



Mouse Antibody Array Kit

User Manual

Cat #:MA6320, MA6410-MA6412

Panomics, Inc.

Mouse Antibody Array Kit User Manual

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INTRODUCTION

Overview The completion of entire genome sequences for a number of species has propelled science into a new era. Our goal now is to characterize the proteome—the total complement of proteins for a specie. Although the human genome encodes approximately 30,000 genes, the human proteome is thought to include more than a million protein variants, arising from differential gene splicing and post-translational modifications (1). Identifying and characterizing all of those variants is an extensive undertaking that demands new high-throughput technologies.

Because of variable rates in protein translation, modification and degradation, mRNA levels do not correlate well with protein levels (2-3). Thus, assessing a cell's protein content requires a method for directly monitoring the protein themselves. One such method is the antibody array, an adaptation of the high-throughput cDNA microarray technology (4-9). Antibody arrays have proven to be perfectly suited to profiling protein expression. One benefit is the specificity of antigen-antibody interactions, which confers high selectivity to these arrays. Another is the extreme sensitivity inherent to the enzyme-linked immunosorbent assay (ELISA) on which these arrays are based.

Panomics Mouse Antibody Arrays elevates protein detection to a new level. With this system, you can rapidly and accurately profile the expression of multiple secreted proteins in the picogram per mL range. Serum, plasma, and cell culture supernatants from mouse samples can be used with these kits.

Valuable Tool for Studying Biomarkers

Secreted proteins such as cytokines, chemokines growth factors are of particular significance because they play a crucial role in both the development and the daily activity of the immune system (7-8). The ever-expanding family of cytokine proteins consists of colony-stimulating factors, chemokines, interferons, interleukins, and lymphokines but the majority are growth and differentiation factors. Acting as inter-cellular chemical messengers, cytokines are secreted from cells to seek out and bind specific high-affinity cell-surface receptors, which initiate the activation of signal transduction pathways within cells (8). Both the cytokine proteins and their receptors are transiently expressed to mediate the immune and inflammatory responses of the body.

The vast network of cytokines and their receptors has recently become a focus of both scientific and clinical research. Cytokines have already been implicated in a host of diseases, including arthritis, acute and chronic liver disease, inflammatory bowel disease, cardiac-related diseases and some forms of cancer (10-11). Clearly, breakdowns in communication between cells can lead to cellular malfunction. Only by increasing our comprehension of the complex inner workings of cytokine signaling will we have the keys to unlock the molecular underpinnings of the disease states. The Mouse Antibody Arrays facilitate study of the cytokine network by allowing you to monitor the relative abundance of certain mouse cytokines from a variety of sources in a high-throughput manner.

Antibody Arrays are an ideal tool for profiling cytokines because they detect the presence of functional cytokines, which represent the levels of *active* protein.

Principle of the Antibody Array Assay

The Cytokine and Angiogenesis Antibody Arrays are based on the sandwich ELISA method for detecting protein (12). First, a capture antibody specific to a particular cytokine protein is immobilized on the array membrane. Second, sample is incubated with the capture antibody, proteins are given time to bind, and any unbound protein is washed away. Then, a second antibody, known as the detection antibody, is introduced. This biotin-conjugated antibody binds to a second epitope on the protein, creating an antibody “sandwich” around the cytokine. Through a biotin-streptavidin interaction, an enzyme is used to visualize the bound cytokine protein. The linkage between capture antibody, target protein, and detection antibody offers a high degree of selectivity and sensitivity.

The assay procedure for these antibody arrays is simple and easy to follow (See Figure 1 on the following page):

- (1) Incubate the sample with the array membrane. During this time, cytokine proteins present in the sample will selectively bind to the anti-cytokine antibodies immobilized on the array.

- (2) Add the biotin-labeled detection antibodies to the array membrane. These anti-cytokine antibodies will specifically bind to the captured proteins immobilized on the array, creating an antibody “sandwich.”

- (3) Add streptavidin-HRP to the array which will bind to the biotin labeled detection antibody/cytokine complex.

- (4) Add a chemiluminescent substrate to detect the antibody “sandwich”. If the cytokine is present in the sample, the chemiluminescent signal from the reaction will generate light at that particular spot and the entire array of spots can be detected using either x-ray film or an imaging system.

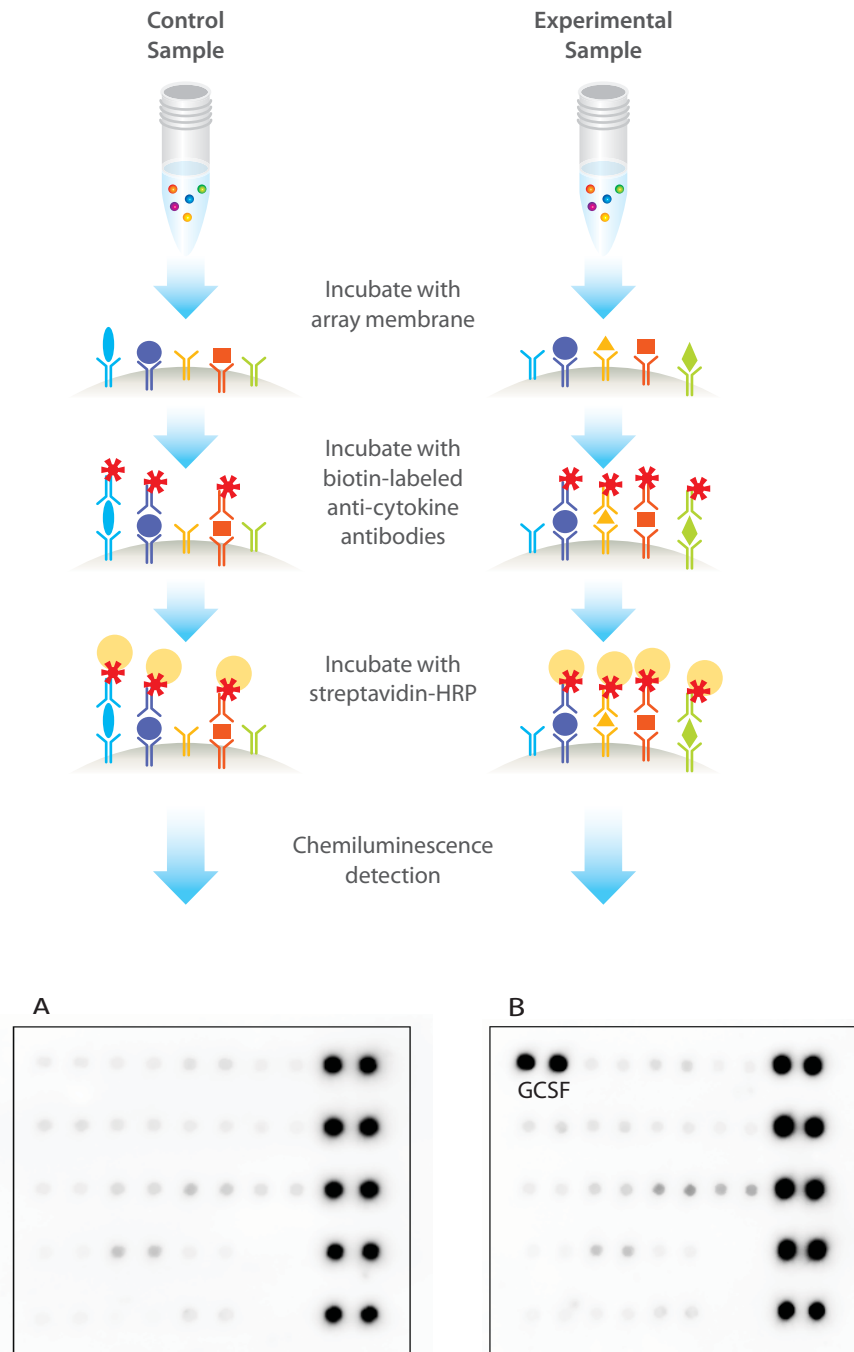


Figure 1: Schematic flow chart of the Mouse Antibody Array assay.

MATERIALS

Kit Configuration The mouse cytokine Antibody Array comes in two sizes.

Products	# Membranes or Assays	Catalog #
Mouse Cytokine Antibody Array 1.0	4	MA6410
Mouse Cytokine Antibody Array 1.0	8	MA6412
Mouse Angiogenesis Antibody Array	4	MA6320

Storage Conditions Upon receipt, store the entire kit at 4 °C. Use within six months of receipt of kit.

Mouse Cytokine Antibody Array membranes (the membranes are 2.8 cm long x 3.2 cm wide; the notch is at the top right corner)
Biotin-Conjugated Anti-Cytokine Mix (each tube contains enough antibodies for 2 assays)
1,000X Streptavidin-HRP Conjugate
1X Blocking Buffer
20X Wash Buffer I – dilute to 1X with dH ₂ O
20X Wash Buffer II – dilute to 1X with dH ₂ O
Detection Buffer A
Detection Buffer B
Eight-Well Tray

Additional Materials Required Required items but not provided in kit

Orbital shaker
Plastic sheet protectors or overhead transparencies
Hyperfilm™ ECL™ (GE Healthcare, Cat.# RPN3114K) OR Chemiluminescence imaging system (e.g., FluorChem® from Alpha Innotech Corp.)

ASSAY PROCEDURE

General Guidelines

- 1.1 Preparation of samples:
 - ◆ Use serum-free conditioned media whenever possible
 - ◆ When serum-containing conditioned media is required, be sure to use serum as a control (many types of serum contain cytokines)
 - ◆ We recommend using 1–2 mL of cell culture supernatants OR 1–2 mL of original or 10-fold diluted sera
 - ◆ If you experience high background, you may further dilute your sample using 1X Wash Buffer II.
- 1.2 Handling array membranes:
 - ◆ Always use forceps to handle membranes by the edge only.
 - ◆ Always place array membranes protein-side up. To do this, use the notched corner (top-right when shorter side of membrane is horizontal) for orientation.
 - ◆ Never allow array membranes to dry during experiments.
 - ◆ Stripping of the array membranes is not recommended.
- 1.3 Incubation:
 - ◆ To avoid drying, completely cover membranes with sample or buffer during incubation steps.
 - ◆ Perform all incubation and wash steps under gentle rotation.
 - ◆ Avoid foaming during incubation steps.

Incubation

IMPORTANT: Be sure that the membrane is fully submerged in assay buffer at all times. NEVER let the membrane dry out.

- 2.1 Place each membrane into the provided eight-well tray with 2 mL of 1X Blocking Buffer. Make sure that the membrane is capture antibody side up by orienting the notch to the top-right corner when the membrane's longer side is vertical (See Appendix A for membrane orientation and notch position). Incubate for 1 to 2 hr at room temperature.
- 2.2 Remove the 1X Blocking Buffer and briefly rinse membrane twice with 4 mL of 1X Wash Buffer II (diluted with dH₂O). Dab the container upside-down on a lint-free towel to remove excess liquid.
- 2.3 Incubate each membrane with 1-2 mL of your samples for 1 to 2 hr at room temperature. Dilute your sample with 1X Wash Buffer II if necessary.
- 2.4 Decant the samples from each container and wash each membrane by adding 4 mL of 1X Wash Buffer I (diluted with dH₂O) and incubate for 5 min at room temperature with shaking. Repeat two more for a total of three washes.
- 2.5 Add 4 mL of 1X Wash Buffer II and incubate for 5 min at room temperature with shaking. Dab the container upside-down on a lint-free towel to remove excess liquid.

- 2.5 Prepare working solution for primary antibody by briefly spinning the tube of Biotin Conjugated Anti-Cytokine/Anti-Angiogenesis Mix. Add 50 μ L of 1X Blocking Buffer to the provided tube of Biotin-Conjugated Anti-Cytokine Mix/Anti-Angiogenesis Mix (*NOTE that this amount is sufficient for two assays*):
- 2.6 Mix gently and add the mixture into 15 mL conical containing 3 mL of 1X Blocking Buffer. From this step on, all membranes can be processed in the same container. If you are using the provided eight-well tray, add 1.5 mL of the diluted Biotin-Conjugated Anti-Cytokine Mix (from Step 2.5) to each membrane. Incubate for 1 to 2 hr at room temperature. Note that the diluted Biotin-Conjugated Anti-Cytokine Mix can be stored for 3 days at 4°C.
- 2.7 Wash as described in Step 2.4 and 2.5.
- 2.8 Prepare 1X Streptavidin-HRP working solution by diluting 1000X Streptavidin-HRP in 1X Blocking Buffer. Every array will use 2 mL of 1X Streptavidin-HRP working solution. For every 2 mL of 1X Wash Buffer II and add 2 μ L of 1000X Streptavidin-HRP. Incubate for 30 to 60 min at room temperature.
- 2.9 Wash as described in Step 2.4 and 2.5.

Detection

IMPORTANT: Do not let the membrane dry out during detection. Once the detection process has begun, it must be completed within 30 min without stopping.

- 3.1 Prepare the detection solution immediately before use by mixing equal amounts of Detection Buffers A and B—200 μ L of Detection Buffer A and 200 μ L of Detection Buffer B for each membrane.
- 3.2 Using forceps to hold the edge, carefully remove each membrane from its tray. Drain the excess Wash Buffer from the membrane by touching the edge against a piece of tissue. Place membrane protein/antibody side-up on a clean plastic sheet protector or overhead transparency.
- 3.5 On a flat and even surface, remove the top plastic sheet of the “sandwiched” membrane and pipette 400 μ L of the mixed working Detection Buffer onto each membrane. Replace the top plastic sheet and ensure that the substrate solution is evenly distributed over the membrane with no air bubbles. Incubate at room temperature for 5 min.
- 3.6 Remove excess substrate by gently applying pressure over the top sheet and using a paper towel to wipe up any excess fluid.
- 3.7 Expose the membranes using either Hyperfilm ECL (2-10 minutes) or a chemiluminescent imaging system (12-15 min), such as the FluorChem Imager from Alpha Innotech Corp. **Note:** *Several different exposure times to film or the imaging system may be needed for an optimal image.*

Interpretation of Results

Figure 2 shows Mouse Cytokine Array 1.0 membrane A incubated with DMEM +5% Fetal Bovine Serum (FBS) and membrane B incubated with the supernatant of NIH3T3 cells grown in DMEM plus 5% FBS. By comparing signal intensities, the relative expression levels of cytokines can be determined. Signal intensities can be quantified by densitometry or by chemiluminescence imaging. The biotin-conjugated antibodies on every membrane serve as positive controls. These control spots can be used to identify the membrane orientation and to compare relative expression levels between different membranes.

For the best possible results, we suggest that you attempt several different exposures to x-ray film or chemiluminescence imaging. We also strongly recommend that you use a negative control, in which the sample is replaced with an appropriate mock buffer. This is especially important for your first experiment using these arrays.

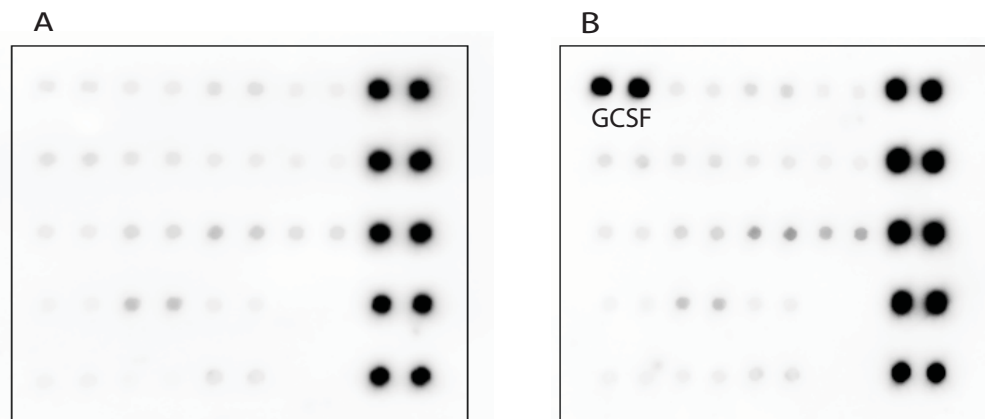


Figure 2. Mouse Cytokine Antibody Array 1.0. Membranes were incubated with DMEM +5% FBS (A) and the supernatant of NIH 3T3 cells grown in DMEM + FBS (B). Images were collected using a Fluorchem Imager (Alpha Innotech). Differences in cytokine expression of GCSF can be observed between the two samples.

Interpretation of Results

Figures 3: Mouse Angiogenesis Antibody Array membranes incubated with tissue extract and conditioned media, respectively. By comparing signal intensities, the relative expression levels of angiogenic factors can be determined. Signal intensities can be quantified by densitometry or by chemiluminescence imaging. The biotin-conjugated antibodies on every membrane serve as positive controls. These control spots can be used to identify the membrane orientation and to compare relative expression levels between different membranes. For the best possible results, we suggest that you attempt several different exposures to x-ray film or chemiluminescence imaging. We also strongly recommend that you use a negative control, in which the sample is replaced with an appropriate mock buffer. This is especially important for your first experiment using these arrays.

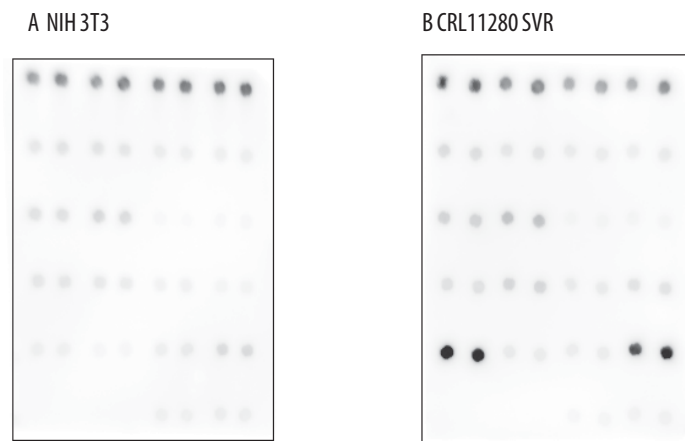


Figure 3: Mouse Angiogenesis Antibody Array incubated with conditioned media. Conditioned media was isolated from 2-day old NIH 3T3 (A) and CRL1280 SVR (B) cells and then incubated with the Mouse Angiogenesis Antibody array membrane. Increased expression of G-CSF and TIMP-1 can be observed in CRL1280 SVR cells. These images were collected using a Fluorchem Imager (from Alpha Innotech).

Troubleshooting Guide

Problem	Cause	Recommendation
High background	High concentration of nonspecific binding compounds in sample	Further dilute samples in 1X Wash Buffer II or decrease incubation time
	Membranes were allowed to dry out during incubation	Make sure that membranes are covered in solution at all times
	X-ray film was overexposed	Decrease exposure time
	Blocking was incomplete	Increase blocking duration
Signal is too weak or nonexistent	Sample is too dilute	Use more concentrated samples for incubation
		Increase incubation time of sample with membrane and/or detection antibodies with membrane
		Increase sample volume or use more cells as starting material
	Dissociation of interacting proteins during incubation	Reduce washing duration
	Other	Reduce concentration of blocking solution by diluting in 1X Wash Buffer II
		Slightly increase concentration of Streptavidin-HRP conjugate or antibody concentration
Use detection solution immediately after mixing		
Signal is uneven	Bubbles formed during the incubation step, or membranes were not completely covered with solution during incubation or detection	Make sure solution is spread evenly over membrane surface without bubbles

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Appendix A:
Schematic
diagram of Mouse
Cytokine and
Angiogenesis
Arrays

Mouse Cytokine Array 1.0

	1	2	3	4	5	6	7	8	9	10
A	G-CSF		IFN-gamma		IL-1-alpha		IL-10		pos	
B	M-CSF		TNF-alpha		IL-2		IL-12		pos	
C	GM-CSF		IP-10		IL-4		IL-13		pos	
D	MIG		Rantes		IL-5		neg		pos	
E	MIP-1-alpha		VEGF		IL-6		neg		pos	

Mouse Angiogenesis Array 1.0

	1	2	3	4	5	6	7	8
A	pos		pos		pos		pos	
B	EGF		IL-1-alpha		Leptin		IFN-gamma	
C	FGFa		IL-1beta		VEGF		IL-12	
D	FGFb		IL-4		TNF-alpha		IP-10	
E	G-CSF		IL-6		TGF-alpha		TIMP-1	
F	Neg		Neg		TGF-beta		TIMP-2	

|----- Activators -----|----- Inhibitors -----|

Note: ** notch is in the top-right corner when the membrane's longer side is vertically aligned; the capture antibodies and controls are spotted in duplicate. Abbreviations: pos = positive control, neg = negative control; all others are standard abbreviations.

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