RayBio® Human Angiogenesis Antibody Array G Series 1000

Patent Pending Technology

User Manual (Revised February 18, 2009)

Combination of Human Angiogenesis Antibody Array G Series 1 & 2
(Cat# AAH-ANG-G1000-4)
RayBio® Human Angiogenesis Antibody Array G Series 1 (Cat# AAH-ANG-G1-4)
RayBio® Human Angiogenesis Antibody Array G Series 2 (Cat# AAH-ANG-G2-4)

Combination of Human Angiogenesis Antibody Array G Series 1 & 2
(Cat# AAH-ANG-G1000-8)
RayBio® Human Cytokine Antibody Array G Series 1 (Cat# AAH-ANG-G1-8)
RayBio® Human Cytokine Antibody Array G Series 2 (Cat# AAH-ANG-G2-8)

Please read the manual carefully before you start your experiment

RayBiotech, Inc.

We Provide You With Excellent
Protein Array Systems And Service

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RayBiotech, Inc.

RayBio® Human Angiogenesis Antibody Array G 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 many genes and signaling pathways. New techniques such as cDNA microarrays have enabled us to analyze the global gene expression [1-3]. 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 [4]. Therefore, it is critical to analyze the protein profile. 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 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.

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 (G series) is a glass chip format. The kit provides a highly sensitive approach to simultaneously detect multiple cytokine expression levels from cell culture supernatant, patient's serum, tissue lysate and other sources. The arrays are manufactured using non-contact arrayer. The experimental procedure is simple and can be performed in any laboratory. The signals from G series arrays are detected using a laser scanner.

The RayBio® Human Angiogenesis Antibody Array G series 1000 can detect 43 human angiogenic factors in single experiment. RayBiotech also provides RayBio® Human Cytokine Antibody Array G series 4000 which is the only product available in the market that can detect 274 human cytokines in single experiment.

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 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 [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.

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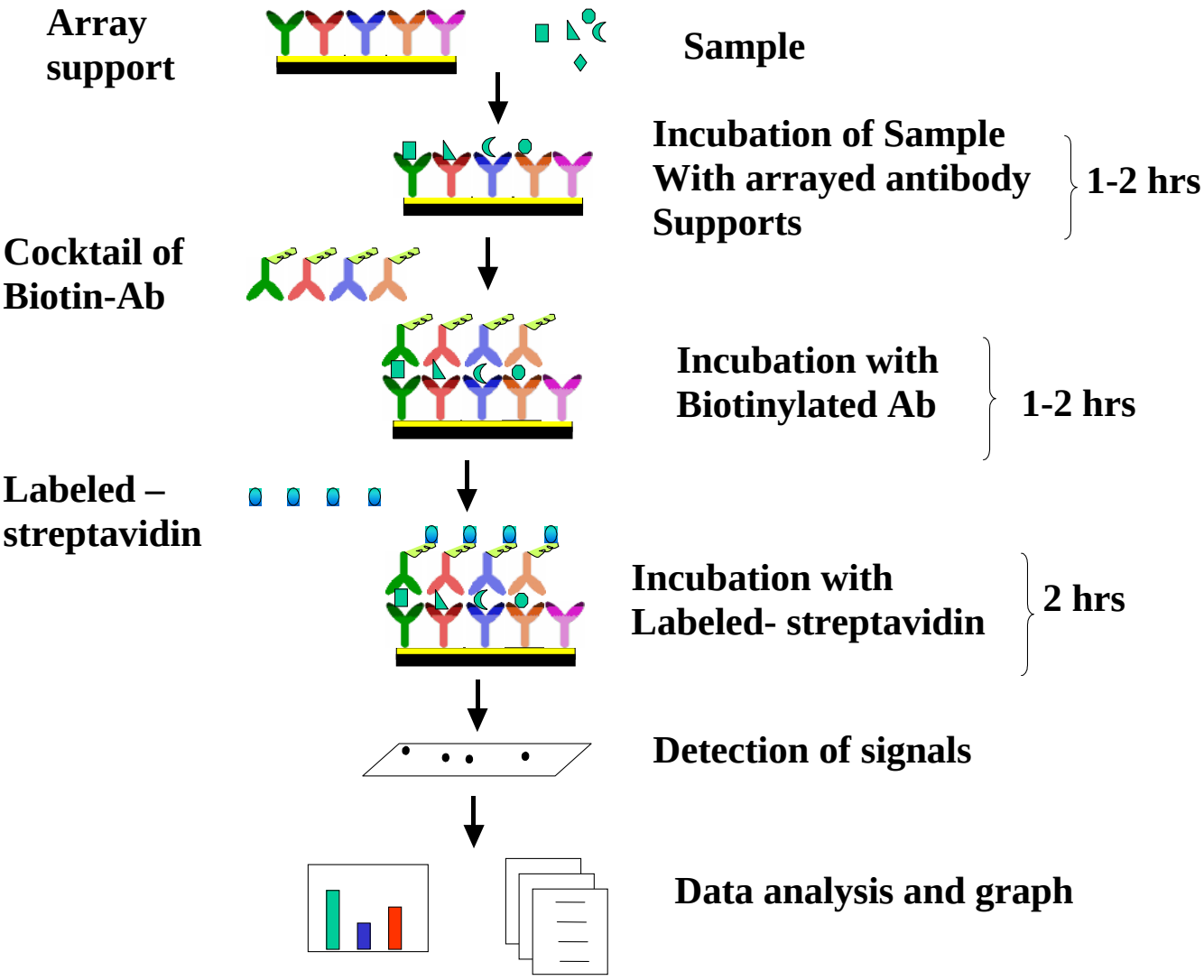
Here's how it works



II. Materials Provided

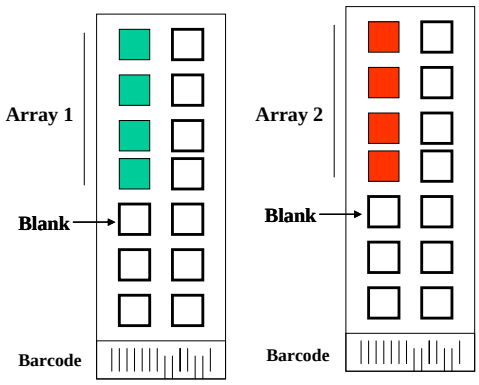
Upon receipt, all components of the RayBio® Human Angiogenesis Antibody Array kit should be stored at -20^{0}C. At -20^{0}C the kit will retain complete activity for up to 6 months. Once thawed, the glass chips, Fluorescent dye-streptavidin, Internal Control and 2X Blocking Buffer should be kept at –20^{0}C and all other component should be stored at 4^{0}C. Use within three months after reagents have been thawed. Please use within six months of purchase.

- RayBio® Human Angiogenesis Antibody Microarray slides (1 slides with 4 or 8 subarrays each: Angiogenesis 1 and Angiogenesis 2)
- Biotin-Conjugated Anti-Cytokines (1 tube/ 4 subarrays: 1 or 2 tubes for Angiogenesis 1 and 1 or 2 tubes for Angiogenesis 2)
- 1,500X Fluorescent Dye-conjugated Streptavidin (Cy3 equivalent, 1 tube)
- 2X Blocking Buffer (5 ml)
- 20X Wash Buffer I (30ml)
- 20X Wash Buffer II (30ml)
- Internal control (powder, 1 tube)
- 2X Cell Lysis Buffer (10 ml)
- RayBio® G series antibody array accessory (including slide incubation chamber, Gasket, Protective cover, Snap-on sides and adhesive film)
- 30 ml tube
- Manual

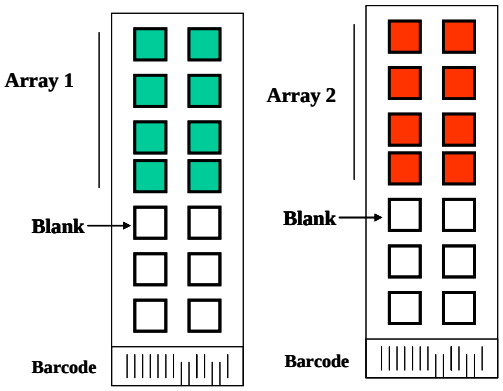
Additional Materials Required

- Orbital shaker
- Laser scanner for fluorescence detection
- Aluminum foil
- Distilled water
- Plastic box

Layout of G series 1000



4 arrays in one glass chip



8 arrays in one glass chip

III. Overview and General Considerations

A. Preparation of Samples

- Use serum-free conditioned media if possible.
- If serum-containing conditioned media is required, use uncultured media 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:
  - 50–100 µl of Conditioned media (undiluted), or
  - 50–100 µl of 2-fold to 5-fold diluted sera or plasma, or
  - 10–200 µg of total protein for cell lysates and tissue lysates.

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

B. Handling glass chips

- The microarray slides are sensitive, do not touch the surface. Grip the slides by the edges only.
- Handle all buffers and slides with powder-free gloves.
- Avoid breaking glass slide.
- Handle glass chip in clean environment.

C. Incubation

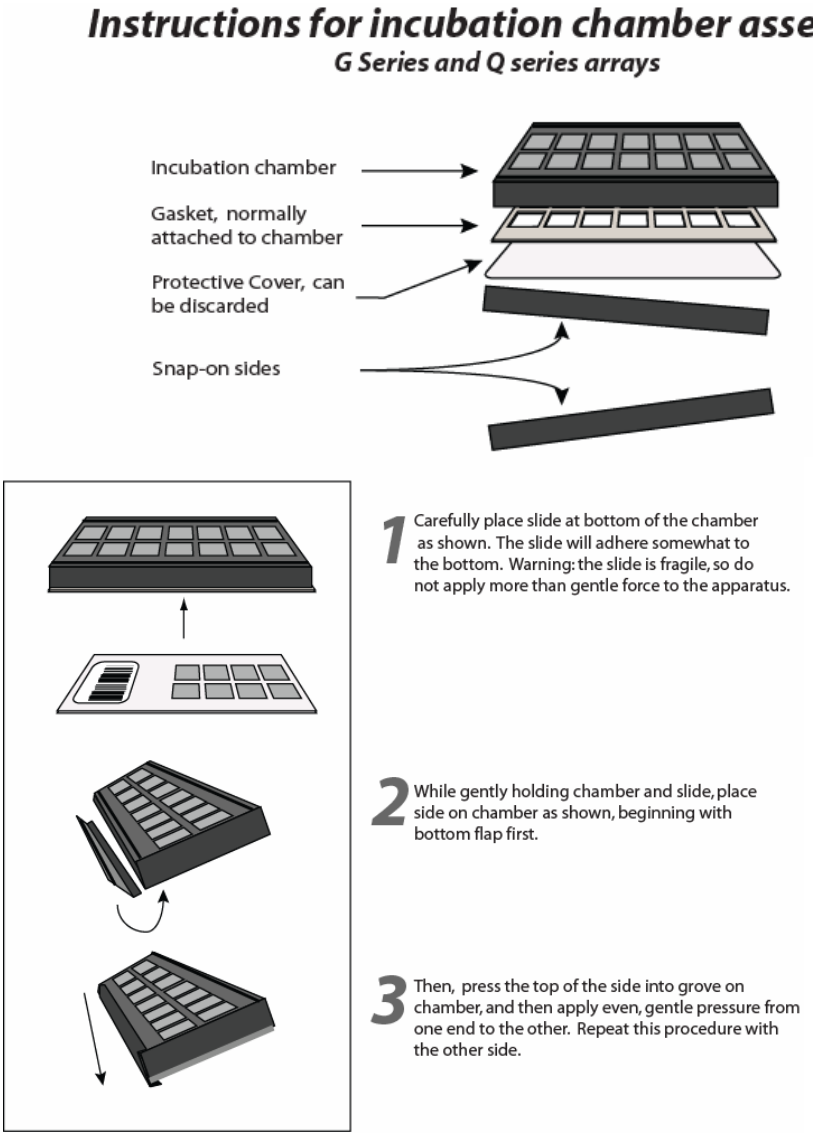
- Completely cover array area with sample or buffer during incubation, and cover the incubation chamber with adhesive film or plastic sheet protector to avoid drying.
- Avoid foaming during incubation steps.
- Perform all incubation and wash steps under gentle rotation.
- Cover the incubation chamber with adhesive film during incubation, particularly when incubation is more than 2 hours or 50 µl of sample or reagent is used.
- Avoid cross-contamination from overflowing solution to neighboring wells.
- Several incubation steps such as step 3 (blocking), step 4 (sample incubation), step 9 (biotin-Ab incubation) or step 12 (Fluorescent dye-streptavidin incubation) may be done at 4°C for overnight. Please make sure to cover the incubation chamber tightly to prevent evaporation.

IV. Protocol

A. Blocking and Incubation

1. Take the glass chip out from the box. Let air dry for 2 hours.

2. Assemble the glass chip into incubation chamber and incubation frame as shown below. (Note: if you slide has be assembled, you can go to step 3 directly).



3. Add 100 µl 1 X Blocking Buffer into each well and incubate at room temperature for 30 min to block slides. Dilute 2X Blocking Buffer with H_2O. Make sure no bubbles are in the well.

Note: only add reagents to wells printed with antibodies.

4. Decant Blocking Buffer from each well. Add 50 to 100 µl of each sample to array 1 and array 2. Incubate arrays with sample at room temperature for 1 to 2 hours. Dilute sample using 1X Blocking Buffer if necessary. We strongly recommend including Internal Control (IC) in your assay. Add 100 µl of Blocking Buffer to IC tube, mix well and transfer 1 µl of IC to each well (50 to 100 µl of sample).

Note: when you transfer IC, use 0.1 µl to 2 µl pipettor.

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

Note: We recommend using 50 to 100 µl of conditioned media or 50 to 100 µl of 2-5 fold diluted serum or plasma or 10-200 µg of protein for cell lysates and tissue lysates. ***Dilute the lysate at least 10 fold with 1 X blocking buffer to make a total volume of 50 to 100 µl. Make sure there are no bubbles in the wells.***

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

5. Decant the samples from each well, and wash 3 times with 150 µl of 1X Wash Buffer I at room temperature with gentle shaking. 2 min per wash. Dilute 20X Wash Buffer I with H_2O. Completely remove Wash Buffer I in each wash step.

Note: avoid solution flowing into neighboring wells.

6. Put the glass chip with frame into a box with 1X Wash Buffer I (cover the whole glass slide and frame with Wash Buffer I), and wash 2 times at room temperature with gentle shaking for 10 min per wash.

7. Decant the 1X Wash Buffer I from each well, Put the glass chip with frame into the box with 1X Wash Buffer II (cover the whole glass slide and frame with Wash Buffer II), and wash 2 times at room temperature with gentle shaking for 5 min per wash. Remove all of Wash Buffer II in the well. Dilute 20X Wash Buffer II with H_2O.

8. Prepare working solution for biotin-conjugated antibodies. After brief spinning,

A. Add 300 µl of 1x blocking buffer to the Biotin-Conjugated Antibody 1 tube. Mix gently.

B. Add 300 µl of 1x blocking buffer to the Biotin-Conjugated Antibody 2 tube. Mix gently.

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

9. Add 70 µl of diluted biotin-conjugated antibodies to each corresponding well (70 µl of diluted biotin-conjugated antibodies 1 to each subarray 1, 70 µl of diluted biotin-conjugated antibodies 2 to each subarray 2). Incubate at room temperature for 2 hours.

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

10. Wash as directed in steps 5 and then wash 3 times with 150 µl of 1X Wash Buffer II at room temperature with shaking. 2 min per wash. Completely remove wash buffer II in each wash step.

11. Add 70 µl of 1,500 fold diluted Fluorescent dye-conjugated streptavidin (after brief spinning, add 1.5 ml of Blocking Buffer to Fluorescent dye-conjugated streptavidin tube) to each subarray. Cover the incubation chamber with Adhesive film. Cover the plate with aluminum foil to avoid exposure to light or incubate in dark room.

12. Incubate at room temperature for 1 to 2 hours.

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

13. Wash with Wash Buffer I **twice** as directed in steps 5.

B. Fluorescence Detection

1. Decant excess Wash Buffer from wells.

2. Disassemble the slide out of the incubation frame and chamber.

3. Place the whole slide in 30 ml centrifuge tube provided, add enough Wash Buffer I (about 20 ml) to cover the whole slide and gently shake at room temperature for 10 minutes. Decant Wash Buffer I. Repeat Wash Buffer I once. Wash with Wash Buffer II (about 20 ml) with gentle shake at room temperature for 10 minutes. Or wash using slide chamber. Rinse the slide with distilled H_2O.

4. Remove water droplets by centrifuge at 1,000 rpm for 3 minutes and then let slide dry completely in air at least 20 minutes (protect from light). Make sure the slides are absolutely dry before the scanning procedure.

5. Image the signals using laser scanner such as Axon GenePix using cy3 or "green" channel (Excitation frequency = 532 nm).

Note: we recommend scanning slides right after experiment. You also can store the slide at –20^{0}C in dark for several days. If you do not have a laser scanner, we can provide service for you. Just simply send your slide to us and we will take care of it.

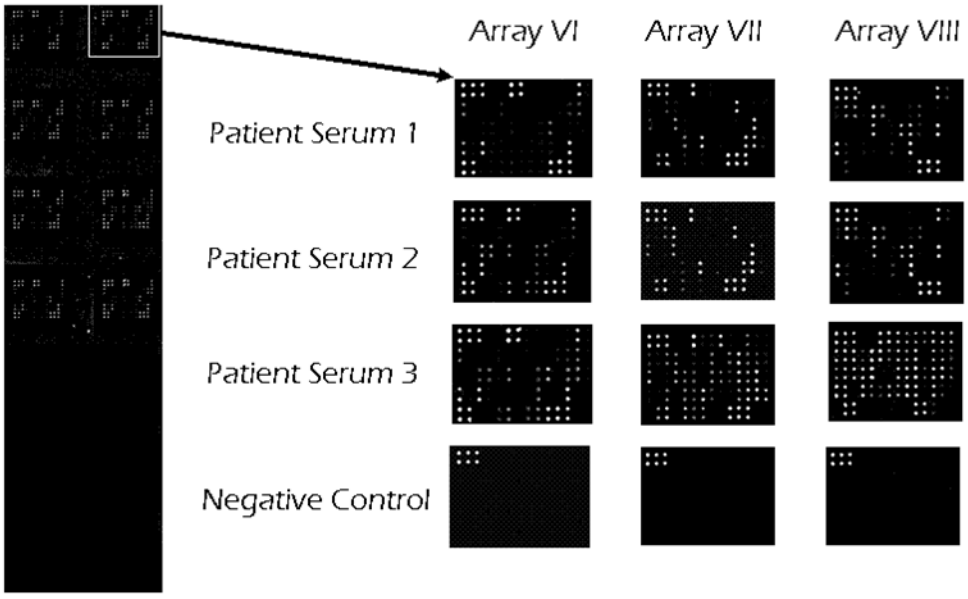
V. Interpretation of Results:

The following figure shows RayBio® Human Cytokine Antibody Array G series 2000 probed with different cell culture supernatant. The images were captured using laser scanner. The biotin-conjugated protein produces positive signals, which can be used to identify the orientation and to compare the relative expression levels among the different wells. The internal control (IC) can also be used to normalize the signal intensities among array membranes in different experiments.

The signal intensities obtained from laser scanner can simply be imported into our analysis tool. The analysis tool will help you:

- Locate your signal intensities to antibody array map
- Link the protein to website for more detailed information on the particular protein
- Protein list sorting
- Average signal intensities
- Subtract background
- Normalize the data from different samples
- Obtain protein level comparison charts among different samples

This analysis tool is very simple and affordable, which will not only assist in compiling and organizing your data, but also reduces your calculations to a "copy and paste" step.



If you do not use our **RayBio® Analysis Tool,** you can locate the cytokines by referring to RayBio® Human Angiogenesis Antibody Array G series 1000. Please keep in mind that G series 1000 consists of two individual arrays; human angiogenesis antibody array G 1 and human angiogenesis antibody array G 2. Refer to corresponding map (see next page).

Normalization and comparison

For biomarker discovery or for analysis of large number of arrays, great attention must be paid to the normalization. Our antibody array design includes several controls for normalization and comparison of arrays performing in different membranes and different experiments (for more information please read the reference 17).

Positive control. Positive control is biotinylated protein. It can be used to normalize the streptavidin incubation step. If the positive signals from different array membranes are similar, positive control is a simple and effective way for normalization.

Internal control. RayBio® antibody arrays also include spiking-in protein serving as internal control (IC). The spiking-in proteins do not have cross-reactivity with protein in the array. It can be used to normalize the entire process.

Negative control. Negative control is BSA. Normally, it should only give a background reading.

Data Extraction Tips:

- Ignore any comet tails
- Define the area for signal capture for all spots as 110-120 micron diameter, using the same area for every spot.
- Use median signal value, not the total or the mean
- Use local background correction (also median value).
- Exclude obvious outlier data in its calculations.

Using these guidelines, along with using PMT, brightness and contrast settings that reduce the background as much as possible, we get very good results with interassay and intraassay CV <20%, even with some imperfections in the antibody spots.

Threshold of significant difference in expression: Any ≥1.5-fold increase or ≤0.65-fold decrease in signal intensity for a single analyte between samples, provided that both signals are well above background (Mean background + 2 standard deviations, accuracy ≈ 95%).

RayBio® Human Angiogenesis Antibody Array G Series 1000

Combine Human Angiogenesis Antibody Array G series 1 and 2 to detect 43 angiogenic factors in one experimant

RayBio® Human Angiogenesis Antibody Array G series 1

(simultaneous detection of 20 angiogenic factors)

	a	b	c	d	e	f	g	h
1	POS-1	POS-2	POS-3	NEG	NEG	Angiogenin	EGF	ENA-78
2	POS-1	POS-2	POS-3	NEG	NEG	Angiogenin	EGF	ENA-78
3	b FGF	GRO	IFN-gamma	IGF-I	IL-6	IL-8	LEPTIN	MCP-1
4	b FGF	GRO	IFN-gamma	IGF-I	IL-6	IL-8	LEPTIN	MCP-1
5	PDGF-BB	PIGF	RANTES	TGF-beta1	TIMP-1	TIMP-2	Thrombopoietin	VEGF
6	PDGF-BB	PIGF	RANTES	TGF-beta1	TIMP-1	TIMP-2	Thrombopoietin	VEGF
7	VEGF-D	IC1	IC2	IC3	NEG	NEG	NEG	NEG
8	VEGF-D	IC1	IC2	IC3	NEG	NEG	NEG	NEG

RayBio® Human Angiogenesis Antibody Array G series 2

(simultaneous detection of 23 angiogenic factors)

	a	b	c	d	e	f	g	h
1	POS-1	POS-2	POS-3	NEG	NEG	Angiopoietin-1	Angiopoietin-2	Angiostatin
2	POS-1	POS-2	POS-3	NEG	NEG	Angiopoietin-1	Angiopoietin-2	Angiostatin
3	Endostatin	G-CSF	GM-CSF	I-309	IL-10	IL-1alpha	IL-1beta	IL-2
4	Endostatin	G-CSF	GM-CSF	I-309	IL-10	IL-1alpha	IL-1beta	IL-2
5	IL-4	I-TAC	MCP-3	MCP-4	MMP-1	MMP-9	PECAM-1	Tie-2
6	IL-4	I-TAC	MCP-3	MCP-4	MMP-1	MMP-9	PECAM-1	Tie-2
7	TNF-alpha	u PAR	VEGF R2	VEGF R3	IC-1	IC-2	IC-3	NEG
8	TNF-alpha	u PAR	VEGF R2	VEGF R3	IC-1	IC-2	IC-3	NEG

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.

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**.

Human Custom Antibody Array List (285 proteins)

4-1BB/TNFRSF9	CNTF	GDNF	IL-18 R alpha	MIP-1 alpha	SCF
ACE-2	Cripto-1	GITR	IL-18 R beta	MIP-1 beta	SCF R
Activin A	CRP	GITR Ligand	IL-1ra	MIP-1 delta	SDF-1 alpha
Adiponectin/Acrp30	CTACK/CCL27	GM-CSF	IL-2	MIP-3 alpha	SDF-1 beta
Adipsin/Factor D	CTLA-4	GRO	IL-2 R alpha	MIP-3 beta	sgp130
AFP	CXCL16	GRO-a	IL-2 R beta	MMP-1	Shh N
AgRP(ART)	DAN	Growth Hormom	IL-2 R gamma	MMP-2	Siglec-5
ALCAM	Decorin	HB-EGF	IL-21 R	MMP-3	Siglec-9
Angiogenin	DKK-1	HCC-4/CCL16	IL-22	MMP-7	sTNF RII
Angiopoietin-1	DKK-3	hCGa, intact	IL-28A/IFN-lambda	MMP-8	sTNT RI
Angiopoietin-2	DKK-4	HGF	IL29/IFN-lambda 1	MMP-9	TACE
Angiostatin	DPPIV/CD26	HVEM	IL-3	MMP-10	TARC
ANGPTL4	DR6	I-309	IL-31	MMP-13	TECK/CCL25
AR (amphiregulin)	Dtk	ICAM-1	IL-4	MPIF-1	TGF-alpha
Axl	E-Cadherin	ICAM-2	IL-5	MSP a Chain	TGF-beta 1
B7-1(CD80)	EDA-A2	ICAM-3	IL-5 R alpha	NAP-2	TGF-beta 2
Bate2 M	EGF	IFN-gamma	IL-6	NCAM-1	TGF-beta 3
BCAM	EGF R	IGFBP-1	IL-6 sR	NGF R	Thyroglobulin
BCMA/TNFRSF17	EG-VEGF/PK1	IGFBP-2	IL-7	Nidogen-1/Entactin	Tie-1
BDNF	ENA-78	IGFBP-3	IL-8	NrCAM	Tie-2
beta IG-H3	Endoglin	IGFBP-4	IL-9	NRG1-beta 1/HRG1-beta 1	TIM-1
Betacellulin (BTC)	Endostatin	IGFBP-5	IL-9 R	NT-3	TIMP-1
bFGF	Eotaxin	IGFBP-6	Insulin	NT-4	TIMP-2
BLC	Eotaxin-2	IGF-I	IP-10	Oncostatin M	TIMP-4
BMP-4	Eotaxin-3	IGF-I sR	I-TAC/CXCL11	Osteopontin	TNF-alpha
BMP-5	EpCAM/TROP1	IGF-II	LAP(TGF-b1)	Osteoprotegerin	TNF-beta
BMP-6	ErbB2	IL-1 alpha	Leptin R	PAI-I	TPO
BMP-7	ErbB3	IL-1 beta	LEPTIN(OB)	PARC	TRAIL R1
b-NGF	Erythropoietin R (EPO R)	IL-1 R4/ST2	LH	P-Cadherin	TRAIL R2
BTC	E-Selectin	IL-1 sRI	LIF	PDGF R alpha	TRAIL R3
CA125	Fas Ligand	IL-1 sRII	LIGHT	PDGF R beta	TRAIL R4
CA15-3	Fas/TNFRSF6	IL-10	LIMPII/SR-B2	PDGF-AA	Trappin-2/Elafin
CA19-9	Fcr RIIB/C	IL-10 R alpha	Lipocalin-2/NGAL	PDGF-AB	TREM-1
Carbonic Anhydrase IX(CA9)	Ferritin	IL-10 R beta	L-Selectin	PDGF-BB	TROY
Cardiotrophin-1 (CT-1)	FGF-4	IL-11	Lymphotactin	PECAM-1	TSH
Cathepsin S	FGF-6	IL-12 p40	LYVE-1	Platelet Factor 4	TSLP
CCL14a/HCC-1	FGF-7	IL-12 p70	Marapsin/Pancreasin	PlGF	u PAR
CCL21/6ckine	FGF-9	IL-13	MCP-1	Procalcitonin/Calcitonin	Ubiquitin+1
CCL28/VIC	FLRG	IL-13 Ra1	MCP-2	Prolactin	VCAM-1
CD14	Flt-3 Ligand	IL-13 Ra2	MCP-3	PSA-free	VE-Cadherin
CD23/Fc epsilon RII	Follistatin	IL-15	MCP-4	PSA-total	VEGF
CD27	Fractalkine	IL-16	MCSF	P-selectin	VEGF R2
CD30	FSH	IL-17	M-CSF R	RAGE	VEGF R3
CD40	Furin	IL-17B	MDC	RANK	VEGF-C
CD40 Ligand	Galectin-7	IL-17C	MICA	RANTES	VEGF-D
CEA	GCP-2	IL-17F	MICB	Resistin	
CEACAM-1	GCSF	IL-17R	MIF	S-100b	
CK beta 8-1	GDF-15/MIC-1	IL-18 BPa	MIG	SAA	

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. Peptide
8. Protein arrays
9. EIA

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
8. EIA

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	Inadequate detection	Check laser power and PMT parameters
	Inadequate reagent volumes or improper dilution	Check pipetters and ensure correct preparation
	Short incubation times	Ensure sufficient incubation Time and change sample incubation step to overnight
	Too low protein concentration in sample	Don't make too low dilution Or concentrate sample
	Improper storage of kit	Store kit at suggested temperature
High background	Excess of biotinylated antibodies	Make sure correct amount of antibodies
	Excess of streptavidin	Make sure correct amount of streptavidin
	Inadequate detection	Check laser power And PMT parameters
	dust	Work in clean environment
	Insufficient wash	Increase wash time and use more wash buffer
Uneven signal	Bubbles formed during incubation	Avoid bubble formation during incubation
	Arrays are not completed Covered by reagent	Completely cover arrays with solution

VII. Selected References Using RayBiotech Products

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