Focal Points

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Cytokine Antibody Array Applications with UVP BioImaging Systems

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Introduction

All cell functions, including cell proliferation, cell death and differentiation, as well as maintenance of health status and development of disease, are controlled by many genes and signaling pathways. New techniques such as cDNA microarrays have enabled RayBiotech to analyze the global gene expression. However, almost all cell functions are executed by proteins, which cannot be studied by DNA and RNA alone. Experimental analysis clearly shows a disparity between the relative expression levels of mRNA and their corresponding proteins. Therefore, it is critical to analyze the protein profile. Currently, 2D acrylamide SDS page coupled with mass spectrometry is the mainstream approach to analyzing multiple protein expression levels. However, the requirement of sophisticated devices and the lack of quantitative measurements greatly limit its broad application. Thus, no simple, cost effective and rapid method of analysis of multiple protein expression levels has been available to researchers until now.

RayBiotech's RayBio Human Cytokine Antibody Arrays is the first commercially available protein array system. By using the RayBiotech system, scientists can rapidly and accurately identify the expression profiles of multiple cytokines in several hours inexpensively.

The RayBiotech kit provides a simple array format and highly sensitive approach to simultaneously detect multiple cytokine expression levels from conditioned media, patient's sera and other sources. Arrays I, III, IV and V are specifically designed for conditioned media, serum and plasma. Arrays 1.1, 3.1, 4.1 and 5.1 are designed for cell lysate and certain tissue lysates.

Cytokines are traditionally detected by ELISA, however, RayBiotech's approach has several advantages over ELISA. First and most importantly is that this approach can detect many cytokines simultaneously. Secondly, sensitivity is greatly increased. As little as 4 pg/ml of MCP-1 can be detected using the protein array format. In contrast, at least, 40 pg/ml of MCP-1 is required to produce a clear signal in an ELISA assay. Furthermore, the detection range is much greater than ELISA. For example, the detection range of IL-2 varies from 25 to 250,000 pg/ml using RayBiotech technology, whereas the detection range varies only within 100-1000 fold in a typical ELISA. Therefore, the detection range is at least 100-fold greater with protein array compared with ELISA. The variation is lower than ELISA as well. As determined by densitometry, the variation between two spots ranged from 0 to 10% in duplicated experiments. In contrast, variation (about 20%) in ELISA is much higher. Finally, the system is much quicker and can be much easier to adapt to high-throughput format.

Pathway-specific array systems allow investigators to focus on the specific problem and are becoming an increasingly powerful tool in cDNA microarray system. RayBiotech's first protein array system, known as RayBio Human Cytokine Antibody Arrays, is particularly useful compared with the human cytokine cDNA microarray system. Besides the ability to detect protein expression, RayBiotech's system is a more accurate reflection of active cytokine levels because it only detects secreted cytokines, and no amplification step is needed. Furthermore, it is much simpler, faster, environmentally friendlier, and more sensitive.

Simultaneous detection of multiple cytokines undoubtedly provides a powerful tool to study cytokines. Cytokines play an important role in innate immunity, apoptosis, angiogenesis, cell growth and differentiation. Cytokines are involved in most disease processes, including cancer and cardiac diseases. The interaction between cytokines and the cellular immune system is a dynamic process. The interactions of positive and negative stimuli, and positive as well as negative regulatory loops are complex and often involve multiple cytokines.

Materials

Upon receipt, the array membranes and 2X Blocking Buffer should be kept at –20^{O}C. All other components should be stored at 4^{O}C. Please use within three months of purchase.

- RayBio Human Cytokine Antibody Array membranes (2/4/8 membranes)
- Biotin-Conjugated Anti-Cytokines (1/2/4 tubes, each tube for two membranes)
- 1,000X HRP-Conjugated Streptavidin (24 µl)
- 2X Blocking Buffer (15/25ml)
- 20X Wash Buffer I (10/20ml)
- 20X Wash Buffer II (10/20ml)
- Detection Buffer A (1.5/2.5ml)*
- Detection Buffer B (1.5/2.5ml)*
- 2X Cell Lysis Buffer (10/20ml)*
- Detection Buffer C (1.5/2.5ml)**
- Detection Buffer D (1.5/2.5ml)**
- Eight-Well Tray (1 each)
- Manual

* For RayBio Human Cytokine Antibody Arrays 1.1, 3.1, 4.1, 5.1
** For RayBio Human Cytokine Antibody Arrays I, III, IV and V

Use array I, III, IV and V for conditioned medium, serum, plasma, and urine. Use array 1.1, 3.1, 4.1 and 5.1 for cell lysates and certain tissue lysates.

Additional Materials Required:

- Small plastic boxes or containers
- Orbital shaker
- Plastic sheet protector or Saran Wrap
- AutoChemi Chemiluminescence imaging system

Figure 1 – AutoChemi Imaging System

Overview and General Considerations

A. Preparation of Samples

Use serum-free conditioned media if possible. If serum-containing conditioned media is required, use serum as control since many types of sera contain cytokines.

For cell lysates and tissue lysates, we recommend using 1X Cell Lysis Buffer to extract proteins from cell or tissue (e.g. using homogenizer). After extraction, spin the sample and save supernatant for experiment. Determine protein concentration. Dilute 2X Cell Lysis Buffer with H_2O (we recommend adding proteinase inhibitors to Cell Lysis Buffer before use).

We recommend using:
1 ml of Conditioned media
or 1 ml of original or 10-fold diluted sera or plasma
or 50-500 µg of protein for cell lysates and tissue lysates.

If you experience high background, you may further dilute your sample.

B. Handling Array Membranes

Always use forceps to handle membranes, and grip the membranes by the edges only.

Never allow array membranes to dry during experiments.

Incubation

Completely cover membranes with sample or buffer during incubation, and cover eight-well tray with lid to avoid drying.

Avoid foaming during incubation steps.

Perform all incubation and wash steps under gentle rotation.

Several incubation steps such as step 2 (blocking), or step 3 (sample incubation), or step 7 (biotin-Ab incubation) or step 10 (HRPstreptavidin incubation) may be done at 4^{O}C for overnight.

Protocol

A. Blocking and Incubation

1. Place each membrane into the provided eight-well tray ("-" means the antibody printed side). Use arrays I, III, IV and V for conditioned medium, serum and plasma, and arrays 1.1, 3.1, 4.1 and 5.1 for cell lysate and certain tissue lysate.
2. Add 2 ml 1X Blocking Buffer and incubate at room temperature for 30 min to block membranes. Dilute 2X Blocking Buffer with H_2O.
3. Decant Blocking Buffer from each container, and incubate membranes with 1ml* of sample at room temperature for 1 to 2 hours. Dilute sample using 1X Blocking Buffer if necessary.

   Note: We recommend using 1 ml of Conditioned media or 1 ml of original or 10-fold diluted sera or plasma or 50-500 µg of protein for cell lysates and tissue lysates. Dilute lysate at least 10 folds with 1X blocking buffer.

   Note: The amount of sample used depends on the abundance of cytokines. More of the sample can be used if signals are too weak. If signals are too strong, the sample can be diluted further.

   Note: Incubation may be done at 4^{O}C for overnight.
4. Decant the samples from each container, and wash 3 times with 2 ml of 1X Wash Buffer I at room temperature with shaking. 5 min per wash. Dilute 20X Wash Buffer I with H_2O.
5. Wash 2 times with 2 ml of 1X Wash Buffer II at room temperature with shaking. 5 min per wash. Dilute 20X Wash Buffer II with H_2O.
6. Prepare working solution for primary antibody.
7. Add 100 µl of 1x blocking buffer to the Biotin-Conjugated Anti-Cytokines tube. Mix gently and transfer all mixture to a tube containing 2 ml of 1x blocking buffer.

   Note: the diluted biotin-conjugated antibodies can be stored at 4^{O}C for 2-3 days.
8. Add 1 ml of diluted biotin-conjugated antibodies to each membrane. Incubate at room temperature for 1-2 hours.

   Note: incubation may be done at 4^{O}C for overnight.
9. Wash as directed in steps 4 and 5.
10. Add 2 ml of **1,000** fold diluted HRP-conjugated streptavidin (e.g. add **2** µl of HRP-conjugated streptavidin to **1998** µl 1X Blocking Buffer) to each membrane.

    Note: mix tube containing 1,000X HRP-Conjugated Streptavidin well before use since precipitation may form during storage.
11. Incubate at room temperature for 2 hours.

    Note: incubation may be done at 4^{O}C for overnight.
12. Wash as directed in steps 4 and 5.

B. Detection

Note: Do not let the membrane dry out during detection. The detection process must be completed within 40 minutes without stopping.

Proceed with detection reaction.

For RayBio Human Cytokine Antibody Arrays **I, III, IV and V**, use 1X Detection Buffer ***C*** and ***D***. Add 250 µl of 1X Detection Buffer ***C*** and 250 µl of 1X Detection Buffer ***D*** for one membrane; mix both solutions; Drain off excess wash buffer by holding the membrane vertically with forceps. Place membrane protein side up ("-" mark is on the protein side top left corner) on a clean plastic sheet (provided in the kit). Pipette the mixed Detection Buffer on to the membrane and incubated at room temperature for ***2*** minutes. Ensure that the detection mixture is completely and evenly covering the membrane without any air bubbles.

For RayBio Human Cytokine Antibody Arrays **1.1, 3.1, 4.1 and 5.1,** use Detection Buffer ***A*** and ***B***. Add 250 µl of 1X Detection Buffer ***A*** and 250 µl of 1X Detection Buffer ***B*** for one membrane; mix both solutions; Drain off excess wash buffer by holding the membrane vertically with forceps. Place membrane protein side up ("-" mark is on the protein side top left corner) on a clean plastic sheet (provided in the kit). Pipette the mixed Detection Buffer on to the membrane and incubated at room temperature for ***1*** minute. Ensure that the detection mixture is completely and evenly covering the membrane without any air bubbles.

1. Drain off excess detection reagent by holding the membrane vertically with forceps and touching the edge against a tissue. Gently place the membrane, protein side up, on a piece of plastic sheet ("-" mark is on the protein side top left corner). Cover another piece of plastic sheet on the array. Gently smooth out any air bubble. Avoid using pressure on the membrane.
2. Expose to x-ray film (Kodak x-omat AR film) and detect signal using film developer, or detect signal directly from membrane using chemiluminescence imaging system.
3. Expose the membranes for 40 Seconds. Then re-expose the film according to the intensity of signals. If the signals are too strong (background too high), reduce exposure time (e.g. 5-30 seconds). If the signals are too weak, increase exposure time (e.g. 5-20 min or overnight. Or re-incubate membranes overnight with 1x HRP-conjugated streptavidin, and redo detection in the second day.
4. Save membranes in –20^{O}C to –80^{O}C for future references.

Results

In a series of experiments designed to create profiles of cytokines released following exposure from a variety of amphotericin B formulations, Turtinen (key reference) reported that chemiluminescent membrane based antibody arrays gave results that correlated well with ELISA based assays. In addition to the low cost per data point, increased sensitivity and greater dynamic range, the chemiluminescent membrane arrays are easily analyzed by CCD based 12 bit image capture with the UVP AutoChemi system.

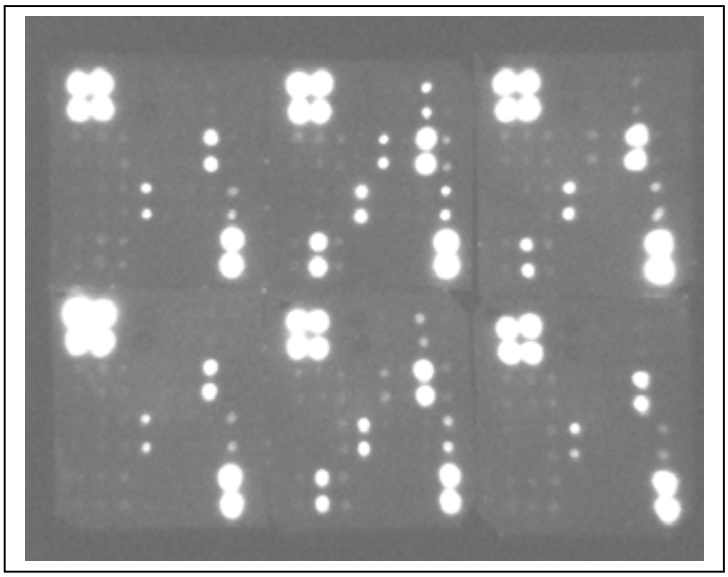

Figure: 2. *RayBio Human Cytokine Anitbody Array 1 Chemiluminescent image. Cytokines, each in duplicate, were detected on a membrane antibody array using chemiluminescence. Using a 90 sec exposure, the image was captured with a UVP AutoChemi (AC1) imaging system set at f1.2 and analyzed with UVP imaging software using grey scale levels.*

The biotin-conjugated IgG produces positive signals, which can be used to identify the orientation and to compare the relative expression levels among the different membranes.

One important parameter is background. To obtain the best results, take several exposures. We also strongly recommend using a negative control in which the sample is replaced with an appropriate mock buffer according to the array protocol, particularly during your first experiment.

By comparing the signal intensities, relative expression levels of cytokines can be made. The intensities of signals can be quantified by densitometry. Positive control can be used to normalize the results from different membranes being compared. The signals also can be detected and quantitated by using a chemiluminescence imaging device.

The RayBio Analysis Tool is a program specifically designed for analysis of RayBio Cytokine Antibody Arrays. This tool will not only assist in compiling and organizing your data, but also reduces your calculations to a "copy and paste."

RayBio Human Cytokine Antibody Array I and 1.1 Map

	A	b	c	d	e	f	g	h
1	Pos	Pos	Neg	Neg	GCSF	GM-CSF	GRO	GRO-α
2	Pos	Pos	Neg	Neg	GCSF	GM-CSF	GRO	GRO-α
3	IL-1α	IL-2	IL-3	IL-5	IL-6	IL-7	IL-8	IL-10
4	IL-1α	IL-2	IL-3	IL-5	IL-6	IL-7	IL-8	IL-10
5	IL-13	IL-15	IFN-γ	MCP-1	MCP-2	MCP-3	MIG	RANTES
6	IL-13	IL-15	IFN-γ	MCP-1	MCP-2	MCP-3	MIG	RANTES
7	TGF-β1	TNF-α	TNF-β	Blank	Blank	Blank	Blank	Pos
8	TGF-β1	TNF-α	TNF-β	Blank	Blank	Blank	Blank	Pos

Troubleshooting Guide

Problem	Cause	Recommendation
Weak signal or no signal	1. Taking too much time for Detection.	1. The whole Detection process must be completed in 30 min.
	2. Film developer does not work properly.	2. Fix film developer.
	3. Did not mix HRPstreptavidin well before use.	3. Mix tube containing 1,000X HRP-Conjugated Streptavidin well before use since precipitation may form during storage.
	4. Sample is too diluted.	4. Increase sample volume, (e.g. using undilute sample) or using more cells (e.g. seed 2 million cells. After 1 or 2 days, change complete medium with low serum medium and collect conditioned medium 2 day later. Use about 1 to 2 ml of conditioned medium for experiment).
	5. Other.	1. Reduce blocking concentration by diluting in 1X Wash Buffer II.
		2. Slightly increase HRP concentrations.
		3. Slightly increase biotin-antibody concentrations.
		4. Expose film for overnight to detect weak signal.
Uneven signal	1. Bubbles formed during incubation.	1. Remove bubble during incubation.
	2. Membranes were not completely covered by solution.	2. Completely cover membranes with solution.
High background	1. Exposure is too long.	1. Decrease exposure time.
	2. Membranes were allowed to dry out during experiment.	2. Completely cover membranes with solution during experiment.
	3. Sample is too concentrated.	3. Use more diluted sample.

Key Reference

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