

# RayBio<sup>®</sup> Human Cytokine Antibody Array G-Series 4000

Patent Pending Technology

## User Manual (Revised June 6, 2014)

RayBio<sup>®</sup> Human Cytokine Antibody Array G-Series 4000  
Cat# AAH-CYT-G4000-4

RayBio<sup>®</sup> Human Cytokine Antibody Array G-Series 4000  
Cat# AAH-CYT-G4000-8

RayBio<sup>®</sup> Human Cytokine Antibody Array G-Series  
Testing Service  
Cat# AAH-SERV-G

Please read manual carefully  
before starting experiment



We provide you with excellent Protein Array systems and services

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Tel: (Toll Free) 1-888-494-8555 or +1-770-729-2992; Fax: +1-770-206-2393;  
Website: [www.raybiotech.com](http://www.raybiotech.com) Email: [info@raybiotech.com](mailto:info@raybiotech.com)

RayBiotech, Inc., the Protein Array Pioneer Company, strives to research and develop new products to meet demands of the biomedical community. RayBiotech's patent-pending technology allows detection of up to 1,000 cytokines, chemokines and other proteins in a single experiment. Our format is simple, sensitive, reliable, reproducible and cost-effective.

Our product offerings include:

1. Protein (antigen) Arrays
2. RayBio<sup>®</sup> Cytokine Antibody Arrays
  - C Series (Membrane, chemiluminescence detection)
  - G-Series (Glass slide, fluorescence detection)
3. Pathway- and disease-focused antibody arrays
  - Angiogenesis Antibody Arrays
  - Apoptosis Antibody Arrays
  - Atherosclerosis Antibody Arrays
  - Chemokine Antibody Arrays
  - Growth Factor Antibody Arrays
  - Inflammation Antibody Arrays
  - MMP Antibody Arrays
  - Obesity Antibody Arrays
4. Quantibody<sup>®</sup> Multiplex ELISA Arrays
5. RayBio<sup>®</sup> L-Series Biotin Label-based Antibody Arrays
6. RayBio<sup>®</sup> E-Series Competition-based Antibody Arrays
7. RayBio<sup>®</sup> Phosphorylation Antibody Arrays
  - Receptor Tyrosine Kinases
  - EGFR and ErbB family (site-specific phosphorylation)
8. Over 1,300 different ELISA kits
9. EIA (Competitive ELISA) kits
10. Cell-based Phosphorylation Assay
11. Over 20,000 different antibodies
12. Recombinant proteins
13. Peptide
14. Recombinant antibodies



# Protocol for RayBio<sup>®</sup> Human Cytokine Antibody Array G-Series 4000

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## TABLE OF CONTENTS

I.	Introduction.....	1
II.	Product Information.....	5
	A. Storage Recommendations.....	5
	B. RayBio <sup>®</sup> G-Series Glass Slide Layout .....	5
	C. Additional Materials Required.....	5
	D. Materials Provided .....	6
	E. How It Works .....	7
III.	Helpful Tips and General Considerations.....	7
	A. Preparation and Storage of Samples.....	7
	B. Handling Glass Slides.....	9
	C. Incubations and Washes.....	10
	D. Data Extraction Tips.....	10
IV.	Protocol.....	10
	A. Preparation and Storage of Reagents.....	10
	B. Blocking and Incubations.....	11
	C. Fluorescence Detection.....	14
V.	Interpretation of Results.....	15
	A. Explanation of Control Spots.....	15
	B. Typical Results using G-Series Arrays.....	15
	C. Background Subtraction.....	16
	D. Normalization of Array Data.....	16
	E. Threshold of Significance.....	17
VI.	Antibody Array Maps.....	18
VII.	Troubleshooting Guide.....	23

RayBio<sup>®</sup> Cytokine Antibody Arrays are patent-pending technology.  
RayBio<sup>®</sup> is the trademark of RayBiotech, Inc.

## I. Introduction

New techniques such as cDNA microarrays have enabled us to analyze global gene expression<sup>1-3</sup>. However, almost all cell functions are executed by proteins, which cannot be studied simply through DNA and RNA techniques. Experimental analysis clearly shows disparity can exist between the relative expression levels of mRNA and their corresponding proteins<sup>4</sup>. Therefore, analysis of the proteomic profile is critical.

The conventional approach to analyzing multiple protein expression levels has been to use 2-D SDS-PAGE coupled with mass spectrometry<sup>5,6</sup>. However, these methods are slow, expensive, labor-intensive and require specialized equipment<sup>7</sup>. Thus, effective study of multiple protein expression levels can be complicated, costly and time-consuming. Moreover, these traditional methods of proteomics are not sensitive enough to detect most cytokines (typically at pg/ml concentrations).

Cytokines, broadly defined as secreted cell-cell signaling proteins distinct from classic hormones or neurotransmitters, play important roles in inflammation, innate immunity, apoptosis, angiogenesis, cell growth and differentiation<sup>7</sup>. They are involved in most diseases, including cancer, obesity and inflammatory and cardiac diseases.

Simultaneous detection of multiple cytokines undoubtedly provides a powerful tool to study cytokines. Regulation of cellular processes by cytokines is a complex, dynamic process, often involving multiple proteins. Positive and negative feedback loops, pleiotropic effects and redundant functions, spatial and temporal expression of or synergistic interactions between multiple cytokines, even regulation via release of soluble forms of membrane-bound receptors, all are common mechanisms modulating the effects of cytokine signaling<sup>8-14</sup>. As such, unraveling the role of individual cytokines in physiologic or pathologic processes generally requires consideration and detection of multiple cytokines rather than of a single cytokine.

RayBio® G-Series Cytokine Antibody Arrays have several advantages over detection of cytokines using single-target ELISA:

1. More Data, Less Sample: Antibody arrays provide high-content screening using about the same sample volume as for ELISA.
2. Global View of Cytokine Expression: Antibody array screening improves the chances for discovering key factors, disease mechanisms or biomarkers related to cytokine signaling.
3. Greater Sensitivity: As little as 4 pg/ml of MCP-1 can be detected using the G-Series array format. In contrast, our similar MCP-1 ELISA assay has a sensitivity of 40 pg/ml of MCP-1.
4. Increased Range of Detection: ELISA assays typically detect a concentration range of 100- to 1000-fold, however, RayBiotech arrays can detect IL-2 at concentrations of 25 to 250,000 pg/ml, a range of 10,000-fold.
5. Better Precision: As determined by densitometry, the inter-array Coefficient of Variation (CV) of spot signal intensities is 5-10%, comparing favorably with ELISA testing (CV = 10-15%).

The RayBio® G-Series Cytokine Antibody Array is a glass slide that is a highly sensitive approach to simultaneously detect multiple cytokine expression levels from diverse sample types. The experimental procedure is simple and can be performed in any laboratory. The signals from G-Series arrays are detected using a laser scanner.

Larger, multi-array G-Series Human Cytokine Antibody Array Kits, such as the G1000, can detect hundreds of cytokines in a single experiment. For example, the Human G1000 arrays can detect up to 120 cytokines, the Human G2000 arrays can detect up to 174 cytokines, and the Human G4000 can detect up to 274 cytokines.

RayBiotech, The Protein Array Pioneer Company, introduced the first protein arrays to the market in 2001 and continues to lead in the development of innovative protein array technologies. For a list of publications demonstrating the usefulness of this easy-to-use array format, see Section VIII.

1. Tang X, Marciano DL, Leeman SE, Amar S. LPS induces the interaction of a transcription factor, LPS-induced TNF- $\alpha$  factor, and STAT6(B) with effects on multiple cytokines. *PNAS*. 2005;102(14): 5132-5137.
2. Xu Y, Kulkosky J, Acheampong E, et al.. HIV-1-mediated apoptosis of neuronal cells: Proximal molecular mechanisms of HIV-1-induced encephalopathy. *PNAS*. 2004;101(18): 7070-7075.
3. El-Hage N, Gurwell JA, Singh IN, et. al. Synergistic increases in intracellular Ca(2+), and the release of MCP-1, RANTES, and IL-6 by astrocytes treated with opiates and HIV-1 Tat. *Glia*. 2005;50(2): 91-106.
4. Oh HS, Moharita A, Potian JG, et al. Bone Marrow Stroma Influences Transforming Growth Factor- $\beta$  Production in Breast Cancer Cells to Regulate c-myc Activation of the Preprotachykinin-I Gene in Breast Cancer Cells. *Cancer Res*. 2004;64: 6327-6336.
5. Osorio Y, Ghiasi H. Recombinant Herpes Simplex Virus Type 1 (HSV-1) Codelivering Interleukin-12p35 as a Molecular Adjuvant Enhances the Protective Immune Response against Ocular HSV-1 Challenge. *J Virol*. 2005;79(6): 3297-3308.
6. Mullick A, Elias M, Picard S, Bourget L, et al. Dysregulated Inflammatory Response to *Candida albicans* in a C5-Deficient Mouse Strain. *Infect Immunity*. 2004;72(10): 5868-5876.
7. Maruvada P, Wang W, Wagner PD, Srivastava S. Biomarkers in molecular medicine: cancer detection and diagnosis. *Biotechniques*. 2005;38: S9-S15.
8. Bartek J, Hodny Z, Lukas J. Cytokine loops driving senescence. *Nature Cell Biol*. 2008;10: 887-889.
9. Minami K, Yanagawa Y, Iwabuchi K, et al. Negative feedback regulation of T helper type 1 (Th1)/Th2 cytokine balance via dendritic cell and natural killer T cell interactions. *Blood*. 2005;106: 1685-1693.
10. Ozaki K, Leonard WJ. Cytokine and Cytokine Receptor Pleiotrophy and Redundancy. *J Biol Chem*. 2002;277: 29355-29358.
11. Ragab AA, Nalepka JL, Bi Y, Greenfield EM. Cytokines synergistically induce osteoclast differentiation: support by immortalized or normal calvarial cells. *Am J Physiol Cell Physiol*. 2002;283(3): C679-C687.
12. Devalaraja MN, Richmond A. Multiple chemotactic factors: fine control or redundancy. *Trends Pharmacol Sci*. 1999;20(4): 151-156.
13. Heaney ML, Golde DE. Soluble Cytokine Receptors. *Blood*. 1996;87(3): 847-857.

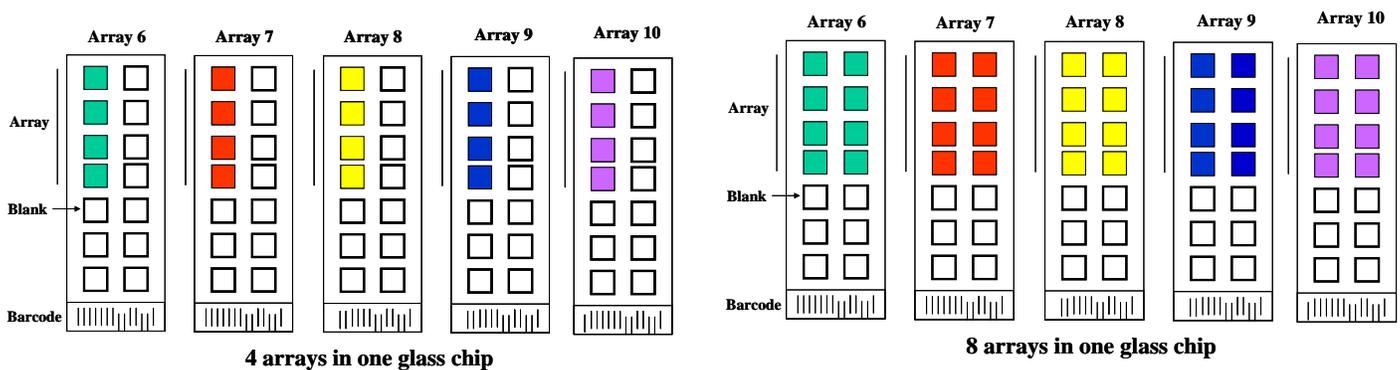
## II. Product Information

### A. Storage Recommendations:

For best results, we recommend storing the entire kit at  $-20^{\circ}\text{C}$  or  $-80^{\circ}\text{C}$  upon arrival and using the kit within 6 months of receipt. RayBiotech warranties this product for 6 months if stored in this manner.

Once thawed, store glass slides and 1X Blocking Buffer at  $-20^{\circ}\text{C}$  or  $-80^{\circ}\text{C}$  and all other component at  $4^{\circ}\text{C}$ . After thawing, the entire kit should be used within 3 months. RayBio<sup>®</sup> Antibody Array kits are robust and will retain full activity even if accidentally stored at room temperature (RT) for up to 24 hours.

### B. RayBio<sup>®</sup> G-Series Glass Slide Layout



### C. Additional Materials Required

- Small plastic boxes or containers
- Pipettors, pipette tips and other common lab consumables
- Orbital shaker or oscillating rocker
- Aluminum foil
- Gene microarray scanner or similar laser fluorescence scanner (see pages 9 & 14)

## D. Materials Provided

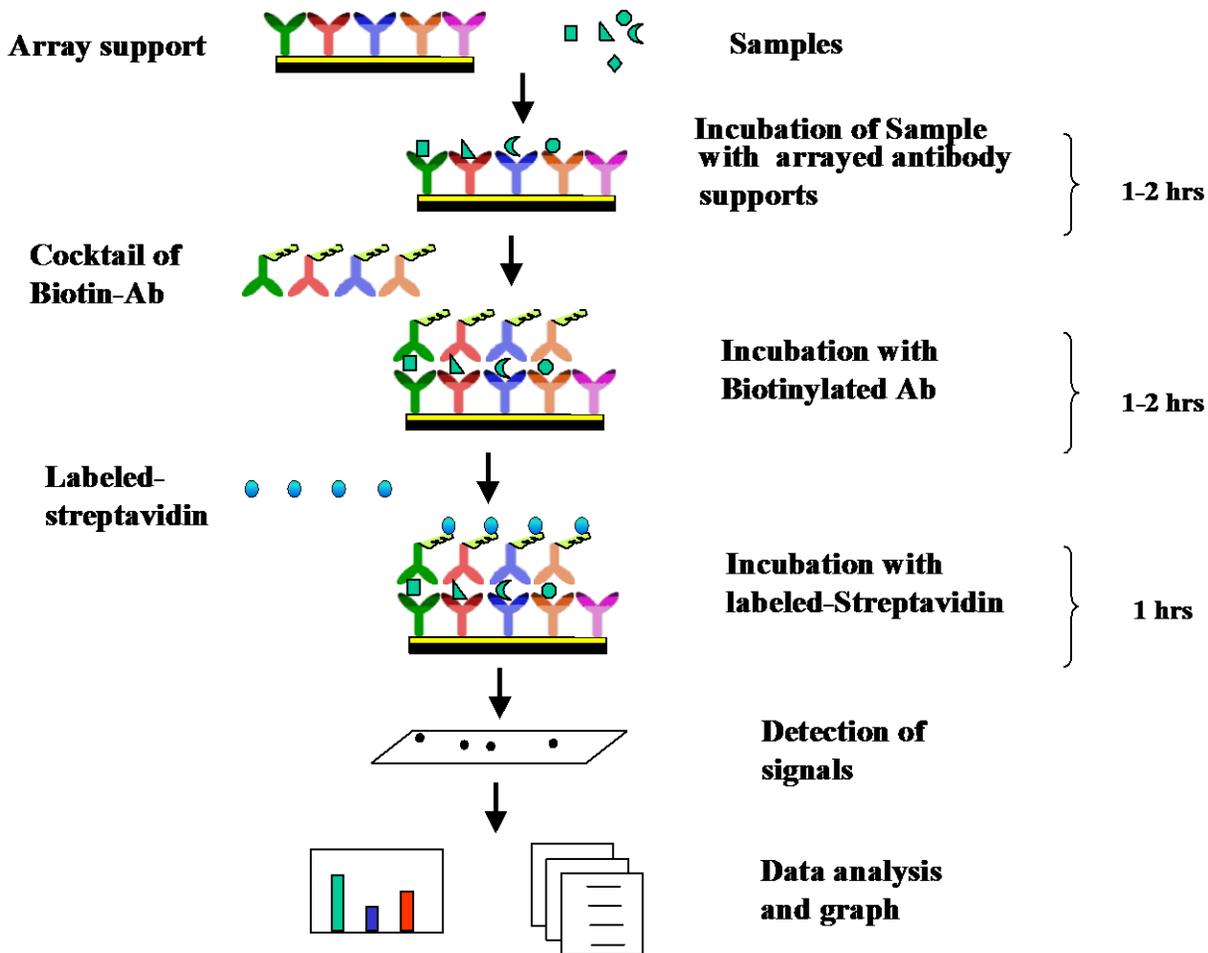
Item	Description	AAH-CYT-G4000-4	AAH-CYT-G4000-8
AAH-CYT-G6	RayBio <sup>®</sup> Human Cytokine Array G6 Glass Slide*	1 slide with 4 Sub-arrays*	1 slide with 8 Sub-arrays*
AAH-CYT-G7	RayBio <sup>®</sup> Human Cytokine Array G7 Glass Slide*	1 slide with 4 Sub-arrays*	1 slide with 8 Sub-arrays*
AAH-CYT-G8	RayBio <sup>®</sup> Human Cytokine Array G8 Glass Slide*	1 slide with 4 Sub-arrays*	1 slide with 8 Sub-arrays*
AAH-CYT-G9	RayBio <sup>®</sup> Human Cytokine Array G9 Glass Slide*	1 slide with 4 Sub-arrays*	1 slide with 8 Sub-arrays*
AAH-CYT-G10	RayBio <sup>®</sup> Human Cytokine Array G10 Glass Slide*	1 slide with 4 Sub-arrays*	1 slide with 8 Sub-arrays*
0103002 - HG6	Biotin-Conjugated Human G6 Anti-Cytokines	1 ea	2 ea
0103002 - HG7	Biotin-Conjugated Human G7 Anti-Cytokines	1 ea	2 ea
0103002 - HG8	Biotin-Conjugated Human G8 Anti-Cytokines	1 ea	2 ea
0103002 - HG9	Biotin-Conjugated Human G9 Anti-Cytokines	1 ea	2 ea
0103002 - HG10	Biotin-Conjugated Human G10 Anti-Cytokines	1 ea	2 ea
0103004-H	1,500X HiLyte Plus™ 555 Streptavidin-Fluor†	1 ea	2 ea
0103004-B	1X Blocking Buffer	20 ml	40 ml
0103004-W‡	20X Wash Buffer I ‡	30 ml	60 ml
0103004-W‡	20X Wash Buffer II ‡	30 ml	60 ml
Other Kit Components: 2X Cell Lysis Buffer (10 ml), Manual, Adhesive Plastic Strips, 30 ml Centrifuge Tube			

\* Kit contains 5 pre-assembled glass slides with either 4 or 8 printed sub-arrays per slide (in sealed plastic envelope)  
**[NOTE: In some cases, 2 slides x 4 sub-arrays/slide may be substituted in kits containing 8 sub-arrays]**

† This fluor is patent-pending technology from Anaspec, Inc.

‡ Wash Buffers are sold as sets

## E. How It Works



## III. Helpful Tips and General Considerations

### A. Preparation and Storage of Samples

#### 1. General Considerations:

- Freeze samples as soon as possible after collection.
- Avoid multiple freeze-thaw cycles. If possible, sub-aliquot your samples prior to initial storage.
- Spin samples hard (5-10 minutes at 10K to 15K RPM) immediately prior to incubation of samples with array.
- Optimal sample concentrations may need to be determined empirically based on the signal intensities of spots and background signals obtained.
- Most samples will not need to be concentrated. If concentration is required, we recommend using a spin-column concentrator with a chilled centrifuge.

## 2. Recommended Sample Volumes and Dilution Factors

***NOTE: All sample dilutions should be made using 1X Blocking Buffer. For all sample types, final volume = 50-100 µl per sub-array.***

- **Cell Cultured Media:** Neat (no dilution needed)
- **Serum & Plasma:** 5-fold to 10-fold dilution
- **Most other Body Fluids:** Neat or 2-fold to 5-fold dilution
- **Cell and Tissue Lysates:** Minimum 5-fold to 10 fold to equal concentrations of total protein in each lysate sample.
  - You must determine the total protein concentration of each lysate/homogenate. We recommend using the BCA method (available from Pierce); it is insensitive to detergents commonly found in lysis buffers.
  - Minimum Recommended Dilution of Lysates (prior to sample incubation): 5-fold to 10 fold with 1X Blocking Buffer. Dilute all lysate samples to the same final concentration of total lysate protein in 1X Blocking Buffer to 100 µl final volume.
  - To start, we recommend using 10-100 µg of total protein in 100 µl of 1X Blocking Buffer (final volume) per sub-array.
  - Optimal amounts of total lysate protein may range from 5-500 µg per sub-array. Based upon background and spots intensities, you may increase or decrease the amount of protein used in subsequent experiments.
- **Other Liquid Sample Types:** Most often Neat or 2-fold to 5-fold. However, optimal dilutions should be determined empirically.

## 3. Sample Preparation

For tips on sample preparation, please visit our Website:

<http://www.raybiotech.com/Tech-Support/SampleTips.pdf>

### B. Handling Glass Slides

- Do not remove glass slide from assembly until Step 16.
- Hold the slides by edges only; do not touch the surface.
- Handle all buffers and slides with powder-free gloves.
- Dry glass slide completely before proceeding to Step 3.
- Handle and dry glass slide in clean environment.

- Avoid breaking glass slide when removing chamber assembly.

### C. Incubations and Washes

- Cover incubation chamber with adhesive film (included in kit) to prevent evaporation, particularly during incubation or wash steps >2 h or with liquid volumes <100 µl per well.
- Perform all incubation and wash steps under gentle rotation or rocking motion (~0.5 to 1 cycle/s).
- Wash steps in Wash Buffer II and all incubation steps may be performed overnight at 4°C.
  - Overnight sample incubations are the most effective at increasing sample spot intensities.
- Avoid cross-contamination of samples to neighboring wells
- To remove Wash Buffers and other reagents from chamber wells, you may invert the Glass Slide Assembly to decant, and aspirate the remaining liquid.
- In Wash Steps 6, 12 and 15, you may gently flush wells several times using a wash bottle filled with Wash Buffer I.

### D. Scanning and Data Extraction Tips: (see also page 18)

For tips on scanning and data extraction, please visit our Website:  
<http://www.raybiotech.com/Tech-Support/ScanningTips.pdf>

For a list of recommended scanners, please visit our Website:  
[http://www.raybiotech.com/files/Tech-Support/Laser Scanners for Glass Slide Arrays.pdf](http://www.raybiotech.com/files/Tech-Support/Laser%20Scanners%20for%20Glass%20Slide%20Arrays.pdf)

## IV. Protocol

### A. Preparation and Storage of Reagents

***NOTE: During this protocol, prepare reagents immediately prior to use and keep working dilutions of all reagents on ice at all times.***

1. Blocking Buffer is supplied at 1X concentration. No dilution is required.

2. Wash Buffers I and II are supplied at 20X concentration.
  - a). For each glass slide (4 or 8 sub-arrays/slide), dilute 6 ml of 20X concentrate with deionized H<sub>2</sub>O to a final volume of 120 ml each of Wash Buffer I & Wash Buffer II.
  - b). Wash buffer reagents at working dilution (1X) can be stored at 4°C for up to 1 month. Stock solutions at 20X can be stored 4°C for up to 3 months.
  
3. Biotin-conjugated Anti-Cytokines are supplied at high concentration in a small liquid bead (typically ~2-5 µl).
  - a). Spin down each tube prior to reconstitution, as the concentrated liquid bead may have moved to the top of the tube during handling.
  - b). Prepare each stock reagent by adding 300 µl 1X Blocking Buffer to Biotin-Conjugated Anti-Cytokines. Mix well.
  - c). 1X Biotin-Conjugated Anti-Cytokines may be stored for 2-3 days at 4°C.
  
4. Streptavidin-Fluor is supplied at 1500x concentration.
  - a). Mix the tube containing 1500X Streptavidin-Fluor well before use, as precipitants may form during storage.
  - b). Add 100 µl of 1X Blocking Buffer to tube containing 1500X Streptavidin-Fluor. Mix well.
  - c). Quantitatively transfer all of Streptavidin-Fluor reagent from the original tube to a larger one, and dilute with 1X Blocking Buffer to a final volume of 1500 µl (ie, 1.5 ml).
  - d). Wrap tube containing Streptavidin-Fluor with aluminum foil.
  - e). This working dilution can be stored for 3-5 days at 4°C.

## B. Blocking and Incubations

***NOTE: Please carefully read Section III of this manual before proceeding***

***NOTE: Prepare all reagents immediately prior to use as described above (Section IV.A) and before proceeding.***

- 1) Remove packaged glass slides from freezer. Place unopened packages on the benchtop. Wait for about 15 min for entire chamber assembly to equilibrate to room temperature (RT). Then open packages, remove the chamber assemblies and place them in laminar flow hood to dry for 1-2 hours.

***NOTE: Be sure each glass slide is completely dry before proceeding.***

- 2) If necessary, assemble glass slides into incubation chamber and frame as shown below and on page 12. (Note: if you slide is already assembled, you can proceed directly to Step 3).
- 3) Add 100  $\mu$ l 1 X Blocking Buffer into each well and incubate at RT for 30 min to block slides.

***NOTE: Only add reagents or samples to wells printed with antibodies (see diagram on page 5).***

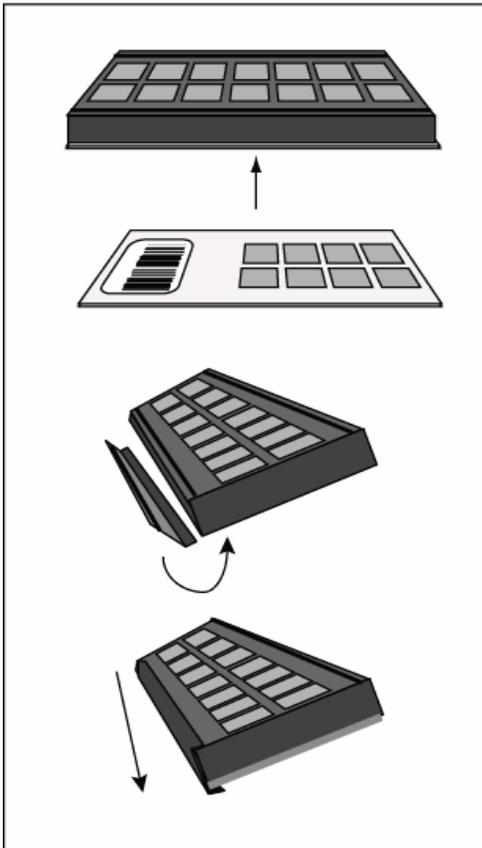
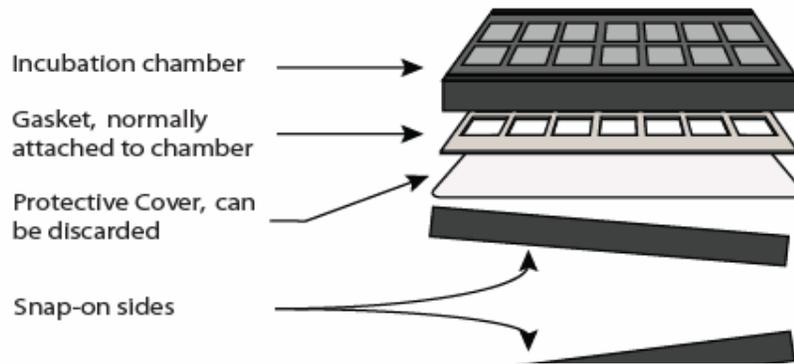
- 4) Decant, then aspirate remaining Blocking Buffer from each well.

***NOTE: To aspirate liquid samples or reagents from wells, gently place the pipette tip only in the corners of the well. Do not scrape the pipette tip across the surface of the slide.***

- 5) Add 50 to 100  $\mu$ l of sample to each sub-array. Be sure to load each sample on all 5 slides (Cytokine Array G6, G7, G8, G9 & G10). Cover the incubation chambers with Adhesive film (included in kit). Incubate arrays with sample at RT for 2 hours. Dilute sample using 1X Blocking Buffer if necessary.
- 6) Remove adhesive film, and carefully aspirate samples from sub-arrays, touching only the corners with your pipette tip.

***NOTE: Try to keep samples from flowing into neighboring wells.***

## Instructions for incubation chamber assembly G Series and Quantibody Arrays



**1** Carefully place slide at bottom of the chamber as shown. The slide will adhere somewhat to the bottom. Warning: the slide is fragile, so do not apply more than gentle force to the apparatus.

**2** While gently holding chamber and slide, place side on chamber as shown, beginning with bottom flap first.

**3** Then, press the top of the side into groove on chamber, and then apply even, gentle pressure from one end to the other. Repeat this procedure with the other side.

- 7) Wash 3 x 2 min with 150  $\mu$ l 1X Wash Buffer I at RT. Be sure to completely remove liquid each time and use fresh buffer for each wash. Decant final wash solution before proceeding to next step.
- 8) Obtain a clean container (eg, pipette tip box or slide staining jar) and place glass slide assemblies into the container. Add enough 1X Wash Buffer I to submerge both glass slides with chamber assemblies intact (approx. 30-50 ml) and remove all bubbles in wells. Wash 10 min at RT with gentle rocking or shaking.

- 9) Remove glass slide assemblies from container and invert to decant liquid. Decant buffer from container and replenish with 1X Wash Buffer I. Submerge both chamber assemblies and wash 10 min at RT with gentle rocking or shaking.
- 10) Remove glass slide assemblies from container and invert to decant liquid. Decant buffer from container. Repeat Steps 8 & 9 with Wash Buffer II.
- 11) Remove glass slide assemblies from container and invert to decant liquid, then carefully aspirate wash buffer from wells, touching only the corners with your pipette tip.

***Important Note: Be sure to use the correct Anti-Cytokine mixture with the correct array slide (e.g., use Human G6 Anti-Cytokine mixture for the slide designated as Human G6, not Human G7).***

- 12) Add 70  $\mu$ l of 1X Biotin-conjugated Anti-Cytokines to each sub-array. Cover incubation chamber with Adhesive film (included in kit). Incubate at RT for 2 hours with gentle rocking or shaking.
- 13) Carefully aspirate all of the Biotin-conjugated Anti-Cytokine reagent from each well. Wash as described in Step 7 above, first with Wash Buffer I then with Wash Buffer II, making sure to completely remove buffer between washes and after final wash.
- 14) Add 70  $\mu$ l of 1X Streptavidin-Fluor to each sub-array. Cover the incubation chambers with Adhesive film (included in kit), then cover entire assemblies with aluminum foil to avoid exposure to light or incubate in dark room. Incubate at RT for 2 hours with gentle rocking or shaking.
- 15) Remove aluminum foil and adhesive film. Carefully aspirate the Streptavidin-Fluor reagent from each well. Wash as described in Step 7 above, first with Wash Buffer I then with Wash Buffer II, making sure to completely remove buffer between washes and after final wash.

- 16) Remove the glass slides from the frame assemblies. Place both slides in 30 ml centrifuge tube provided, or slide staining jar. Add enough Wash Buffer I to cover both slides (about 20 ml) and gently rock or shake at RT for 10 min.
- 17) Decant buffer and repeat wash as described in Step 16 (1 x 10 min with Wash Buffer I).
- 18) Decant buffer and repeat wash as described in Step 16, but this time using Wash Buffer II for only 2-3 minutes.
- 19) Decant buffer, remove the glass chip from the tube, then gently rinse the slide with de-ionized H<sub>2</sub>O using a plastic wash bottle.
- 20) Remove water droplets by applying suction gently with a pipette tip.

***NOTE: Be careful not to touch the array portions of the slide with your pipette tip, only touch the sides of the slide.***

#### C. Obtaining Fluorescent Signal Intensities:

- 21) Allow glass chip to dry in a laminar flow hood for 20 minutes or until slide is completely dry. Place chip under an aluminum foil tent to protect it from light. Make sure the slides are absolutely dry before scanning or storage.
- 22) You may proceed immediately to scanning (Step 23), or you may scan at a later time. You may store the slides at RT indefinitely, provided they are protected from strong light.

***Note: Unlike most Cy3 fluors, the HiLyte Plus™ Fluor 555 very stable at RT and resistant to photobleaching. However, please protect glass slides from strong light and temperatures above RT.***

23) Scan the glass slides with a laser scanner (such as Innopsys' InnoScan®) using Cy3 or "green" channel (excitation frequency = 532 nm). For tips on scanning, visit our Website: <http://www.raybiotech.com/Tech-Support/ScanningTips.pdf>

***NOTE: If you do not have a laser scanner, for a nominal fee you can send your slide to us for scanning and data extraction using Innopsys' InnoScan, and we will return the results to you. Using using alternate protocols, RayBio® G-Series arrays are also compatible with Li-Cor's Odyssey and other microarray scanners.***

## V. Interpretation of Results:

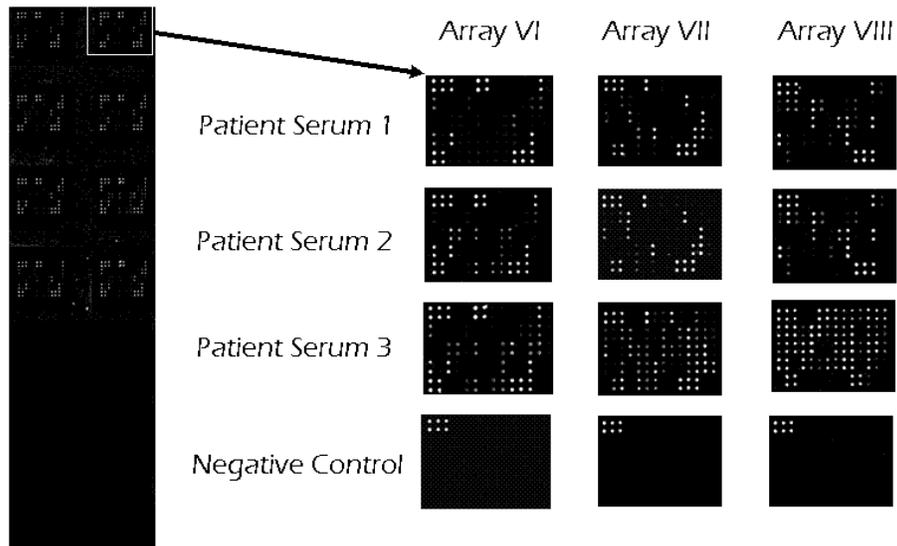
### A. Explanation of Controls Spots

Positive Controls (POS1, POS2, POS3) are equal amounts of biotinylated IgGs printed directly onto each array. All other variables being equal, Positive Control intensities should be the same for each sub-array. This allows for normalization based upon the relative fluorescence signal responses to a known control, much as "housekeeping" genes or proteins can normalize results in PCR or Western blots, respectively.

Negative Control (NEG) spots are printed with a protein-containing buffer. Their signal intensities represent non-specific binding of Biotin-conjugated anti-Cytokines and/or Streptavidin-Fluor. Negative control signal intensities are usually very close to background signals in each sub-array.

### B. Typical results from RayBio® G-Series Antibody Arrays

The following figure shows typical results obtained using RayBio® Antibody Array G-Series Arrays. The images were captured using a GenePix 4000B scanner.



In this example, sera from several patients were incubated with Human Cytokine Arrays 6, 7 & 8, (sold together as Human Cytokine Array G-Series 2000, AAH-CYT-G2000-4 or AAH-CTY-G2000-8) and processed using this standard protocol.

The 6 strong signals of the Positive Control spots in the upper-left corner are useful for proper orientation of the array image. If scanned using optimal scan settings, 3 distinct Positive Control signal intensities will be seen: POS1>POS2>POS3. If all of these signals are of similar intensity, try increasing or decreasing laser power and/or signal gain settings.

Once you have obtained fluorescence intensity data, you should subtract the background and normalize to the Positive Control signals before proceeding to analysis.

### C. Background Subtraction:

Most laser fluorescence scanner software can automatically measure the local background around each spot. As with spot signal intensities, we recommend using MEDIAN background signals. If your resulting fluorescence signal intensity reports do not include these values (eg, a column labeled as “MED532-

B532”), you may need to subtract the background manually or change the default settings on your scanner’s data report menu.

#### D. Normalization of Array Data:

To normalize signal intensity data, one sub-array is defined as "reference" to which the other arrays are normalized. This choice can be arbitrary. For example, in our Analysis Tool Software, the array represented by data entered in the left-most column each worksheet is the default “reference array.”

You can calculate the normalized values as follows:

$$X(Ny) = X(y) * P1/P(y)$$

Where:

P1 = mean signal intensity of POS spots on reference array

P(y) = mean signal intensity of POS spots on Array "y"

X(y) = mean signal intensity for spot "X" on Array "y"

X(Ny)= normalized signal intensity for spot "X" on Array "y"

The RayBio<sup>®</sup> Analysis Tool software is available for use with data obtained using RayBio<sup>®</sup> G-Series Arrays. You can copy and paste your signal intensity data (with and without background) into the Analysis Tool, and it will automatically normalize signal intensities to the Positive Controls.

To order the Analysis Tool, please contact us at +1-770-729-2992 or [info@raybiotech.com](mailto:info@raybiotech.com) for more information.

## E. Threshold of significant difference in expression:

After subtracting background signals and normalization to Positive controls, comparison of signal intensities for antigen-specific antibody spots between and among array images can be used to determine relative differences in expression levels of each analyte (ie, protein detected) between samples or groups.

Any  $\geq 1.5$ -fold increase or  $\leq 0.65$ -fold decrease in signal intensity for a single analyte between samples or groups may be considered a measurable and significant difference in expression, provided that both sets of signals are well above background (Mean background + 2 standard deviations, accuracy  $\approx 95\%$ ).

***NOTE: In the absence of an external standard curve for each analyte, there is no means of assessing absolute or relative concentrations of different analytes in the same sample using immunoassays. If you wish to obtain quantitative data (ie, concentrations of the various analytes in your samples), try using our Quantibody® Multiplex ELISA arrays instead.***

### 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.
- Scan all slides at same PMT

## VI. Antibody Array Maps for Human G-4000 (274 cytokines):

### A. RayBio® Human Cytokine Antibody Array G-Series 6

Detects 60 human cytokines in one experiment

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	POS1	POS2	POS3	NEG	NEG	ANG	BDNF	BLC	BMP4	BMP6	Ckβ 8-1	CNTF	EGF	CCL11
2	POS1	POS2	POS3	NEG	NEG	ANG	BDNF	BLC	BMP4	BMP6	Ckβ 8-1	CNTF	EGF	CCL11
3	CCL24	CCL26	FGF6	FGF7	Flt-3L	CX3CL1	GCP-2	GDNF	CSF2	I-309	IFN-γ	IGFBP1	IGFBP2	IGFBP4
4	CCL24	CCL26	FGF6	FGF7	Flt-3L	CX3CL1	GCP-2	GDNF	CSF2	I-309	IFN-γ	IGFBP1	IGFBP2	IGFBP4
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	MCP1	MCP2	MCP3	MCP4	M-CSF	MDC	MIG	MIP-1δ	MIP-3α	NAP-2	NT-3	PARC
8	Leptin	LIGHT	MCP1	MCP2	MCP3	MCP4	M-CSF	MDC	MIG	MIP-1δ	MIP-3α	NAP-2	NT-3	PARC
9	PDGF-BB	RANTES	SCF	SDF-1	TARC	TGF-β1	TGF-β3	TNF-α	TNF-β	NEG	NEG	NEG	NEG	NEG
10	PDGF-BB	RANTES	SCF	SDF-1	TARC	TGF-β1	TGF-β3	TNF-α	TNF-β	NEG	NEG	NEG	NEG	NEG

- **Notes on Human G6 Array Map:** ANG = Angiogenin, OSM = Oncostatin M, THPO = Thrombopoietin, CCL11 = Eotaxin, CCL24 = Eotaxin-2, CCL26 = Eotaxin-3, Flt-3L = Flt-3 Ligand, CX3CL1 = Fractalkine, CSF2 = GM-CSF, CSF1 = M-CSF
- TGF-β1 reacts only with active form of TGF-β1

## B. RayBio® Human Cytokine Antibody Array G-Series 7

Detects 60 human cytokines in one experiment

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	POS1	POS2	POS3	NEG	NEG	Acrp30	AgRP	ANGPT2	AREG	Axl	bFGF	β-NGF	BTC	CCL28
2	POS1	POS2	POS3	NEG	NEG	Acrp30	AgRP	ANGPT2	AREG	Axl	bFGF	β-NGF	BTC	CCL28
3	CTACK	Dtk	EGFR	ENA-78	Fas	FGF4	FGF9	CSF3	GITRL	GITR	GRO	GRO-α	HCC-4	HGF
4	CTACK	Dtk	EGFR	ENA-78	Fas	FGF4	FGF9	CSF3	GITRL	GITR	GRO	GRO-α	HCC-4	HGF
5	ICAM1	ICAM3	IGFBP3	IGFBP6	sIGF-1R	IL1R4	IL-1 R1	IL-11	IL-12 p40	IL-12 p70	IL-17	IL-2 Rα	sIL-6R	IL-8
6	ICAM1	ICAM3	IGFBP3	IGFBP6	sIGF-1R	IL1R4	IL-1 R1	IL-11	IL-12 p40	IL-12 p70	IL-17	IL-2 Rα	sIL-6R	IL-8
7	ITAC	XCL1	MIF	MIP-1α	MIP-1β	MIP-3β	MSP-α	NT-4	OPG	OSM	PLGF	sgp130	sTNFR2	sTNFR1
8	ITAC	XCL1	MIF	MIP-1α	MIP-1β	MIP-3β	MSP-α	NT-4	OPG	OSM	PLGF	sgp130	sTNFR2	sTNFR1
9	TECK	TIMP1	TIMP2	THPO	TRAIL R3	TRAIL R4	uPAR	VEGF-A	VEGF-D	NEG	NEG	NEG	NEG	NEG
10	TECK	TIMP1	TIMP2	THPO	TRAIL R3	TRAIL R4	uPAR	VEGF-A	VEGF-D	NEG	NEG	NEG	NEG	NEG

### Notes on Array Map:

- Acrp30 = Adiponectin, AgRP = Agouti-related Protein, ANGPT2 = Angiopoietin-2, AREG = Amphiregulin, bFGF = basic FGF (FGF2), BTC = Betacellulin, CCL28 = MEC, CSF3 = G-CSF, GITRL = GITR Ligand, XCL1 = Lymphotoxin, OPG = Osteoprotegerin, OSM = Oncostatin M, PLGF = Placental Growth Factor, THPO = Thrombopoietin
- GRO recognizes CXCL1, CXCL2 and CXCL3 (GRO-α, GRO-β and GRO-γ, respectively)
- sIGF-1R, sIL-6R, sTNFR1, sTNFR2, sgp120, TRAIL R3 and TRAIL R4 recognize soluble receptors.
- VEGF-A detects VEGF(165 aa) and VEGF(121 aa).

## C. RayBio® Human Cytokine Antibody Array G-Series 8

Detects 54 human cytokines in one experiment

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	POS1	POS2	POS3	NEG	NEG	Activin A	ALCAM	B7-1 (CD80)	BMP-5	BMP-7	CTF1	CD14	CXCL16	DR6
2	POS1	POS2	POS3	NEG	NEG	Activin A	ALCAM	B7-1 (CD80)	BMP-5	BMP-7	CTF1	CD14	CXCL16	DR6
3	Endoglin	ErbB3	SELE	FASLG	ICAM2	IGF-II	IL-1 R2	IL-10 R $\beta$	IL-13 R $\alpha$ 2	IL-18 BP $\alpha$	IL-18 R $\beta$	MMP3	IL-2 R $\beta$	IL-2R $\gamma$
4	Endoglin	ErbB3	SELE	FASLG	ICAM2	IGF-II	IL-1 R2	IL-10 R $\beta$	IL-13 R $\alpha$ 2	IL-18 BP $\alpha$	IL-18 R $\beta$	MMP3	IL-2 R $\beta$	IL-2R $\gamma$
5	IL-21R	IL-5 R $\alpha$	IL-9	IP-10	LAP	Leptin R	LIF	SELL	CSF1R	MMP1	MMP13	MMP9	MPIF1	NGFR
6	IL-21R	IL-5 R $\alpha$	IL-9	IP-10	LAP	Leptin R	LIF	SELL	CSF1R	MMP1	MMP13	MMP9	MPIF1	NGFR
7	PDGF-AA	PDGF-AB	PDGF R $\alpha$	PDGF R $\beta$	PECAM1	Prolactin	SCFR	SDF-1 $\beta$	Siglec-5	TGF- $\alpha$	TGF- $\beta$ 2	Tie-1	Tie-2	TIMP4
8	PDGF-AA	PDGF-AB	PDGF R $\alpha$	PDGF R $\beta$	PECAM1	Prolactin	SCFR	SDF-1 $\beta$	Siglec-5	TGF- $\alpha$	TGF- $\beta$ 2	Tie-1	Tie-2	TIMP4
9	CDH5	VEGF R2	VEGF R3	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
10	CDH5	VEGF R2	VEGF R3	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG

### Notes on Array Map:

- CTF1 = Cardiotrophin-1, SELE = E-Selectin, FASLG = Fas Ligand, LAP = Latency-associated Peptide (inhibitor of TGF- $\beta$ 1), SELL = L-Selectin, CSF1R = M-CSF Receptor, CDH5 = VE-Cadherin (Cadherin 5)
- SDF-1 $\beta$  recognizes the beta chain of SDF-1

## D. RayBio® Human Cytokine Antibody Array G-Series 9 Map:

Detects 51 Human Cytokines in one experiment

	A	B	C	D	E	F	G	H	I	J	K	L
1	POS1	POS2	POS3	NEG	NEG	Adipsin	BCAM	CD30	CD40	Fc-γ RIIb/C	Ferritin	FLRG
2	POS1	POS2	POS3	NEG	NEG	Adipsin	BCAM	CD30	CD40	Fc-γ RIIb/C	Ferritin	FLRG
3	FSTN	Furin	LGALS7	GDF15	hGH	IL-10 Rα	IL-22	IL-28A	IL-29	IL-31	Insulin	LH-β
4	FSTN	Furin	LGALS7	GDF15	hGH	IL-10 Rα	IL-22	IL-28A	IL-29	IL-31	Insulin	LH-β
5	LIMPII	Lyve-1	Marapsin	MICA	MICB	MMP2	MMP7	MMP8	MMP10	NCAM1	NID1	NrCAM
6	LIMPII	Lyve-1	Marapsin	MICA	MICB	MMP2	MMP7	MMP8	MMP10	NCAM1	NID1	NrCAM
7	NRG-β1	OPN	PAI-I	PF4	PSA (total)	RAGE	RANK	Resistin	SAA	Siglec-9	TACE	KIM1 / TIM1
8	NRG-β1	OPN	PAI-I	PF4	PSA (total)	RAGE	RANK	Resistin	SAA	Siglec-9	TACE	KIM1 / TIM1
9	TRAIL R2	Trappin-2	TREM1	TSH-β	TSLP	VCAM1	VEGF-C	XEDAR	NEG	NEG	NEG	NEG
10	TRAIL R2	Trappin-2	TREM1	TSH-β	TSLP	VCAM1	VEGF-C	XEDAR	NEG	NEG	NEG	NEG

### Notes on Array Map:

- FLRG = Follistatin-like 3 (secreted glycoprotein), FSTN = Follistatin, LGALS7 = Galectin-7, hGH = (human) Growth Hormone, LH-β = Luteinizing Hormone (beta subunit), NRG-1β = neuregulin 1 type IV beta 1a, OPN = Osteopontin, PF4 = Platelet Factor 4, RAGE = Receptor for Advanced Glycosylation End Products, TSHβ = Thyroid Stimulating Hormone (beta subunit)

## E. RayBio® Human Cytokine Antibody Array G-Series 10 Map:

Detects 49 human cytokines in one experiment

	A	B	C	D	E	F	G	H	I	J	K	L
1	POS1	POS2	POS3	NEG	NEG	4-1 BB	ACE-2	AFP	ANGPT 1	Angio-statin	ANGPTL4	B2M
2	POS1	POS2	POS3	NEG	NEG	4-1 BB	ACE-2	AFP	ANGPT 1	Angio-statin	ANGPTL4	B2M
3	BCMA	β-IG-H3	CA125	CA15-3	CA19-9	CA9	CTSS	CCL14	CCL21	CD23	CD40LG	CEA
4	BCMA	β-IG-H3	CA125	CA15-3	CA19-9	CA9	CTSS	CCL14	CCL21	CD23	CD40LG	CEA
5	CEACAM1	Cripto-1	CRP	DAN	Decorin	Dkk-1	Dkk-3	Dkk-4	DPP4	CDH1	EDA-A2	EG-VEGF
6	CEACAM1	Cripto-1	CRP	DAN	Decorin	Dkk-1	Dkk-3	Dkk-4	DPP4	CDH1	EDA-A2	EG-VEGF
7	EpCAM	HER2 / ErbB2	EPO R	FSH-β	HB-EGF	hCG (intact)	HVEM	IL-13 Rα1	IL-17B	IL-17C	IL-17F	IL-17R
8	EpCAM	HER2 / ErbB2	EPO R	FSH-β	HB-EGF	hCG (intact)	HVEM	IL-13 Rα1	IL-17B	IL-17C	IL-17F	IL-17R
9	CALCA	PSA (free)	S-100b	ShhN	TG	Ubiquitin	Blank	NEG	NEG	NEG	NEG	NEG
10	CALCA	PSA (free)	S-100b	ShhN	TG	Ubiquitin	Blank	NEG	NEG	NEG	NEG	NEG

### Notes on Array Map:

- ANGPT1 = Angiopoietin-1, ANGPTL4 = Angiopoietin-like 4, B2M = β-2 Microglobulin, BCMA = TNFRSF17 (B-cell Maturation Antigen), β-IG-H3 = TGFBI (Transforming Growth Factor, beta-induced), CA9 = Carbonic Anhydrase IX, CTSS = Cathepsin S, CD40LG = CD40 Ligand, CRP = C-reactive Protein, CDH1 = E-Cadherin, EPO R = Erythropoietin Receptor, FSH-β = Follicular Stimulating Hormone (beta subunit), DPP4 = Dipeptidyl-Peptidase 4 (DPPIV), CDH1 = E-Cadherin, CALCA = Pro-Calcitonin, ShhN = Sonic Hedgehog (N-terminus), TG = Thyroglobulin

Customized RayBio® Cytokine Antibody Arrays are available upon request. Select your cytokines of interest from our list of validated antibody pairs, and we will produce the customized array for you. For more information, please visit our website, [www.raybiotech.com](http://www.raybiotech.com).

## VII. Troubleshooting guide

<b>Problem</b>	<b>Cause</b>	<b>Recommendation</b>
No signal for any spots, including Positive Controls	Global detection failure	Adjust scanner settings or re-assemble slide into holder, wash slide 2 x 5 min with 150 $\mu$ l Wash Buffer II and repeat Steps 12-21.
Similar signal intensities for POS1/2/3	Improper laser power and/or PMT setting	Repeat scan using higher and/or lower laser power or PMT settings
High background signals	Incomplete washes	Carefully follow wash protocols, and/or increase wash times
	Sample concentration is too high	Repeat using lower sample concentration
	Fluor and/or Anti-Cytokines are too concentrated	Review protocol for dilution of reagents
Uneven background and/or missing spots	Bubbles present on slide during incubations	Be sure to completely remove all bubbles from slide surface
	Evaporation during incubation steps	Cover chamber assembly during washes and incubations
	Pooling/precipitation of sample or reagent; Incomplete washes.	Cover chamber assembly and use a rocker or shaker during washes and incubations; carefully follow wash protocols.
	Sample is too concentrated	Repeat experiment using more dilute sample
Randomly scattered high-intensity spots	Dust or other particulates	Dry slides in laminar flow hood and/or use clean containers and powder-free gloves.

Weak or no signals antigen-specific pots + Low Background	Sample is too dilute	Repeat experiment using higher sample concentration
	Improper dilution of Anti-Cytokines or Streptavidin-Fluor	Re-assemble slide into holder, wash 2 x 5 min with 150 µl Wash Buffer II and repeat Steps 12-21. Spin down reagents before diluting and mix well.
	Other Tips	Rescan at higher laser power or signal gain setting
		Repeat using higher sample concentration and/or incubate with sample O/N at 4°C
		Increase concentration of and/or length of incubation with Biotin-conjugated Anti-Cytokine (+ add'l large volume wash following Biotin-Ab incubation)
		Review proper storage conditions for kit components

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