Protein Array
Assay System

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Introduction

Nitrocellulose is the predominant immobilization surface for protein microarray applications due to its many functional advantages and, most importantly, its high protein binding capacity. As a substrate for protein microarrays, porous nitrocellulose film slides (Film-Slides) have practical performance advantages over functionalized glass and other non-porous 2D surfaces, including higher binding capacity and retention of native protein conformation and biological activity (1, 2). To obtain the full advantage of this substrate for microarray applications requires the use of optimized reagents designed for use with this surface.

The Protein Array Assay System includes the key reagents necessary for maximal use of our ONCYTE® nitrocellulose film slides and can be used in various protein microarray applications such as antibody and antigen capture arrays (see Figure 1).

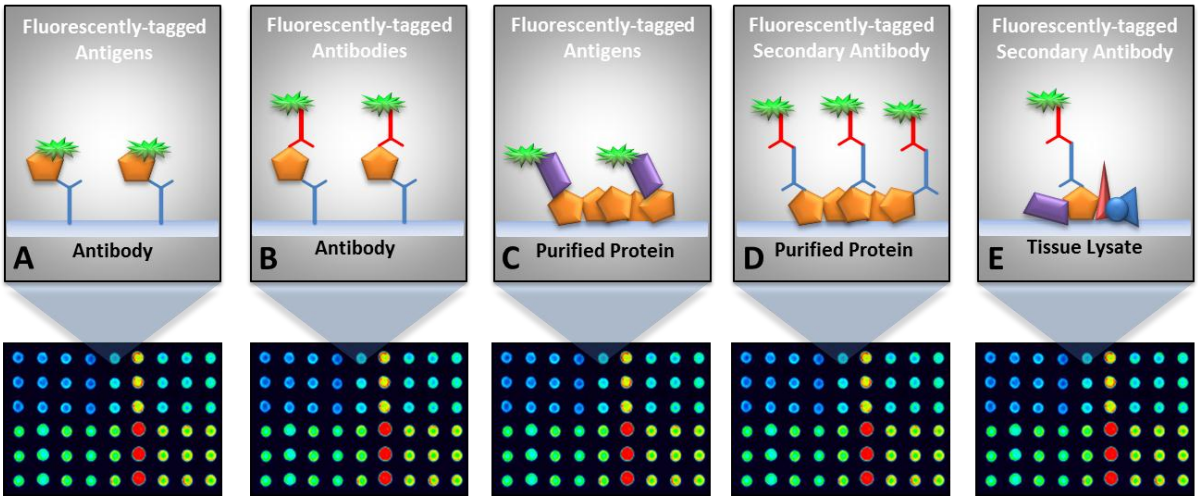


Figure 1. Commonly used protein microarrays configurations. (A) Antibody arrays can be used to capture specific antigens which are directly labeled with a hapten or (B) used in sandwich ELISA-like assays. (C) Purified or recombinant proteins can be arrayed to study protein-protein interaction or (D) to probe serum samples for antibodies. (E) Reverse-Phase Protein Arrays (RPPA) are used to profile dozens or hundreds of arrayed samples (e.g. cell or tissue lysates) for the presence of selected antigens.

For performing protein microarray assays, care must be taken to provide a stable environment for proteins before and during arraying; during microarray storage; and during immunoassays. In this Kit, we provide our GBL Protein Arraying Buffer, Super G Plus Preservation Buffer, and Super G Blocking Buffer that were optimized from protein microarrays with nitrocellulose.

Nitrocellulose films have a long history of use in Western, Northern, and dot- (immuno-) blots for reliable immobilization and capture of biomolecules. They have also been used in the manufacture of lateral flow immunoassays, such as pregnancy tests, in the diagnostic industry. The 3-dimensional structure of ONCYTE® porous nitrocellulose slides show up to 500 times the binding capacity of conventional 2-dimensional surfaces and hydrogel surfaces. To augment this capacity, we have designed our Protein Arraying Buffer to give optimal protein binding to nitrocellulose and for

producing consistent spot size and morphology on this surface. In addition, this buffer was designed to minimize protein aggregation in protein stocks during printing and during storage for optimal use of precious samples.

ONCYTE® Film-slides provide a very broad linear dynamic range for detection, up to 7 orders of magnitude. This is due to their high binding capacity and low fluorescence backgrounds which are important for the development of quantitative protein microarray assays for research and diagnostic applications. To maintain low backgrounds for optimal signal-to-noise and low LOD, we have designed Super G Blocking Buffer to minimize non-specific assay background commonly observed with nitrocellulose blocked with ineffective reagents.

The advantages of nitrocellulose also stem from the nature of the protein-to-matrix interaction, which allows for retention of molecular structure and function of the bound material. Binding of biomolecules to nitrocellulose occurs through combined weak intermolecular forces, probably dominated by hydrophobic and van der Waals forces (1 - 3). To enhance this functional advantage, we have designed Super G Plus Preservation Buffer to treat arrayed proteins for effective retention of immunological recognition during prolonged microarray storage.

Taken together, the optimized reagents in this kit are intended to work with our ONCYTE Films and with our ProPlate® Microarray Incubation Chambers to provide excellent protein microarray results using forward-phase microarray methods. For reverse phase protein arrays (4, 5) we offer a separate reagent RPPA Kit (Cat. #PD105905) designed for optimal performance with that application.

References:

1. Van Oss CJ, Good RJ, Chaudhury MK. Mechanism of DNA (Southern) and protein (Western) blotting on cellulose nitrate and other membranes. J Chromatography. 1987 Mar 27;391(1):53-65.
2. Kingsmore SF. Multiplexed protein measurement: technologies and applications of protein and antibody arrays. Nat Rev Drug Discovery. 2006 Apr;5(4):310-20. Review.
3. Tang Y, *et al*. (2003). Blood genomic expression profile for neuronal injury. J Cereb Blood Flow Metab.: 23(3):310-9.
4. Tibes R. *et al.* (2006). Reverse phase protein array: validation of a novel proteomic technology and utility for analysis of primary leukemia specimens and hematopoietic stem cells. Mol. Cancer Ther. 5:2512–21.
5. Sevecka M. *et al.* (2011). Lysate microarrays enable high-throughput, quantitative investigations of cellular signaling. Mol. Cell Proteomics Apr;10(4):M110.005363.

Intended Use and Storage

Intended Use

This Protein Array Assay System is designed to provide users with reagents necessary for the preparation and use of protein microarrays derived from various sources including purified antibodies and antigens. The reagents in this system supplement the use of ONCYTE® porous nitrocellulose film slides and are provided in quantities for processing up to 20 film slides with our ProPlate Microarray Incubation Chambers.

Microarray Preparation: This system provides our Protein Arraying Buffer designed for use with porous nitrocellulose. The arraying buffer is supplied as a 10x solution and ready to use once diluted to the 1x working concentration. The buffer may be supplemented with inhibitors (not included) if necessary. Microarray printing may be performed directly after protein reconstitution or after storage of the protein samples.

Protein Preservation: Super G Plus Preservation Buffer is provided for the treatment of printed nitrocellulose film slides prior to storage.

Nitrocellulose Blocking: Super G Blocking Buffer is provided for the treatment of printed nitrocellulose film slides prior to and during the immunoassay.

Microarray Assay: Reagents are provided for the processing of printed nitrocellulose film slides, including blocking of the nitrocellulose film with Super G and wash buffers.

For Research Use Only

Storage Conditions

Upon receipt, the kit contents should be placed at 4°C for storage. All reagents may be stored at the recommended temperature for up to 12 months. Some reagents may precipitate during shipping and/or after storage. Mix contents well and allow them to come to room temperature before using. For buffers *other than Super G or Super G Plus* which may have precipitated, place vials into a warm water bath with occasional mixing until contents are fully re-dissolved (usually within 10 minutes). *Do not heat Super G or Super G Plus reagents.*

Kit Contents

Store at 4°C

Reagent Box 1 and 2
3× Super G Blocking Buffer (100 ml)
3 × Super G Plus Preservation Buffer (100 ml)
PBST (100 ml)*
PBS (100 ml)*
Protein Array Buffer (10 ml)
Spot Tuning Solution (1.25 ml)

Notes:

*Due to their high concentrations, these buffers may be most likely to precipitate during shipping/storage. If necessary, follow general recommendations above to re-dissolve buffers prior to using. Once diluted to a 1x working concentration, buffer contents will not precipitate. *Do not heat Super G or Super G Plus, keep at 4° C.*

Materials and Reagents Not Provided in this Kit

Materials
ONCYTE® porous nitrocellulose film slides
ProPlate® Microarray Incubation Chambers

Arraying Reagents
Protease and Phosphatase Inhibitors (see Appendix for recommendations)
Sterile, protease-free water (Sigma W4502, or equivalent)

Assay Reagents
Primary and Secondary Antibodies
Sterile, protease-free water (Sigma W4502, or equivalent)
BSA (Sigma A4737, or equivalent)

Protocols

Protein Reconstitution:

GBL Protein Arraying Buffer is supplied as a 10x concentrated solution and requires dilution to a 1x working concentration with protease-free water prior to use. This arraying buffer was formulated for use with porous nitrocellulose, to enhance protein binding, minimize protein aggregation, and promote proper protein folding. For optimal results, proteins should be reconstituted in 1x GBL Protein Arraying Buffer directly from lyophilized proteins. Mix by gentle pipetting on ice to and allow proteins to fully solubilize for at least 1 hour.

Alternatively, if protein stocks are already suspended in PBS and are not available lyophilized, stocks may be mixed 1:1 with 2x GBL Protein Arraying Buffer. For protein stocks already reconstituted in other spotting buffers, results may vary depending on the buffer components and may produce suboptimal results even when mixed 1:1 with 2x GBL Protein Arraying Buffer.

Depending on needs and condition of protein preparation (e.g. crude cell extracts), it is possible to also supplement the 1x GBL Arraying Buffer with protease or phosphatase inhibitors, if desired (see Appendix). Upon protein reconstitution, protein stocks should be kept on ice until spotting and/or storage. Minimize freeze/thaw cycles by freezing aliquots of protein stock solutions in volumes appropriate for individual use.

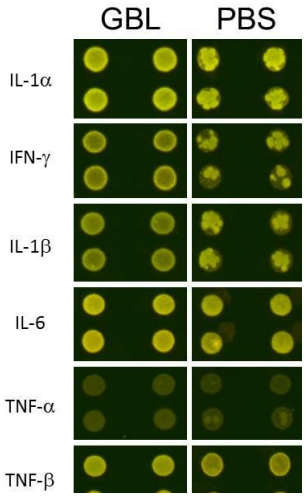


Figure 2. Effect of GBL Protein Arraying Buffer on spot signal and morphology. Microarray image of various immobilized cytokine proteins spotted in parallel on ONCYTE AVID porous nitrocellulose slides. Cytokines were reconstituted in 1x PBS or GBL Protein Arraying Buffer from freshly reconstituted 2x stock solutions to ensure equivalent spotting concentrations (1 mg/ml spotting concentration). Slides were assayed with primary antibodies directed against their antigen followed by secondary labeling with Alexa-fluor 555 and 647 antibodies.

Typical protein spotting concentrations may vary widely between microarray applications and for binding surfaces. For example, protein concentrations for microarrays produced on planar surfaces are typically 0.1 to 0.5 mg/ml. For a 400 pL microarray deposition volume, these concentrations saturate binding sites for most surface types and higher concentrations would not provide additional binding. ONCYTE porous nitrocellulose has a very high protein binding capacity (40 µg/cm^2) which far exceeds that of other microarray surfaces and, thus, allows spotting concentrations up to approximately 50 mg/ml (dependent on deposition volume) before saturation of binding sites is observed. Reconstituting proteins to a working concentration of 0.5 to 2 mg/ml is recommended, but higher concentrations may yield improved results for some applications.

Spot size with GBL Protein Arraying Buffer on porous nitrocellulose should be assessed for individual arraying systems but typical diameters for single 400 pL depositions are 130 - 140 µm for AVID, NOVA, and SuperNOVA films. Spot size is determined by various factors including the microarray pen type, volume deposited, and arraying conditions (temperature

and humidity). For applications requiring larger spot sizes, there are multiple methods available to increase the diameter further. One option is to perform multiple sequential depositions per microarray spot during arraying. A second option is to supplement GBL Protein Arraying Buffer with the supplied Spot Tuning Solution. This buffer was designed to provide increased spot size of up to 150% over the non-supplemented buffer. The values in Table 1 provide a guide for spot size increase expected per volume of Spot Tuning Solution added to 10 ml GBL Protein Arraying Buffer.

Table 1: Spot Tuning Solution Spot Diameter Guide

	AVID		**NOVA**		**SuperNOVA**	
Diameter Increase	Spot Diameter (µm)**	Added Vol. (µl)*	Spot Diameter (µm)**	Added Vol. (µl)*	Spot Diameter (µm)**	Added Vol. (µl)*
-	125	-	110	-	125	-
25%	155	20	135	10	160	15
50%	185	55	165	25	190	30
75%	220	115	190	40	220	50
100%	-	-	220	60	250	70
125%	-	-	245	85	280	120
150%	-	-	270	125	-	-

Notes:

*Volume of Spot Tuning Solution to add to 10 ml 1x GBL Protein Arraying Buffer.

**Estimated spot diameter for a 300 pL deposition volume.

Microarray Printing and Storage

Transfer appropriate volumes of protein stocks to 96- or 384-well spotting plates compatible with your arraying system. Keep microarray plates cooled and sealed until spotting run commences to prevent protein degradation and evaporation. If possible, use a chilled plate holder to minimize sample evaporation and minimize protein degradation during arraying. GBL Protein Array Buffer was formulated to work under variable spotting humidity conditions with minimal variation in spot morphology. For optimal results, when possible, spotter humidity should be maintained at 50 - 60%.

General Recommendations for Printing and Handling of Slides:

- Do not pre-treat ONCYTE® porous nitrocellulose film slides prior to spotting.
- Humidity control (if available) set to 50 – 60%.
- Sample plate lids can be utilized during spotting (if possible with your spotter) to minimize sample evaporation.

- Sample cooling with a chilled plate holder is recommended (if possible with your spotter) to minimize sample evaporation and minimize protein degradation.
- Pre-incubate slides in the spotter at desired humidity for 1 hour prior to printing.
- Allow printed arrays to sit in humidified spotter for 1 hour post-printing.
- Store slides in the slide box provided (light blue GBL box). Other slide boxes and trays may contain materials which can off-gas and significantly impact microarray results.
- Store slides overnight at 4°C to maximize protein binding before use. Post-spotting incubation may vary for different proteins and should be tested for specific applications.
- For long-term storage, store slides desiccated at -20°C or -80°C.
- For optimal results, slides should be treated with the supplied Super G Plus Preservation Buffer prior to storage (see next section).

Super G Plus Blocking and Preservation Protocol

Super G Plus Preservation Buffer is a non-protein based reagent designed to block non-specific protein binding on porous nitrocellulose substrates and preserve protein immunoreactivity over prolonged storage of printed microarrays. It is supplied as a 1X solution, ready to use out of the bottle, for microarray applications.

Cover the slide surface to be hybridized completely with Super G Plus Preservation Buffer. For best results, submerge the slide completely in preservation buffer. This may be performed with small volumes in chambers such as ProPlates, or may be performed in bulk using glass staining jars. Enough Super G Plus is supplied in this kit (300 ml total) to incubate 20 slides in a glass staining jar if desired. No agitation is recommended during blocking. It is recommended to incubate the slide for 1 hour in Super G Plus before washing, though incubation time should be optimized for your specific application.

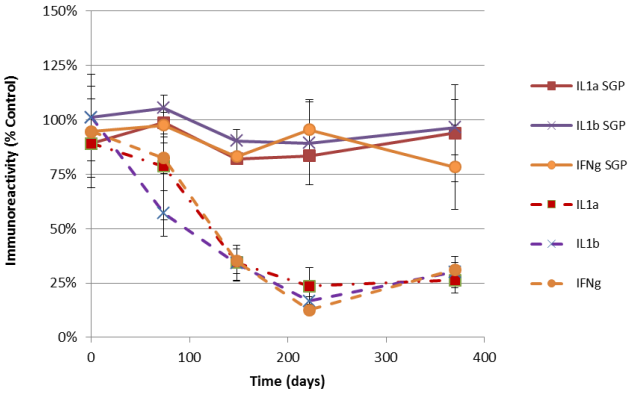


Figure 3. Effect of Super G Plus Preservation Buffer on cytokine immunoreactivity after prolonged storage. Storage was performed under accelerated conditions at 38°C for up to 35 days equivalent to 1 year storage at 4°C. Controls were assayed immediately after preservation incubations. Assays were performed with individual anti-antigen antibodies and detected by fluorescence at 532nm (N=4 arrays).

For blocking/preservation of printed microarrays:

- Submerge dry microarrays (20 slides) into 150 ml Super G Plus in a glass staining jar.
- Incubate slides without agitation for 1 hour.
- After the blocking/preservation incubation time, agitate slides on an orbital shaker (15 min).
- Transfer slides into FRESH 150 ml Super G Plus and agitate further on an orbital shaker (15 min).

For immediate use of printed microarrays:

- Wash microarrays sequentially 3 times with PBST for 5 min with agitation (105 rpm) on an orbital shaker.
- Arrays are ready for immunoassay at this point.
- DO NOT allow slides to dry (skip remaining steps).

For storage of printed microarrays:

- DO NOT WASH arrays after blocking/preservation.
- Remove microarray slides from preservation buffer (this may be performed in batches, depending on drying method). Additional time in the preservative will not be detrimental to the microarrays.
- Place individual slides in 50 ml conical screw-cap tubes (1 slide per tube or 2 slides back-to-back).
- Place balanced tubes with slides into a centrifuge and spin dry slides at 200 rpm for 1 min.
- Remove slides from centrifuge tubes and allow to air dry.
- Place slides in an airtight slide box with desiccant.
- For long-term storage, place (with desiccant) slide at 4 to -20°C.

Notes: Utilizing centrifugation allows for even drying of Super G Plus over the slide surface by removing excess reagent from the surface (slides will not be fully dry). After air drying, the nitrocellulose slide surface will appear slightly darker and the surface will be sticky. This is a normal characteristic of Super G Plus.

If centrifugation is not used, drying may appear uneven and the nitrocellulose surface may appear translucent in various places where there was excess reagent. This does not alter the effectiveness of the preservative. These surfaces will function properly and will appear evenly white again once the preservative is removed during downstream processing.

For optimal results, slides should not be pre-treated with other reagents before using Super G Plus.

Post-Storage Rehydration Procedure:

Remove slides from refrigerator/freezer and allow slides to come to room temperature while desiccated to avoid condensation on slide surface. Rehydrate slides by washing with 1X PBST (or buffer similar in composition to your assay buffer) for 30 minutes with agitation prior to proceeding with the assay.

Notes: *Do not allow slides to dry once they have been rehydrated.*

Super G Blocking Buffer:

Super G Blocking Buffer is a non-protein based reagent designed to block non-specific protein binding on porous nitrocellulose substrates. It has been developed specifically for minimizing nitrocellulose backgrounds, and is supplied as a 1X solution, ready to use out of the bottle, for microarray applications. It has been included in this kit to supplement blocking after Super G Plus; to be used during the immunoassay as a wash buffer; and to be included in assay incubation buffers to further minimize background levels. Recommendations regarding incubation times and concentrations are listed but optimization of all of these parameters is recommended for specific applications.

If arrays have been treated with Super G Plus, it is not typically necessary to perform an additional blocking incubation with Super G prior to commencing with the immunoassay. For some applications requiring lower backgrounds, additional blocking with Super G upon microarray rehydration may be performed prior to the immunoassay. For additional blocking, it is recommended to incubate slides for 15-60 minutes before washing. After blocking with Super G, wash slide(s) with PBST (or buffer similar in composition to your assay buffer) for 3 x 5 minutes with agitation prior to proceeding with the assay.

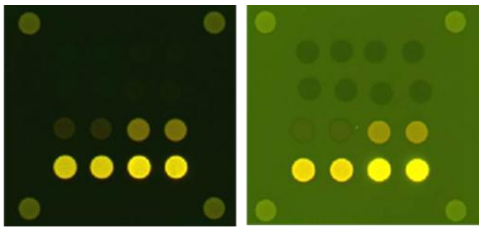


Figure 4. Effect of optimized blocking with Super G Blocking Buffer. Shown are arrays blocked with Super G or PBST for 1 hr prior to immunoassay.

Immunoassay Protocol:

Note: For optimal results, it is VERY important to perform the blocking and preservation steps as described in previous sections. Listed below are 2 sample protocols for either an antibody capture assay or an antigen capture assay. These protocols are intended as general guidelines but assay parameters (incubation times, incubation buffer, etc.) should be optimized for individual applications.

Incubation Buffer with Super G: As a general incubation buffer, 1x PBST supplemented with 10% Super G Blocking Buffer is recommended. (For example, dilute 10 ml of Super G Blocking Buffer into 90 ml of 1x PBST.) Additionally, this may be supplemented with BSA (1-5%).

Washing between incubations with Super G: Washing with Super G Blocking Buffer between incubation steps is recommended and leads to decreased backgrounds. A typical wash routine may be 1x PBST (5 min), followed by 1x Super G (5 min), followed by 1x PBST (5 min).

Antibody Capture Assay:

1. Prior to beginning immunoassays, slides should already be blocked/preserved as per previous sections with Super G Plus or Super G. As per those protocols, slides should be washed 3 x 5 min with PBST with agitation (50 rpm).
2. Attach a ProPlate chamber, if not already attached for blocking step and fill with appropriate volume of 1x PBST.
3. Incubate with sample containing antigens of interest (e.g. patient serum) with agitation on an orbital shaker (50 rpm). Typically 15-60 minutes.
4. Wash with 1x PBST (5 min.) with agitation (50 rpm).
5. Wash with 1x Super G (5 min.) with agitation (50 rpm).
6. Wash with 1x PBST (5 min.) with agitation (50 rpm).
7. Incubate with detection antibody or antibody cocktail in Incubation Buffer with agitation on an orbital shaker (50 rpm). Typically 15-60 minutes.
8. Wash with 1x PBST (5 min.) with agitation (50 rpm).
9. Wash with 1x Super G (5 min.) with agitation (50 rpm).
10. Wash with 1x PBST (5 min.) with agitation (50 rpm).
11. Incubate with fluorescently- or biotin-labeled secondary antibody in Incubation Buffer agitation on an orbital shaker (50 rpm). Typically 15-60 minutes.
12. Wash with 1x PBST (3 x 5 min. with buffer changes) with agitation (50 rpm).
13. *OPTION*: Depending on the detection method where the secondary antibody was biotinylated, perform additional assay amplification incubations such as streptavidin-HRP conjugation, Tyramide Signal Amplification, etc.
    a. Wash with 1x PBST (2 x 5 min. with buffer change) between steps.
    b. After final incubation, wash with 1 x PBST (3 x 5 min).
14. Wash with 1x PBS (3 x 5 min. with buffer changes) with agitation (50 rpm).
15. Rinse with fresh deionized water (1 min) with agitation (50 rpm).
16. Allow slides to dry at room temperature for 10 min. prior to data acquisition.

Antigen Capture Assay:

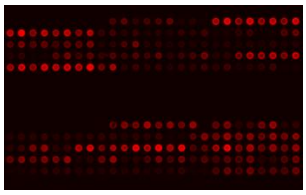
1. Prior to beginning immunoassays, slides should already be blocked/preserved as per previous sections with Super G Plus or Super G. As per those protocols, slides should be washed 3 x 5 min with PBST with agitation (50 rpm).
2. Attach a ProPlate® chamber, if not already attached for blocking step and fill with appropriate volume of 1x PBST.
3. Incubate with serum (e.g. for autoimmune antibody detection) with agitation on an orbital shaker (50 rpm). Typically 15-60 minutes.
4. Wash with 1x PBST (5 min.) with agitation (50 rpm).
5. Wash with 1x Super G (5 min.) with agitation (50 rpm).

6. Wash with 1x PBST (5 min.) with agitation (50 rpm).
7. Incubate with fluorescently- or biotin-labeled secondary antibody in Incubation Buffer with agitation on an orbital shaker (50 rpm). Typically 15-60 minutes.
8. Wash with 1x PBST (3 x 5 min. with buffer changes) with agitation (50 rpm).
9. *OPTION*: Depending on the detection method where the secondary antibody was biotinylated, perform additional assay amplification incubations such as streptavidin-HRP conjugation, Tyramide Signal Amplification, etc.
    a. Wash with 1x PBST (2 x 5 min. with buffer change) between steps.
    b. After final incubation, wash with 1 x PBST (3 x 5 min).
10. Wash with 1x PBS (3 x 5 min. with buffer changes) with agitation (50 rpm).
11. Rinse with fresh deionized water (1 min) with agitation (50 rpm).
12. Allow slides to dry at room temperature for 10 min. prior to data acquisition.

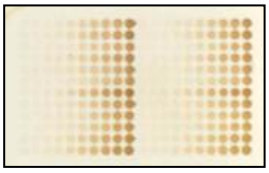
Data Acquisition and Microarray Analysis

There are various detection methods available for data acquisition from protein microarray assays. These include colorimetric, fluorescence, and chemiluminescence. The equipment necessary for each detection method will vary and you should consult to each specific manufacturer's instructions for proper use. Sample images from microarray assays with various forms of detection are shown below.

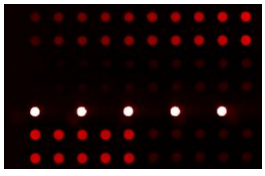
Additionally, each method is subject to variability introduced during the assay or during data acquisition and should be considered when collecting and analyzing data. Inclusion of proper controls during sample spotting will aid in microarray data analysis and control of assay- and detection-induced variability.



Microarray image generated from an RPPA with fluorescence detection using a conventional fluor (Cy5). The method utilized tyramide signal amplification prior to labeling with Cy5. Microarray was imaged with a Molecular Devices GenePix 4400 microarray scanner with excitation at 635nm.



Microarray image generated from an RPPA with colorimetric detection. The method utilized tyramide signal amplification followed with HRP detection with DAB. Microarray was imaged with a desktop flatbed scanner.



Microarray image generated from an RPPA with fluorescence detection using a near IR dye (IRDye800). The method utilized no signal amplification prior to labeling. Microarray was imaged with an InnoScanIR microarray scanner (Innopsys Inc.) with excitation at 785nm.

Appendices

Appendix A – Protease and Phosphatase Inhibitors

Inhibitor cocktails may also be purchased from commercial sources. Two recommended inhibitor cocktails are listed below. Working concentrations are 1 tablet per 10 ml Arraying Buffer volume.

Complete™ Mini Protease Inhibitor Cocktail tablet
(Roche Applied Sciences, Cat. # 11 836 153 001)

PhosSTOP™ Phosphatase Inhibitor Cocktail
(Roche Applied Sciences, Cat. # 04 906 837 001)

Appendix B – Working Reagent Formulas:

GBL Protein Arraying Buffer
Supplied as a 10x concentrated solution. Needs to be diluted with water or available sample buffer with or without additional inhibitors.

Spot Tuning Solution
Supplied as a concentrated solution. Mix with 1x GBL Protein Arraying Buffer as needed for desired spot size. See Table 1.

Super G Blocking Buffer
Supplied as a 1x solution, ready to use.

Super G Plus Preservation Buffer
Supplied as a 1x solution, ready to use.

1x PBST
(Diluted from 10x concentrated solution)
10 mM Sodium Phosphate, pH 7.4
150 mM Sodium Chloride
0.1 % Tween-20

1x PBS
(Diluted from 10x concentrated solution)
10 mM Sodium Phosphate, pH 7.4
150 mM Sodium Chloride