RayBio® Rat Cytokine Antibody Array

Patent Pending Technology

User Manual (Revised 06/14/09)

RayBio® Rat Cytokine Antibody Array 1 (Cat# AAR-CYT-1)
RayBio® Rat Cytokine Antibody Array 2 (Cat# AAR-CYT-2)

RayBio® Custom Ray Cytokine Antibody Array (Cat# AAR-CUST)

RayBio® Rat Cytokine Antibody Array Service (Cat# AAR-SERV)

Please read manual carefully before starting experiment

RayBiotech, Inc.

We Provide You with Excellent
Protein Array Systems and Service

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RayBiotech, Inc.

RayBio® Rat Cytokine Antibody Array Protocol

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Cytokine protein arrays are RayBiotech patent-pending technology.

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I. 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 a multitude of genes and signaling pathways. New techniques such as cDNA microarrays have enabled us to analyze global gene expression [1-3]. However, almost all cell functions are executed by proteins, which cannot be studied simply through DNA and RNA techniques. Experimental analysis clearly shows a disparity between the relative expression levels of mRNA and their corresponding proteins [4]. Therefore, analysis of the protein profile is critical. Currently, two-dimensional polyacrylamide SDS page coupled with mass spectrometry is the mainstream approach to analyzing multiple protein expression levels [5,6]. However, the requirement of sophisticated devices and the lack of quantitative measurements greatly limit their broad application. Thus, effective study of multiple protein expression levels has been complicated, costly are time-consuming until now.

Our RayBio® Rat Cytokine Antibody Array is the first commercially available cytokine protein array system [7-11]. 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 format and highly sensitive approach to simultaneously detect multiple cytokine expression levels from conditioned media, patient's sera, cell lysate, tissue lysates and other sources.

The RayBio® Mouse Cytokine Antibody Array C series 1000 can detect 96 mouse cytokines in single experiment. RayBiotech also provides RayBio® Human Cytokine Antibody Array C series 4000 which is the only product available in the market that can detect 274 human cytokines in single experiment.

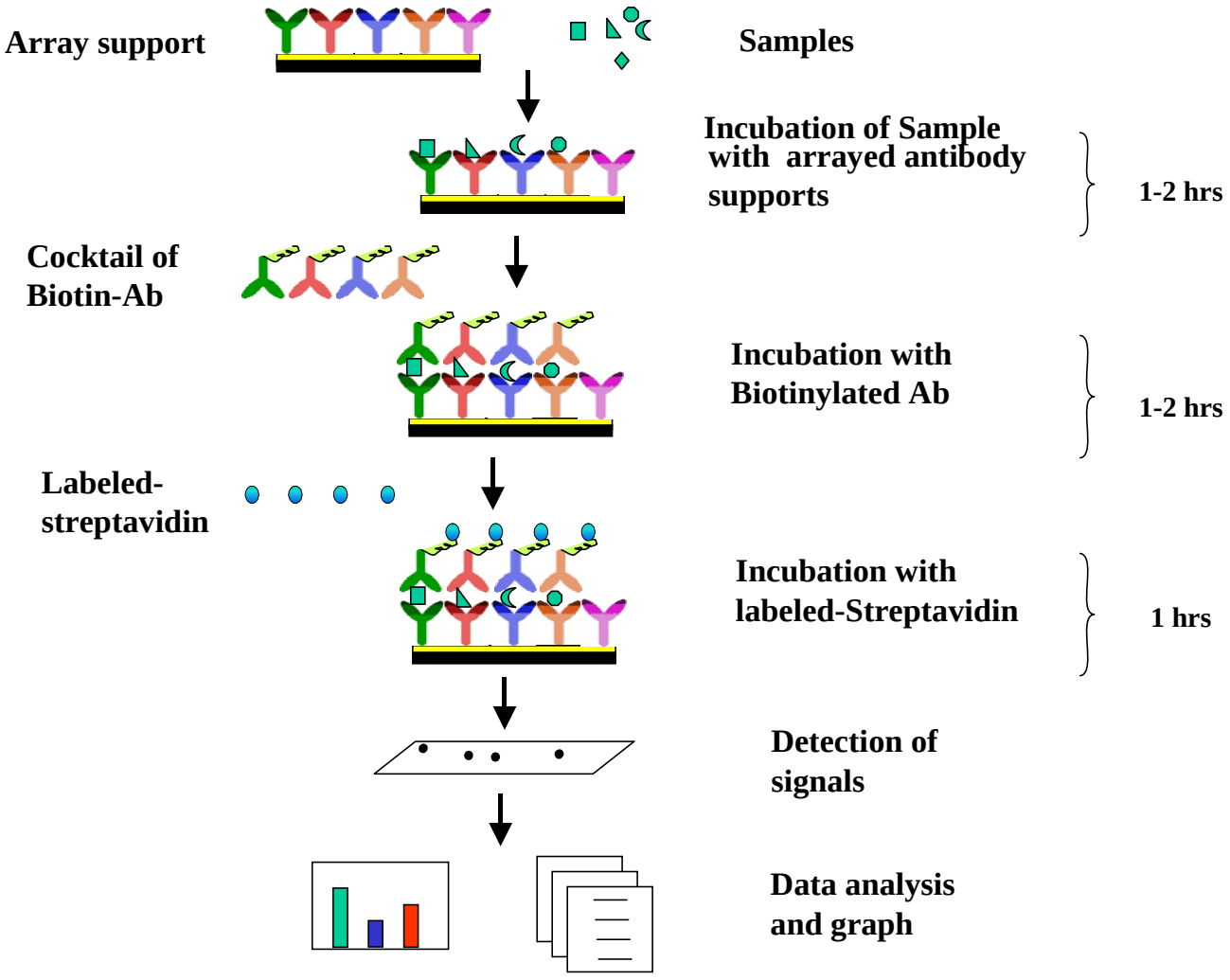
Traditionally, cytokines are detected by using ELISA (enzyme-linked immunsorbent assays); however, RayBiotech's approach has several

advantages over ELISA. First, and most important, our approach can simultaneously detect many cytokines. Secondly, the sensitivity is higher. With this approach, most cytokines can be detected at pg/ml levels. As little as 10 pg/ml of human IL-2 can be detected in the protein array format. Furthermore, the detection range is much greater than ELISA. For example, the detection range of human IL-2 varies from 10 to 100,000 pg/ml, whereas the detection range varies only within 100-1000 fold in a typical ELISA. Therefore, the detection range with protein arrays is greater than ELISA. Additionally, variability is far lower in comparison ELISA. 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 much easier to adapt to high-throughput techniques.

Pathway-specific array systems allow investigators to focus on the specific problem and are becoming an increasingly powerful tool in cDNA microarray systems. RayBiotech's first protein array system, known as RayBio® Rat Cytokine Antibody Array, is particularly useful in comparison with the mouse 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. Cytokines play an important role in innate immunity, apoptosis, angiogenesis, cell growth and differentiation [12]. They 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.

Without doubt, simultaneous detection of multiple cytokines provides a powerful tool to study cytokines.

Here's how it works



II. Materials Provided

Upon receipt, all components of the RayBio® Rat Cytokine Antibody Array kit should be stored at -20^{0}C to -80^{0}C. At -20^{0}C to -80^{0}C the kit will retain complete activity for up to 6 months. Once thawed, the array membranes and 1X Blocking Buffer should be kept at –20^{0}C and all other components should be stored at 4^{0}C. After thawing the reagents, the kit must be used within three months, and please use the kit within six months of purchase.

- RayBio® Rat Cytokine Antibody Array membranes (2/4/8 membranes)
- Biotin-Conjugated Anti-Cytokines (1/2/4 vials) (each vial is for two array membranes)
- 1,000X HRP-Conjugated Streptavidin (50 µl)
- 1X Blocking Buffer (15/25ml)
- 20X Wash Buffer I (10/20ml)
- 20X Wash Buffer II (10/20ml)
- 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

Additional Materials Required

- Small plastic boxes or containers
- Orbital shaker
- Plastic sheet protector or SaranWrap
- Kodak x-omat AR film (REF 165 1454) and film processor

or

Chemiluminescence imaging system

III. Overview and General Considerations

A. Preparation of Samples

- Use serum-free conditioned media if possible.
- If serum-containing conditioned media is required, use the serum as a 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 down and save the supernatant for your experiment. Determine the 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
  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 the array membranes to dry during experiments.

C. Incubation

- Completely cover the membranes with sample or buffer during incubation, and cover the eight-well tray with a 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), step 3 (sample incubation), step 7 (biotin-Ab incubation) or step 10 (HRP-streptavidin incubation) may be done at 4^0C for overnight.

IV. Protocol

A. Blocking and Incubation

1. Place each membrane into the provided eight-well tray (- means the antibody printed side).

2. Add 2 ml 1X Blocking Buffer and incubate at room temperature for 30 min to block membranes.

Note: incubation may be done at 4^0C for overnight.

3. 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 the lysate at least 10 folds with 1 X blocking buffer.***

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

Note: Incubation may be done at 4^0C 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. Please allow 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. Allow 5 min per wash. Dilute 20X Wash Buffer II with H_2O.

6. Prepare working solution for primary antibody.

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 4^0C for 2-3 days.

7. 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 4^0C for overnight.

8. Wash as directed in steps 4 and 5.

9. 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 the tube containing 1,000X HRP-Conjugated Streptavidin well before use since precipitation may form during storage.***

10. Incubate at room temperature for 2 hours.

Note: incubation may be done at 4^0C for overnight.

11. Wash as directed in steps 4 and 5.

B. Detection

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

1. Proceed with the detection reaction.

   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 onto the membrane and incubate at room temperature for ***2*** minutes. Ensure that the detection mixture is completely and evenly covering the membrane without any air bubbles.

2. Drain off any 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 with another piece of plastic sheet on the array. Gently smooth out any air bubbles. Avoid using pressure on the membrane.

3. Expose the array to x-ray film (we recommend to use Kodak x-omat AR film) and detect signal using film developer. Or the signal can be detected directly from the membrane using a chemiluminescence imaging system.

Expose the membranes for 40 seconds and 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.

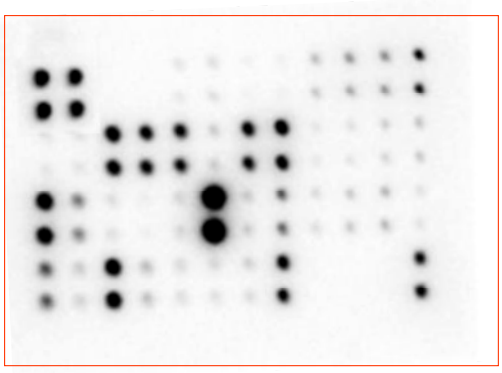
4. Save membranes in –20 ^{0}C to –80 ^{0}C for future reference.

V. Interpretation of Results:

The following figure shows RayBio® Rat Cytokine Antibody Array membranes probed with conditioned media from two different cell lines. Membranes were exposed to Kodak x-omat film at room temperature for 1 minute. 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, we suggest that several exposures be attempted. 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.

Typical results using RayBio® Cytokine Antibody arrays



By comparing the signal intensities, relative expression levels of cytokines can be made. The intensities of signals can be quantified by densitometry. The positive control can be used to normalize the results from the different membranes being compared. The signals also can be detected and quantified 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." Call RayBiotech, Inc. at 770-729-2992 for ordering information.

RayBio® Rat Cytokine Antibody Array 1

(Detect 19 rat cytokines in one experiment)

	a	b	c	d	e	f	g	h
1	Pos	Pos	Neg	Neg	CINC-2	CINC-3	CNTF	Fractalkine
2	Pos	Pos	Neg	Neg	CINC-2	CINC-3	CNTF	Fractalkine
3	GM-CSF	IFN-γ	IL-1α	IL-1β	IL-4	IL-6	IL-10	LIX
4	GM-CSF	IFN-γ	IL-1α	IL-1β	IL-4	IL-6	IL-10	LIX
5	Leptin	MCP-1	MIP-3α	β-NGF	TIMP-1	TNF-α	VEGF	BLANK
6	Leptin	MCP-1	MIP-3α	β-NGF	TIMP-1	TNF-α	VEGF	BLANK
7	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	Pos
8	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	Pos

RayBio® Rat Antibody Array 2

(Detect 34 cytokines in one experiment)

	A	B	C	D	E	F	G	H	I	J	K	L
1	POS	POS	NEG	NEG	Activin A	Agrin	B7-2/CD86	beta-NGF	CINC-1	CINC-2alpha	CINC-3	CNTF
2	POS	POS	NEG	NEG	Activin A	Agrin	B7-2/CD86	beta-NGF	CINC-1	CINC-2alpha	CINC-3	CNTF
3	Fas Ligand	Fractalkine	GM-CSF	ICAM-1	IFN-gamma	IL-1alpha	IL-1beta	IL-1 R6	IL-2	IL-4	IL-6	IL-10
4	Fas Ligand	Fractalkine	GM-CSF	ICAM-1	IFN-gamma	IL-1alpha	IL-1beta	IL-1 R6	IL-2	IL-4	IL-6	IL-10
5	IL-13	Leptin	LIX	L-Selectin	MCP-1	MIP-3alpha	MMP-8	PDGF-AA	Prolactin R	RAGE	Thymus Chemokine-1	TIMP-1
6	IL-13	Leptin	LIX	L-Selectin	MCP-1	MIP-3alpha	MMP-8	PDGF-AA	Prolactin R	RAGE	Thymus Chemokine-1	TIMP-1
7	TNF-alpha	VEGF	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	POS
8	TNF-alpha	VEGF	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	POS

* For use with serum, plasma, condition medium, urine, other body fluids, cell lysates and certain tissue lysates samples

RayBiotech, Inc., the protein array pioneer company, strives to research and develop new products to meet demands of the biomedical community. RayBio's patent-pending technology allows detection of over 180 cytokines, chemokines and other proteins in a single experiment. Our format is simple, sensitive, reliable and cost effective. Products include: Cytokine Arrays, Chemokine Arrays, ELISA kits, Phosphotyrosine kits, Recombinant Proteins, Antibodies, and custom services.

1. Antibody arrays
   Cytokine antibody array

Human cytokine antibody arrays
Mouse cytokine antibody arrays
Rat cytokine antibody arrays

Pathway- or disease-focused antibody arrays
Inflammation antibody array
Angiogensis antibody array
Chemokine antibody array
Growth factor antibody array
MMP antibody array
Atherosclerosis antibody array

Quantibody arrays for quantitative measurement of cytokine and other protein concentraton

Phosphorylation antibody arrays

Biotin label-based antibody arrays for high density antibody arrays.

Antibody analysis tool, software

2. ELISA
3. Cell-based phosphorylation assay
4. Custom antibody arrays
5. Antibody
6. Recombinant protein
7. Protein arrays

RayBiotech also provides excellent custom service:

1. Antibody arrays
2. Protein arrays
3. Peptide synthesis
4. Production of recombinant protein and antibody
5. Peptide arrays
6. Phosphorylation arrays
7. ELISA

Just simply send your samples and we will do the assay for you.

Technology transfer program
Have you developed technologies or reagents interested to the scientific and research community? RayBiotech can help you commercialize your technologies, reagents and dream.

VI. 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 HRP-streptavidin 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 dilute.	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 to x-ray file 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.

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Note:

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Cytokine protein arrays are RayBiotech patent-pending technology.

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