RayBio® Human Cytokine Antibody Array C Series 1000

Patent Pending Technology

User Manual (Revised 05152007)

RayBio® Human Cytokine Antibody Array C series 1000
(Combination of Array 6 & 7 Cat# AAH-CYT-1000)

RayBio® Human Cytokine Antibody Array 6 (Cat# AAH-CYT-6)
RayBio® Human Cytokine Antibody Array 7 (Cat# AAH-CYT-7)

Please read manual carefully before starting experiment

We Provide You with Excellent Protein Array Systems and Service

Tel:(Toll Free) 1-888-494-8555 or 770-729-2992; Fax: 1-888-547-0580;
Website:www.raybiotech.com Email: info@raybiotech.com

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RayBiotech, Inc.

RayBio® Human Cytokine Antibody Array C Series 1000 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® Human Cytokine Antibody Array is the first commercially available 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.

Traditionally, cytokines are detected by using ELISA; however, RayBiotech's approach has several advantages over ELISA. First and most importantly is that our 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

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ELISA. Therefore, the detection range is greater with protein array compared with ELISA. Additionally, 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 techniques.

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 Array, is particularly useful in comparison 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 [12]. 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.

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

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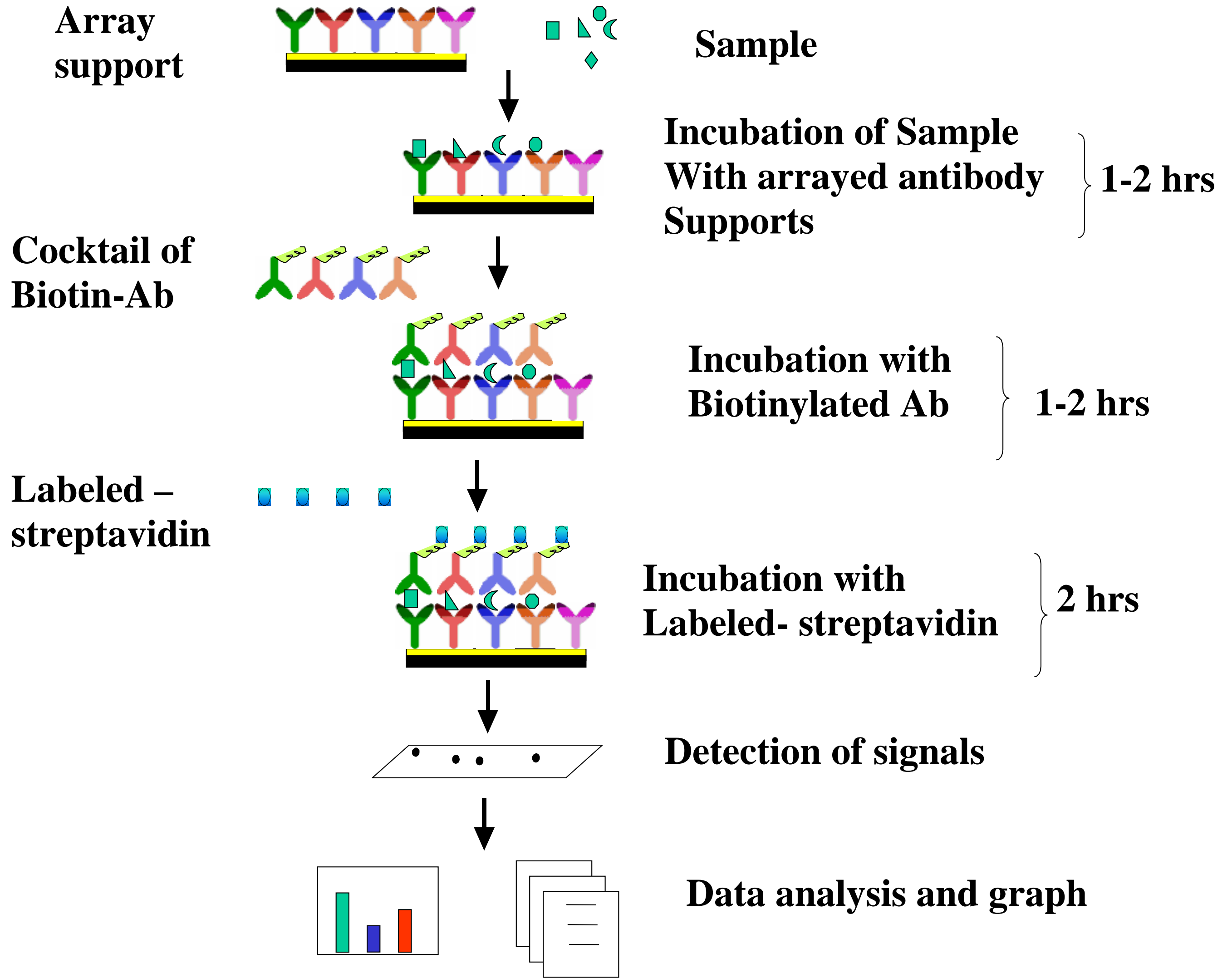
Here's how it works



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II. Materials Provided

Upon receipt, all components of the RayBio® Human 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 component 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® Human Cytokine Antibody Array membranes (2/4/8 array membranes 6 and 2/4/8 array membranes 7).
- Biotin-Conjugated Anti-Cytokines (1/2/4 tubes, each tube for two membranes)
- 1,000X HRP-Conjugated Streptavidin (50 µl)
- 1X Blocking Buffer (25/50 ml)
- 20X Wash Buffer I (10/20 ml)
- 20X Wash Buffer II (10/20 ml)
- 2X Cell Lysis Buffer (10/20 ml)
- Detection Buffer C (1.5/2.5 ml)
- Detection Buffer D (1.5/2.5 ml)
- 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

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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 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 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 8 (biotin-Ab incubation) or step 11 (HRP-streptavidin incubation) may be done at 4^0C for overnight. Please make sure to cover the 8 well plate tightly to prevent evaporation.

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IV. Protocol

A. Blocking and Incubation

1. Place one array membrane **6** (top left corner marked with "**-**") and one array membrane **7** (top left corner marked with "+") into same well of the provided eight-well tray ("-" or "+" marked side is the antibody printed side).

2. Add 2 ml 1X Blocking Buffer and incubate at room temperature for 30 min to block membranes. Add some Blocking Buffer between the two membranes. Make sure there are no bubbles between membranes.

3. Decant Blocking Buffer from each container, and incubate membranes with sample at room temperature for 1 to 2 hours. Dilute sample using 1X Blocking Buffer if necessary.

Note: We recommend using 1.2 ml of conditioned media or 1.2 ml of original or 10-fold diluted sera or plasma or 50-500 ug of protein for cell lysates and tissue lysates. ***Dilute the lysate at least 10 folds with 1 X blocking buffer. Add some samples between array membrane 6, 7 and 8. Make sure there are no bubbles between membranes.***

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 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.

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6. **From this step, place array membrane 6 (marked with “-”) into one well and array membrane 7 (with “+”) into another well**.

7. Prepare working solution for biotin-conjugated antibodies.

   Add 100 µl of 1x blocking buffer to the Biotin-Conjugated Antibody 6 tube. Mix gently and transfer all mixture to a tube containing 2 ml of 1x blocking buffer.

   Add 100 µl of 1x blocking buffer to the Biotin-Conjugated Antibody 7 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.

8. Add 1 ml of diluted biotin-conjugated antibodies to each membrane (1 ml of diluted biotin-conjugated antibodies 6 to array membrane 6 marked with “-” and 1 ml of diluted biotin-conjugated antibodies 7 to array membrane 7 marked with “+”). Incubate at room temperature for 1-2 hours.

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

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12. 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 ("-"or "+" mark is on the protein side top left corner) on a clean plastic sheet (provided in the kit). Transfer 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 ("-" or "+"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 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

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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 references.

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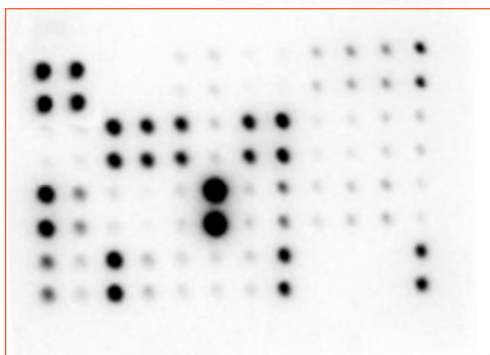
V. Interpretation of Results:

The following figure shows RayBio® Human Cytokine Antibody Array membranes C series 2000 probed with different patient's plasma. 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 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

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RayBio® Human Cytokine Antibody Array 6 (60)

	a	b	c	d	e	f	g	h	i	j	k	l	m	n
1	POS	POS	NEG	NEG	Blank	Angiogenin	BDNF	BLC	BMP-4	BMP-6	CK β 8-1	CNTF	EGF	Eotaxin
2	POS	POS	NEG	NEG	Blank	Angiogenin	BDNF	BLC	BMP-4	BMP-6	CK β 8-1	CNTF	EGF	Eotaxin
3	Eotaxin-2	Eotaxin-3	FGF-6	FGF-7	Fit-3 Ligand	Fractalkine	GCP-2	GDNF	GM-CSF	I-309	IFN-γ	IGFBP-1	IGFBP-2	IGFBP-4
4	Eotaxin-2	Eotaxin-3	FGF-6	FGF-7	Fit-3 Ligand	Fractalkine	GCP-2	GDNF	GM-CSF	I-309	IFN-γ	IGFBP-1	IGFBP-2	IGFBP-4
5	IGF-I	IL-10	IL-13	IL-15	IL-16	IL-1α	IL-1β	IL-1ra	IL-2	IL-3	IL-4	IL-5	IL-6	IL-7
6	IGF-I	IL-10	IL-13	IL-15	IL-16	IL-1α	IL-1β	IL-1ra	IL-2	IL-3	IL-4	IL-5	IL-6	IL-7
7	Leptin	LIGHT	MCP-1	MCP-2	MCP-3	MCP-4	M-CSF	MDC	MIG	MIP-1δ	MIP-3α	NAP-2	NT-3	PARC
8	Leptin	LIGHT	MCP-1	MCP-2	MCP-3	MCP-4	M-CSF	MDC	MIG	MIP-1δ	MIP-3α	NAP-2	NT-3	PARC
9	PDGF-BB	RANTES	SCF	SDF-1	TARC	TGF-β1	TGF-β 3	TNF-α	TNF-β	Blank	Blank	Blank	Blank	POS
10	PDGF-BB	RANTES	SCF	SDF-1	TARC	TGF-β1	TGF-β 3	TNF-α	TNF-β	Blank	Blank	Blank	Blank	POS

RayBio® Human Cytokine Antibody Array 7 (60)

	a	b	c	d	e	f	g	h	i	j	k	l	m	n
1	POS	POS	NEG	NEG	Blank	Acrp30	AgRP	Angiopoietin-2	Amphiregulin	Axl	bFGF	b-NGF	BTC	CCL-28
2	POS	POS	NEG	NEG	Blank	Acrp30	AgRP	Angiopoietin-2	Amphiregulin	Axl	bFGF	b-NGF	BTC	CCL-28
3	CTACK	Dtk	EGF-R	ENA-78	Fas/TNFRSF6	FGF-4	FGF-9	GCSF	GITR-Ligand	GITR	GRO	GRO-α	HCC-4	HGF
4	CTACK	Dtk	EGF-R	ENA-78	Fas/TNFRSF6	FGF-4	FGF-9	GCSF	GITR-Ligand	GITR	GRO	GRO-α	HCC-4	HGF
5	ICAM-1	ICAM-3	IGFBP-3	IGFBP-6	IGF-I SR	IL-1 R4/ST2	IL-1 RI	IL-11	IL-12 p40	IL-12 p70	IL-17	IL-2 R alpha	IL-6 R	IL-8
6	ICAM-1	ICAM-3	IGFBP-3	IGFBP-6	IGF-I SR	IL-1 R4/ST2	IL-1 RI	IL-11	IL-12 p40	IL-12 p70	IL-17	IL-2 R alpha	IL-6 R	IL-8
7	I-TAC	Lymphotactin	MIF	MIP-1α	MIP-1β	MIP-3β	MSP-α	NT-4	Osteoprotegerin	Oncostatin M	PIGF	sgp130	sTNF RII	sTNF-RI
8	I-TAC	Lymphotactin	MIF	MIP-1α	MIP-1β	MIP-3β	MSP-α	NT-4	Osteoprotegerin	Oncostatin M	PIGF	sgp130	sTNF RII	sTNF-RI
9	TECK	TIMP-1	TIMP-2	Thrombopoietin	TRAIL R3	TRAIL R4	uPAR	VEGF	VEGF-D	Blank	Blank	Blank	Blank	POS
10	TECK	TIMP-1	TIMP-2	Thrombopoietin	TRAIL R3	TRAIL R4	uPAR	VEGF	VEGF-D	Blank	Blank	Blank	Blank	POS

Abbreviations: IP-10, Interferon-inducible protein-10; LAP, latency associated peptide (TGF-β1); LIF, leukocyte inhibitory factor. MMP, Matrix Metalloproteinase; Pos, positive control; Neg, negative control. All other are used standard abbreviations.

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

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We also offer Custom Human 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**.

4-1BB/TNFRSF9	adiponectin/Acrp30	AgRP(ART)	ALCAM	ANGIOGENIN	Angiopoietin-1
Angiopoietin-2	Angiostatin	AR (amphiregulin)	Axl	B7-1(CD80)	BDNF
bFGF	BLC	BMP-4	BMP-6	BMP-7	b-NGF
BTC	Cardiotrophin-1	CCL28/VIC	CD27	CD30	CD40
CD40 Ligand	Ck beta 8-1	CNTF	CTACK/CCL27	CTLA-4	CXCL16
Dkk-4	DR6	Dtk	EGF	EGF R	ENA-78
Endostatin	Eotaxin	Eotaxin-2	Eotaxin-3	E-Selectin	Fas/TNFRSF6
FGF-4	FGF-6	FGF-7	FGF-9	Flt-3 Ligand	Follistatin
Fractalkine	GCP-2	GCSF	GDNF	GITR Ligand/TNFSF18	GITR/TNFRF18
GM-CSF	GRO	HB-EGF	HCC-4/CCL16	HGF	HVEM
I-309	ICAM-1	ICAM-2	ICAM-3	IFN-gamma	IGFBP-1
IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-6	IGF-I	IGF-I SR
IGF-II	IL-1 R4/ST2	IL-1 sRI	IL-1 sRII	IL-1 alpha	IL-1 beta
IL-1ra	IL-2	IL-2 R alpha	IL-2 R beta	IL-2 R gamma	IL-2 sR alpha
IL-3	IL-4	IL-5	IL-5 R alpha	IL-6	IL-6 sR
IL-7	IL-8	IL-9	IL-9 R	IL-10	IL-10 R alpha
IL-10 R beta	IL-11	IL-12	IL-12 p40	IL-12 p70	IL-13
IL-13 R alpha	IL-15	IL-16	IL-17	IL-18 BP alpha	IL-18 R alpha
IL-18 R beta	IL-21 R	IP-10	I-TAC/CXCL11	LAP(TGF-b1)	LEPTIN(OB)
LIF	LIGHT	L-Selectin	Lymphotactin	MCP-1	MCP-2
MCP-3	MCP-4	M-CSF	M-CSF R	MDC	MIF
MIG	MIP-1 alpha	MIP-1 beta	MIP-1 gamma	MIP-3 alpha	MIP-3 beta
MMP-1	MMP-2	MMP-3	MMP-9	MMP-10	MMP-13
MPIF-1	MSP	NAP-2	NGF R	NT-3	NT-4
ONCOSTATIN M	Osteoprotegerin	PARC	PDGF R alpha	PDGF R beta	PDGF-AA
PDGF-BB	PECAM-1	PF4	PIGF	Prolactin	P-selectin
RANTES	SCF	SCF R	SDF-1	sgp130	ST2
sTNF RII/TNFRS1B	sTNT RI/TNFRS1A	Tarc	TECK/CCL25	TGF-alpha	TGF-beta
TGF-beta 2	TGF-beta 3	Tie-1	Tie-2	TIMP-1	TIMP-2
TIMP-3	TIMP-4	TNF-alpha	TNF-beta	TPO	TRAIL R1
TRAIL R2	TRAIL s R3/TNFRS10C	TRAIL s R4/TNFRS10D	u PAR	VCAM-1	VE-Cadherin
VEGF	VEGF-D	VEGF R2	VEGF R3		

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

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2. 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

   Antibody analysis tool, software

3. ELISA
4. Cell-based phosphorylation assay
5. Custom antibody arrays
6. Antibody
7. Recombinant protein
8. Cytokine protein arrays
9. Quantibody arrays for quantitative measurement of cytokine and other protein concentration.

10. Phosphorylation antibody arrays

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

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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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