RayBio® Mouse Cytokine Antibody Array 6

Patent Pending Technology

User Manual (Revised June 14, 2009)

RayBio® Mouse Cytokine Antibody Array 6 (Cat# AAM-CYT-6)

RayBio® Mouse Cytokine Antibody Custom Array (Cat# AAM-CUST)
RayBio® Mouse Cytokine Antibody Array Service (Cat#AAM-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® Mouse Cytokine Antibody Array Protocol

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Cytokine Antibody 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® Mouse 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 6 can detect 97 mouse cytokines in a single experiment (same well). All experimental procedures are performed at the same conditions. This is the highest density array in any sandwich-based detection format including antibody arrays and bead-based assays.

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® Mouse 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.

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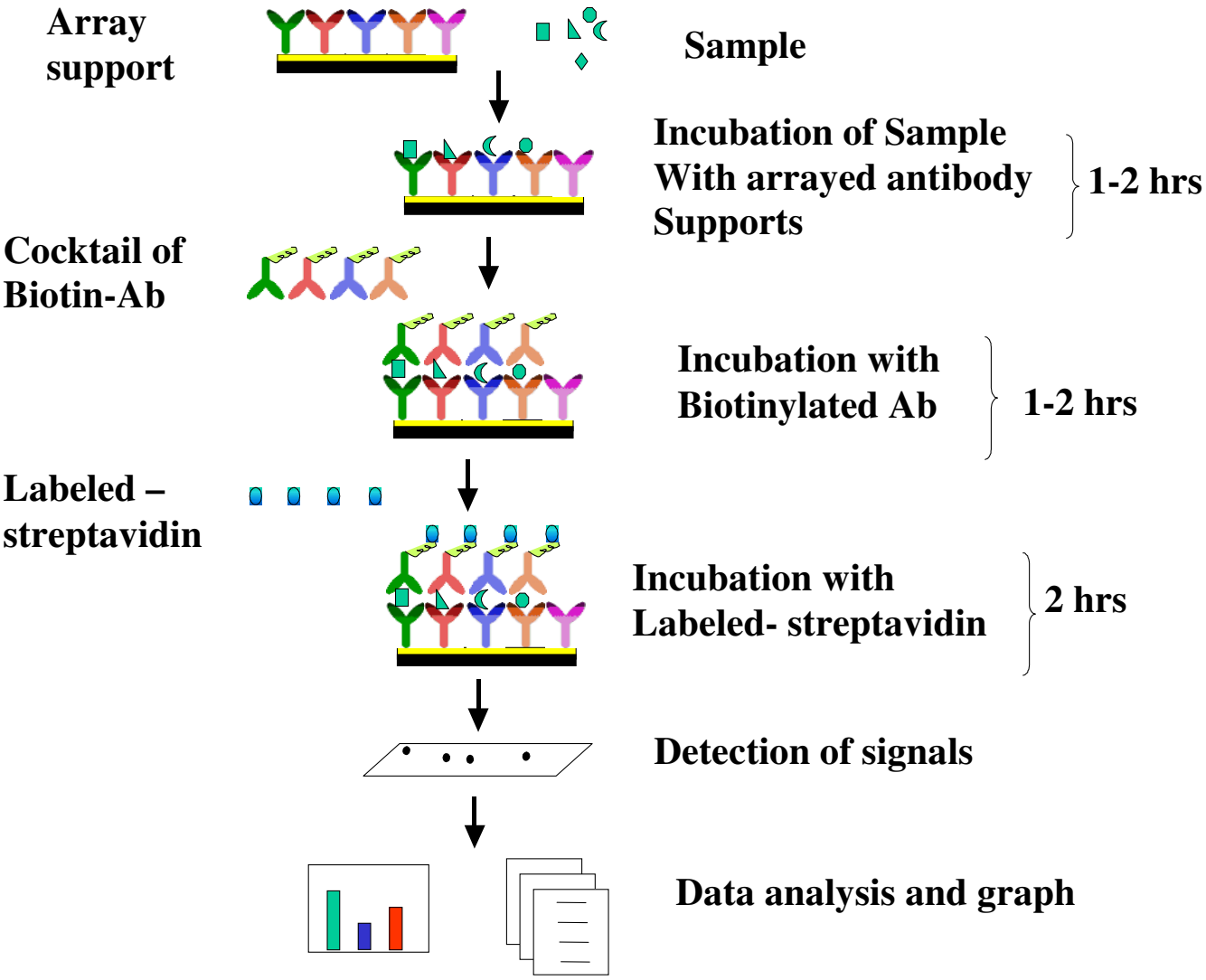
Here's how it works



II. Materials Provided

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

COMPONENT	AAM-CYT-6-2	AAM-CYT-6-4	AAM-CYT-6-8
Mouse Cytokine Array C6	2 membranes	4 membranes	8 membranes
Blocking Buffer	1 vial (25 ml)		2 vials (25 ml/ea)
Biotinylated Antibody Cocktail	1 vial	2 vials	4 vials
1,000X HRP-Streptavidin Concentrate	1 vial (50 µl)		2 vials (50 µl/ea)
20X Wash Buffer I Concentrate	1 vial (10 ml)	1 vial (20 ml)	2 vial (20 ml)
20X Wash Buffer II Concentrate	1 vial (10 ml)	1 vial (20 ml)	2 vial (20 ml)
2X Cell Lysis Buffer Concentrate	1 vial (10 ml)	1 vial (16 ml)	2 vial (16 ml)
Detection Buffer C	1 vial (1.5 ml)	1 vial (2.5 ml)	2 vial (2.5 ml)
Detection Buffer D	1 vial (1.5 ml)	1 vial (2.5 ml)	2 vial (2.5 ml)
4-Well Incubation Tray w/ Lid	1 tray		
Other Kit Components: Plastic Sheets, Array Map Template, User Manual			

Additional Materials Required

- Small plastic boxes or containers
- Orbital shaker
- Plastic sheet protector or Saran Wrap
- 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 media is required, use an uncultured media aliquot as a negative control sample, since many types of sera contain cytokines.
- For cell lysates and tissue lysates, we recommend using RayBio® Cell Lysis Buffer to extract proteins from cell or tissue (e.g. using homogenizer). Dilute 2X RayBio® Cell Lysis Buffer with H_2O (we recommend adding proteinase inhibitors to Cell Lysis Buffer before use). After extraction, spin the sample down and save the supernatant for your experiment. Determine protein concentration.
- We recommend using per membrane:
    - 2 ml of Conditioned media (undiluted), or
    - 2 ml of 2-fold to 5-fold diluted sera or plasma, or
    - 50-500 μg of total protein for cell lysates and tissue lysates (use ~200-250 μg of total protein for first experiment) ***Dilute the lysate at least 10 fold with 1 X blocking buffer.***

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 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°C for overnight.

IV. Protocol

A. Blocking and Incubation

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

2. Add 3 ml Blocking Buffer and incubate at room temperature for 30 min to block membranes. Make sure there are no bubbles between the membranes.

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

3. Decant Blocking Buffer from each container. Incubate membranes with 2 ml* of sample at room temperature for 1 to 2 hours. Dilute sample using Blocking Buffer if necessary.

** Note: We recommend using 2 ml of undiluted cell-culture conditioned media or 2 ml of 2-fold to 5-fold diluted sera or plasma or 50-500 µg of protein for cell lysates and tissue lysates.* ***Dilute the lysate at least 10 fold 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°C for overnight.

4. Dilute 20X Wash Buffer I with H_2O. Decant the samples from each container, and wash 3 times with 3 ml of 1X Wash Buffer I at room temperature with shaking. Please allow 5 min per wash.

5. Dilute 20X Wash Buffer II with H_2O. Wash 2 times with 3 ml of 1X Wash Buffer II at room temperature with shaking. Allow 5 min per wash.

6. Prepare working solution for primary antibody.

   Add 100 µl of Blocking Buffer to the Biotin-Conjugated Anti-Cytokines tube. Mix gently and transfer all mixture to a tube containing 4 ml of Blocking Buffer.

Note: the diluted biotin-conjugated antibodies can be stored at 4°C for 2-3 days.

7. Add 2 ml of diluted biotin-conjugated antibodies to each membrane. Incubate at room temperature for 1-2 hours.

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

8. Wash as directed in steps 4 and 5.

9. Add 3 ml of **1,000** fold diluted HRP-conjugated streptavidin (e.g. add **3** µl of HRP-conjugated streptavidin to **2997** µl 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°C 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 500 µl of 1X Detection Buffer ***C*** and 500 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 covers 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 the array with another piece of plastic sheet. Gently smooth out any air bubbles. Avoid using pressure on the membrane.

3. Expose to x-ray film (we recommend to use Kodak X-Omat AR film) and detect the 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. 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.

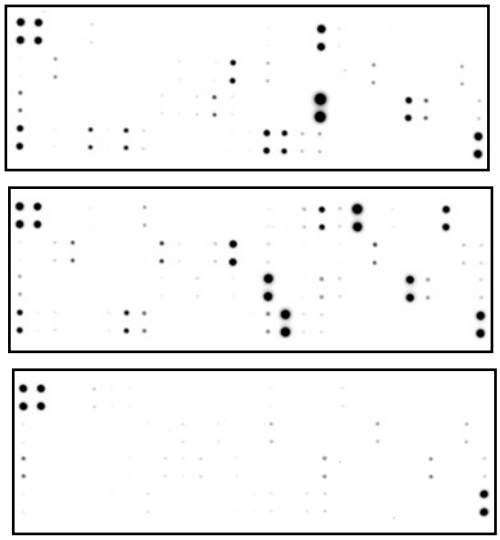
4. Save membranes in –20°C to –80°C for future reference.

V. Interpretation of Results:

The following figure shows RayBio® Mouse 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 array 6.



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 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® Antibody Arrays. This tool will not only assist in compiling and organizing your data, but reduces your calculations to a "copy and paste." Call RayBiotech, Inc. at 770-729-2992 for ordering information.

RayBio® Mouse Cytokine Antibody Array 6 (97)

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	POS	POS	NEG	NEG	6Ckine	ALK-1	Amphiregulin	Axl	BLC	Cardiotrophin-1	CD27	CD27 L	CD30	CD30 L
2	POS	POS	NEG	NEG	6Ckine	ALK-1	Amphiregulin	Axl	BLC	Cardiotrophin-1	CD27	CD27 L	CD30	CD30 L
3	Flt-3 Ligand	Fractalkine	Galectin-1	Gas 6	GCSF	GITR	GITR Ligand	Granzyme B	HAI-1	HGF	IFN gamma	IGFBP-5	IGFBP-6	IGF-II
4	Flt-3 Ligand	Fractalkine	Galectin-1	Gas 6	GCSF	GITR	GITR Ligand	Granzyme B	HAI-1	HGF	IFN gamma	IGFBP-5	IGFBP-6	IGF-II
5	IL-12 p70	IL-13	IL-15	IL-17	IL-17B R	IL-17E	IL-17F	IL-20	IL-21	I-TAC	JAM-A	KC	Leptin	Leptin R
6	IL-12 p70	IL-13	IL-15	IL-17	IL-17B R	IL-17E	IL-17F	IL-20	IL-21	I-TAC	JAM-A	KC	Leptin	Leptin R
7	Osteopontin	Osteoporotegerin	Prolactin	Pro-MMP-9	RANTES	SCF	sTNF RI	sTNF RII	TACI	TARC	TNF alpha	TPO	TRANCE	TROY
8	Osteopontin	Osteoporotegerin	Prolactin	Pro-MMP-9	RANTES	SCF	sTNF RI	sTNF RII	TACI	TARC	TNF alpha	TPO	TRANCE	TROY

	O	P	Q	R	T	U	V	W	X	Y	Z	AA	AB
1	CD36/SR-B3	CTLA-4	CXCL16	Decorin	Dkk-1	E-Cadherin	EGF	Eotaxin	Eotaxin-2	Epigen	E-Selectin	Fas Ligand	Fcg RIIB
2	CD36/SR-B3	CTLA-4	CXCL16	Decorin	Dkk-1	E-Cadherin	EGF	Eotaxin	Eotaxin-2	Epigen	E-Selectin	Fas Ligand	Fcg RIIB
3	IL-1 alpha	IL-1 beta	IL-1ra	IL-2	IL-2 R alpha	IL-3	IL-4	IL-5	IL-6	IL-9	IL-10	IL-11	IL-12 p40
4	IL-1 alpha	IL-1 beta	IL-1ra	IL-2	IL-2 R alpha	IL-3	IL-4	IL-5	IL-6	IL-9	IL-10	IL-11	IL-12 p40
5	L-Selectin	Lungkine	Mad CAM-1	MCP-1	MDC	MFG-E8	MIG	MIP-1alpha	MIP-1gamma	MIP2	MIP-3 alpha	MIP-3 beta	MMP-2
6	L-Selectin	Lungkine	Mad CAM-1	MCP-1	MDC	MFG-E8	MIG	MIP-1alpha	MIP-1gamma	MIP2	MIP-3 alpha	MIP-3 beta	MMP-2
7	TWEAK R	VCAM-1	VEGF	VEGF R1	VEGF R3	VEGF-D	Blank	Blank	Blank	Blank	Blank	Blank	POS
8	TWEAK R	VCAM-1	VEGF	VEGF R1	VEGF R3	VEGF-D	Blank	Blank	Blank	Blank	Blank	Blank	POS

* Columns A to AB are on a single membrane.

Abbreviations: Pos-positive control; Neg-negative control. All others use standard abbreviations.

Note: IL-12 reacts both IL-12p40 and IL-12p70. IL-12p70 only recognizes IL-12p70.

We also offer Custom Mouse Cytokine Antibody Arrays. You can select the cytokines of interest from the following list and we will produce the customized array at an affordable price. For more information, please visit our website **www.raybiotech.com**

RayBio® Mouse Custom Array Antibody List

Choose from 146 cytokines and other proteins

4-1BB	6Ckine	ACE	ALK-1	Amphiregulin	Axl
bFGF	BLC	Cardiotrophin-1	CD27	CD27 Ligand	CD30
CD30 Ligand	CD36	CD40	CD40 Ligand	Chordin	CRG-2
CTACK	CTLA-4	CXCL16	Decorin	DKK-1	DPPIV
Dtk	E-Cadherin	EGF	Endoglin	Eotaxin	Eotaxin-2
Epigen	Epiregulin	E-Selectin	FasLigand	Fc gamma RIIB	Flt-3 Ligand
Fractalkine	Galectin-1	Galectin-3	Galectin-7	GCSF	GITR
GITR Ligand	GM-CFS	Granzyme B	Growth arrest specific 1	Growth arrest specific 6	HAI-1
HGF	HGFR	ICAM-1	IFN gamma	IGFBP-2	IGFBP-3
IGFBP-5	IGFBP-6	IGF-I	IGF-II	IL-1 alpha	IL-1 beta
IL-1 R4/ST2L	IL-10	IL-11	IL-12 p40	IL-12 p70	IL-13
IL-15	IL-17	IL-17 B	IL-17 BR	IL-17 E	IL-17 F
IL-1ra	IL-2	IL-2 R alpha	IL-20	IL-21	IL-28/IFN-lambda
IL-3	IL-3 R beta	IL-4	IL-5	IL-6	IL-6 R
IL-7	IL-9	I-TAC	JAM-A	KC	Leptin R
LEPTIN(OB)	LIX	L-Selectin	Lungkine	Lymphotactin	MAdCAM-1
MCP1	MCP-5	M-CSF	MDC	MFG-E8	MIG
MIP-1alpha	MIP-1gamma	MIP-2	MIP-3 alpha	MIP-3 beta	MMP-2
MMP-3	Neprilysin	Osteopontin	Osteoporotegerin	Pentraxin 3/TSG-14	PF-4
Prolactin	Pro-MMP-9	P-Selectin	RAGE	RANTES	Resistin
SCF	SDF-1alpha	Shh-N	sTNF RI	sTNF RII	TACI
TARC	TCA-3	TECK	Thymus CK-1	TIMP-1	TIMP-2
TNF alpha	TPO	TRANCE	TREM-1	TROY	TSLP
TWEAK R	TWEAK	VCAM-1	VEGF	VEGF-D	VEGF R1
VEGF R2	VEGF R3				

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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