C; however, at higher concentrations of betaine, this chemical can precipitate out of solution when kept at 4 °C. For this reason, it is best to keep the capture printing buffer at room temperature.
5. For the same reasons as listed (*see* **Note 2**) and because BSA has particles that can lead to missing spots, the detection printing buffers are filtered with a sterile 0.45 μm filter prior to mixing with individual stock detection antibody solutions. We do not recommend filtering the solutions once antibodies have been added, because the volumes are too small, in the range of 10–20 μL. However, 1 mL of the detection printing buffer containing glycerol, BSA, and Tween 20 can be filtered using a 3 mm luer-lock filter and a 1 mL sterile luer-lock syringe. A 1.5″ 18 G hypodermic luer-lock needle can be fitted to the 1 mL syringe to pick up the detection printing buffer before filtering. Dispose of the needle in a sharps container according to local waste management regulations.
6. The capture antibody printing buffer was optimized to work well on a 2D reactive aldehyde surface. While other surfaces with higher antibody-binding capacity were identified (e.g., high-capacity epoxy surfaces), antibody-target binding was strongest on the reactive aldehyde surface suggesting that antibodies were less denatured or less crowded.
7. Xenobind microarray slides can be cleaned by sonicating ten slides at a time in distilled water, followed by a quick rinse with distilled water and then pure ethanol using squeeze bottles, immediately followed by drying with a stream of compressed nitrogen. It's very important not to let the ethanol air-dry on the microarray slides, as it can leave visible chemical smudges.
8. The main requirements for the microarray printer are spot absolute positional accuracy in both the printing and in positioning

slides on a deck, the compatibility with the high-viscosity printing buffers, and printing speed. Slower (several hours) printing leads to degradation of assay signal for spots that are printed earlier and therefore gives a worse reproducibility performance for the assay.

9. Excessive plasma treatment of microplates can lead to cross-contamination between wells as liquid films form on the surface, and the optimal plasma processing time should be carefully verified. We observed that when loading printing buffers containing betaine and 2,3-butanediol on a 1536-well plate, contamination between adjacent wells occurred readily. Therefore, the 1536-well plate used for capture antibody solutions is not plasma-treated. The additives present in the detection printing buffer (glycerol and BSA) do not however flow easily onto the plastic surface and therefore require a short 10 s plasma treatment in order to easily be loaded into the 1536-well plate.
10. We prepared a flat PDMS surface that we laid on top of the rotary shaker surface by curing approximately 200 mL of PDMS in a 24.5 cm^2 square petri dish normally used for cell culture. Cured PDMS (Sylgard® 184, Corning) is prepared by mixing a ratio of 1:10 of curing agent to the polymer base, mixing thoroughly by hand before pouring into the petri dish. The dish is allowed to stand for 30 min to allow bubbles to escape and is then cured in a 60 °C oven for a minimum of 8 h. The PDMS can then be removed from the petri dish if needed. This step is optional if multiple microarray slide gaskets can be secured at once to the rotary shaker surface.
11. The presence of more than 5% glycerol in the capture antibody printing solution can significantly hinder binding of the antibodies to the surface.
12. If all detection antibodies within an assay are biotinylated, then all can be detected using a fluorescently labeled streptavidin. Alternatively, detection antibodies can be directly labeled with a fluorescent molecule. If detection antibodies are not labeled, they should (1) have been made in an animal species different than that of the capture antibody and (2) be probed with a secondary antibody made in the same species as that of the capture antibody in order to avoid cross-reactivity to the capture antibody. For example, if the capture antibody of an antibody pair is a mouse IgG and the detection antibody is a goat IgG, then the labeled secondary antibody should be a mouse anti-goat IgG. However all matched antibody pairs should be compatible with the labeled secondary antibody within a subarray. Any other combination (such as a capture antibody that is a goat IgG) will lead to false-positive signals due to the labeling of the capture antibody rather than the detection antibody for this assay.

13. Proteins that are obtained by purification from animal or human samples (e.g., cancer related and other proteins that have particular glycosylation patterns that cannot readily be reproduced by recombinant protein synthesis methods) often contain impurities in the form of unrelated proteins, some of which may be targets in the same subarray. This can lead to significant assay signal and cross-reactivity in the standard curves of other targets even in the absence of those targets' specific recombinant antigens [24].
14. Marker pens that dissolve in either water or organic solvents readily leak onto the slide and may smear the surface with highly fluorescent chemicals. We suggest using the solvent-resistant permanent marker from Thermo Fisher Scientific (laboratory marking pen, ref. #2000) which doesn't leak or smear using this protocol.
15. Alignment of printing between the capture and the detection steps is critical for the success of this assay. For some printers, it is as simple as avoiding re-initialization of the printer between the two printing rounds. For others which suffer from printing position drift with time, a method for calibrating the printing head position is necessary. A microscope calibration slide can be used for this in the following way. First, wash the microscope calibration slide with pure ethanol using a squeeze bottle, and dry it with a stream of compressed nitrogen. Before the capture antibody printing step, quickly print a subarray on top of the calibration area, using a solution of 50% glycerol in PBS. View the printed calibration spots under a microscope and make note of the position of a specific spot which lies within the calibration area, compared to the center of the slide. Before performing the detection antibody printing, redo this procedure. Adjust printing margins to compensate for any misalignment that occurred between the two printing rounds.
16. Individual capture antibodies will bind to the surface with different affinities and at different rates. To increase the amount of capture antibody bound on the surface, increase the concentration of that antibody in its printing solution while keeping the concentration of additives (betaine, 2,3-butanediol) the same. Conversely, if fluorescence signals are too strong or streaking is observed around a specific capture antibody, decrease its concentration in the printing solution while keeping the concentration of additives the same. The streaking is due to the surface being saturated by the capture antibody solution and the presence of unbound capture antibodies which quickly bind to the surface during the initial washing, prior to blocking the microarray slide surface.
17. The proper alignment of detection antibody spots onto their corresponding capture antibody spots requires a very precise

positioning of the microarray slides within a slide deck and the accurate alignment of the printing head prior to both printing rounds (*see* **Note 15**). Therefore it is important that slides can be positioned accurately and reproducibly within the slide deck (with less than 10 μm variability). A spring-clamped slide deck and a proper manual technique for loading slides are essential and can achieve this reproducibility.

18. Gaskets that have dust or a very hydrophilic surface can lead to contamination between wells because of an incomplete seal. In order to thoroughly wash gaskets, our method is to first rinse all parts in a mild soap solution, then rinse many times with distilled water, and finally dip for 10–15 s with shaking in pure ethanol. The gasket parts are then dried with a stream of compressed nitrogen, before being assembled and ready for use. They are then stored in a closed petri dish that prevents dust from falling on them.
19. It is important to wash the microarray slides with wash buffer with enough force, which is why the wash buffer is placed in a squeeze bottle. This helps prevent fluorescent streaks on the microarray surface. If streaking of certain spots is still seen in spite of proper washing, slightly decrease the concentration of the capture antibody in the solution for the streaking spots (*see* **Note 16**).
20. Because paper generates a lot of fluorescent dust particles, even low-dust clean-room paper is avoided when wiping microarray slides or surfaces within the working environment. Instead, we use a microfiber cloth of the like that is used to clean lenses or eyeglasses. Particles generated by microfiber cloth are not fluorescent, and they can easily be removed with a stream of compressed nitrogen.
21. If a small film of liquid is present at the top of the gasket, it can lead to contamination between wells when samples and antigens are shaken. Do not cover the gasket unless this cover has a liquid-tight seal. Any seal that is not tight will also lead to contamination between wells and falsify results.
22. Most antibodies and antigens will degrade at varying speeds after they are dried on the surface. Therefore, spots should be protected with 5% trehalose which was found to slow down degradation and in many cases even prevents it. The actual quantity of trehalose left on the surface after drying with the stream of compressed nitrogen is dependent on the drying method. A stream that is strong, and head-on, was found best to achieve an even surface. If the detection spots spread and lose their shape on the surface during detection antibody printing, then there is too much trehalose on the surface. It is then recommended to use a stronger stream of nitrogen and to hold

the nozzle close to the microarray slide (2–3 cm), although using a slightly less concentrated solution of trehalose is also possible. However, a lower concentration of trehalose can lead to more degradation of antibodies and antigens.

23. Binding of streptavidin to the detection antibodies is not limited by the concentration of streptavidin used in these experiments. However, the streptavidin signal will increase with an increase in incubation time. In order to maximize reproducibility across slides, it is important to incubate the streptavidin solution for exactly the same amount of time per slide. Therefore, if it takes 15 s to wash a single slide, apply the streptavidin solution to each slide with a 15 s delay between each slide. This will ensure that all slides have exactly the same streptavidin incubation time.
24. Saturated pixels are pixels that have the maximum value (or very close to the maximum value of 65,535 in a 16-bit system) and are in fact too high to be recorded by the scanner at the gain used. This leads to a loss of data and falsification of results. If a significant number of pixels are saturated for a given gain, all slides of the experiment should be re-scanned with a lower gain.
25. Fluorophores in a dry state slowly degrade in the presence of air (humidity, oxygen, heat), even in a sealed room where ozone is actively removed. For this reason it is important to scan all the slides in an experiment as quickly as possible to minimize the effect of this degradation on reproducibility. If it is necessary to scan slides multiples times at different gains, then first scan all slides at an initial gain, and then scan all of them at a second higher or lower gain depending on the results, rather than scanning each slide at multiple gains. This will maximize reproducibility between microarray slides.
26. When saving fluorescence images of microarray slides, it is very important to save the image data with high dynamic range (16 bit or 20 bit) while avoiding image compression. Formats such as GIF or JPEG may only accommodate 8-bit images and compress the data with information loss that will likely lead to false results. Image formats such as TIFF accommodate 16-bit and 20-bit images and also allow compression using loss-less LZW algorithms for example.
27. In instances where the number n of technical replicates is very low, the mean and standard deviation of the group of technical replicates is very sensitive to the presence of outliers. Therefore a test or method for removing outliers that is efficient at low n is required. A minimum of three technical replicates is required for proper statistics. Grubbs' test performs well with $n = 3$ or more, while Peirce's criterion only works with $n = 4$ or more.

28. Protein quantification is subject to matrix effects that limit the comparison of quantities of a target obtained in samples within a single dilution. Because of the matrix effects, the concentration values are not considered to be absolute, and a target concentration inferred from the 1:3 dilution may be lower than the one inferred from the 1:50 dilution. Different targets are subject to different matrix effects depending on the sample type and the dilution.

Acknowledgments

We wish to thank the National Science and Engineering Research Council (NSERC) of Canada, the Canadian Cancer Society (CCS), and the Canadian Institute of Health Research (CIHR) for funding. D.J. holds a Canada Research Chair (CRC) in Micro- and Nanobioengineering.

References

1. Pla-Roca M, Leulmi RF, Tourekhanova S, Bergeron S, Laforte V, Moreau E, Gosline SJ, Bertos N, Hallett M, Park M, Juncker D (2012) Antibody colocalization microarray: a scalable technology for multiplex protein analysis in complex samples. Mol Cell Proteomics 11(4):M111 011460. doi:10.1074/mcp.M111.011460
2. Juncker D, Bergeron S, Laforte V, Li H (2014) Cross-reactivity in antibody microarrays and multiplexed sandwich assays: shedding light on the dark side of multiplexing. Curr Opin Chem Biol 18:29–37. doi:10.1016/j.cbpa.2013.11.012
3. Laforte V, Olanrewaju A, Juncker D (2013) Low-cost, high liquid volume silicon quill pins for robust and reproducible printing of antibody microarrays. In: MicroTAS: miniaturized systems for chemistry and life sciences, Freiburg, Germany, 27–31 October 2013. Chemical and Biological Microsystems Society (CBMS), pp. 485–487
4. Bergeron S, Laforte V, Lo PS, Li H, Juncker D (2015) Evaluating mixtures of 14 hygroscopic additives to improve antibody microarray performance. Anal Bioanal Chem 407(28):8451–8462. doi:10.1007/s00216-015-8992-8
5. Frampton JP, White JB, Simon AB, Tsuei M, Paczesny S, Takayama S (2014) Aqueous two-phase system patterning of detection antibody solutions for cross-reaction-free multiplex ELISA. Sci Rep 4:4878. doi:10.1038/srep04878
6. Assarsson E, Lundberg M, Holmquist G, Bjorkesten J, Thorsen SB, Ekman D, Eriksson A, Rennel Dickens E, Ohlsson S, Edfeldt G, Andersson AC, Lindstedt P, Stenvang J, Gullberg M, Fredriksson S (2014) Homogenous 96-plex PEA immunoassay exhibiting high sensitivity, specificity, and excellent scalability. PLoS One 9(4):e95192. doi:10.1371/journal.pone.0095192
7. Fredriksson S, Dixon W, Ji H, Koong AC, Mindrinos M, Davis RW (2007) Multiplexed protein detection by proximity ligation for cancer biomarker validation. Nat Methods 4(4):327–329. doi:10.1038/nmeth1020
8. Fredriksson S, Gullberg M, Jarvius J, Olsson C, Pietras K, Gústafsdóttir SM, Östman A, Landegren U (2002) Protein detection using proximity-dependent DNA ligation assays. Nat Biotechnol 20:473–477
9. Aldo P, Marusov G, Svancara D, David J, Mor G (2016) Simple plex(TM): a novel multi-analyte, automated microfluidic immunoassay platform for the detection of human and mouse cytokines and chemokines. Am J Reprod Immunol 75(6):678–693. doi:10.1111/aji.12512
10. Blank K, Lankenau A, Mai T, Schiffmann S, Gilbert I, Hirler S, Albrecht C, Benoit M, Gaub HE, Clausen-Schaumann H (2004) Double-chip protein arrays: force-based multiplex sandwich immunoassays with increased specificity. Anal Bioanal Chem 379(7–8):974–981. doi:10.1007/s00216-004-2607-0

11. Christendat D, Yee A, Dharamsi A, Kluger Y, Savchenko A, Cort JR, Booth V, Mackereth CD, Saridakis V, Ekiel I, Kozlov G, Maxwell KL, Wu N, McIntosh LP, Gehring K, Kennedy MA, Davidson AR, Pai EF, Gerstein M, Edwards AM, Arrowsmith CH (2000) Structural proteomics of an archeon. Nat Struct Biol 7(10):903–909
12. Kusnezow W, Jacob A, Walijew A, Diehl F, Hoheisel JD (2003) Antibody microarrays: an evaluation of production parameters. Proteomics 3:254–264
13. Berlier JE, Rothe A, Buller G, Bradford J, Gray DR, Filanoski BJ, Telford WG, Yue S, Liu J, Cheung C-Y, Chang W, Hirsch JD, Beechem JM, Haugland RP, Haugland RP (2003) Quantitative comparison of long-wavelength alexa fluor dyes to Cy dyes: fluorescence of the dyes and their bioconjugates. J Histochem Cytochem 51(12):1699–1712
14. Byerly S, Sundin K, Raja R, Stanchfield J, Bejjani BA, Shaffer LG (2009) Effects of ozone exposure during microarray posthybridization washes and scanning. J Mol Diagn 11(6):590–597. doi:10.2353/jmoldx.2009.090009
15. Cox WG, Beaudet MP, Agnew JY, Ruth JL (2004) Possible sources of dye-related signal correlation bias in two-color DNA microarray assays. Anal Biochem 331(2):243–254. doi:10.1016/j.ab.2004.05.010
16. Anderson GP, Nerurkar NL (2002) Improved fluoroimmunoassays using the dye alexa fluor 647 with the RAPTOR, a fiber optic biosensor. J Immunol Methods 271:17–24
17. Becker W (2012) Fluorescence lifetime imaging–techniquesandapplications.JMicrosc247(2):119–136. doi:10.1111/j.1365-2818.2012.03618.x
18. Li H, Bergeron S, Juncker D (2012) Microarray-to-microarray transfer of reagents by snapping of two chips for cross-reactivity-free multiplex immunoassays. Anal Chem 84(11):4776–4783. doi:10.1021/ac3003177
19. Li H, Munzar JD, Ng A, Juncker D (2015) A versatile snap chip for high-density sub-nanoliter chip-to-chip reagent transfer. Sci Rep 5:11688. doi:10.1038/srep11688
20. Sittampalam GS, Coussens NP, Nelson H, Arkin M, Auld D, Bejcek B, Glicksman M, Inglese J, Iversen PW, Li Z, McGee J, McManus O, Minor L, Napper A, Peltier JM, Riss T, Trask OJ, Weidner J (2016) Assay guidance manual. Eli Lilly & Company and the National Center for Advancing Translational Sciences, Bethesda, MD
21. Wilson JJ, Burgess R, Mao YQ, Luo S, Tang H, Jones VS, Weisheng B, Huang RY, Chen X, Huang RP (2015) Antibody arrays in biomarker discovery. Adv Clin Chem 69:255–324. doi:10.1016/bs.acc.2015.01.002
22. Li H, Bergeron S, Annis MG, Siegel PM, Juncker D (2015) Serial analysis of 38 proteins during the progression of human breast cancer tumor in mice using an antibody colocalization microarray. Mol Cell Proteomics 14(4):1024–1037. 10.1074/
23. Wingren C, Borrebaeck CA (2009) Antibody-based microarrays. Methods Mol Biol 509:57–84. doi:10.1007/978-1-59745-372-1_5
24. Gonzalez RM, Seurynck-Servoss SL, Crowley SA, Brown M, Ommen GS, Hayes DF, Zangar RC (2008) Development and validation of sandwhich ELISA microarrays with minimal assay interference. J Proteome Res 7:2406–2414