RayBio® Human Th1/Th2/Th17 Antibody Array C Series

Patent Pending Technology

User Manual (Revised October 8, 2010)

RayBio® Human Th1/Th2/Th17 Antibody Array C Series
Cat# AAH-TH17-1-4

RayBio® Human Th1/Th2/Th17 Antibody Array C Series
Cat# AAH-TH17-1-8

RayBio® Human Cytokine Antibody Array Service
Cat# AAH-SERV

Please read manual carefully
before starting experiment

We provide you with excellent Protein Array systems and services

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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 507 cytokines, chemokines and other proteins in a single experiment. Our format is simple, sensitive, reliable and cost effective.

Our product offerings include:

1. Protein (antigen) Arrays
2. Cytokine Antibody Arrays (Human, Mouse, Rat and Porcine)
    - C-Series (Membrane, chemiluminescence detection)
    - G-Series (Glass chip, fluorescence detection)
3. Pathway- and Disease-focused antibody arrays
    - Angiogensis 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® Multiplex ELISA Arrays
5. L-Series Biotin Label-based Antibody Arrays
6. Phosphorylation Antibody Arrays
    - Receptor Tyrosine Kinases
    - EGFR and ErbB family (site-specific phosphorylation)
7. Over 500 different ELISA kits
8. EIA kits
9. Cell-based phosphorylation assay
10. Over 10,000 different Antibodies
11. Recombinant proteins
12. Peptide

Protocol for RayBio® Human Th1/Th2/Th17 Antibody Array C Series

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I. Introduction

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 disparity can exist between the relative expression levels of mRNA and their corresponding proteins[4]. 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 [5,6]. However, these methods are slow, expensive, labor-intensive and require specialized equipment[7]. 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[7]. They are involved in most disease processes, 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, pleiotrophic 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[8-14]. 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® C Series Cytokine Antibody Arrays have several advantages over detection of cytokines using single-target ELISA kits:

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 C 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%).

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. Literally hundreds of publications have demonstrated the usefulness of this high quality, easy-to-use array format (see Section VIII).

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II. Product Information

A. Materials Provided

Item	Description	AAH-TH17-4	AAH-TH17-8
AAH-TH17-*Y*	RayBio® Human Cytokine Antibody Array Membranes*	1 or 2 paks*	2 paks*
0103002-HTH-1	Biotin-Conjugated Anti-Cytokines	2 ea	4 ea
0103004-H	1,000X HRP-Conjugated Streptavidin	1 ea	1 ea
0103004-B	1X Blocking Buffer	25 ml	50 ml
0103004-W †	20X Wash Buffer I †	10 ml	20 ml
0103004-W †	20X Wash Buffer II †	10 ml	20 ml
0103004-L	2X Cell Lysis Buffer	10 ml	20 ml
0103004-D †	Detection Buffer C †	1.5 ml	2.5 ml
0103004-D †	Detection Buffer D †	1.5 ml	2.5 ml
0103004-T	8-Well Plastic Tray	1	1
Other Kit Components: 8-well Tray, Plastic sheets, Manual, Array Templates, Packing list			

* Packs contains 2 or 4 arrays each
† Wash Buffers and Detection Buffers are sold as Sets
Y = 4 or 8

B. Additional Materials Required

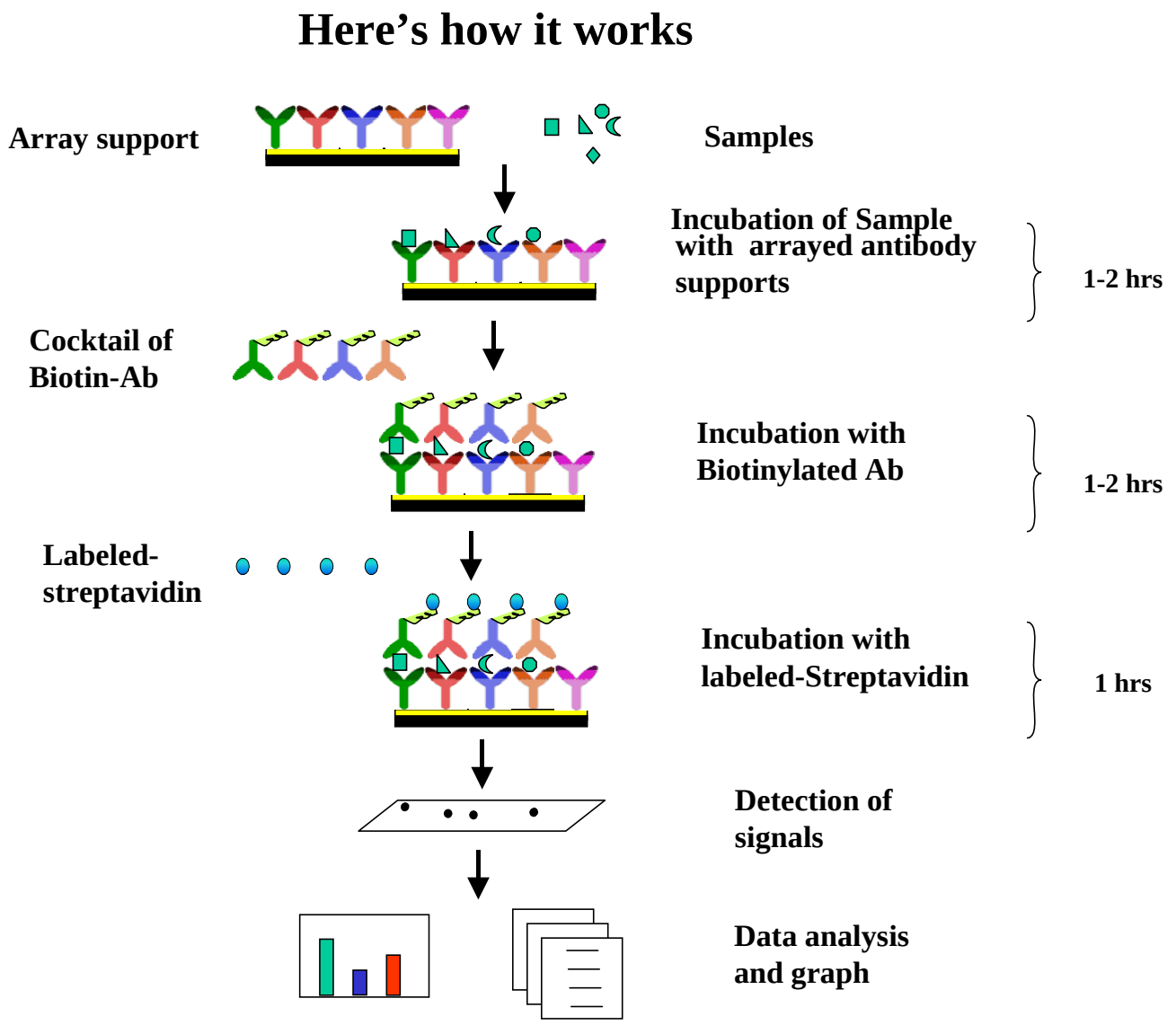
- Small plastic boxes or containers
- Pipettors, pipet tips and other common lab consumables
- Orbital shaker or oscillating rocker
- Saran Wrap
- A chemiluminescent blot documentation system (such as UVP's ChemiDoc-It® or EpiChem II Benchtop Darkroom), X-ray Film and a suitable film processor, or another chemiluminescent detection system.

C. Storage Recommendations:

For best results, store the entire kit at -20°C or -80°C upon arrival. If stored frozen, we recommend using the kit within 6 months, which is the duration of the product warranty period.

Once thawed, store array membranes and 1X Blocking Buffer at -20°C or -80°C, and all other component at 4°C. After thawing, the entire kit should be used within 3 months. Array kits are robust and will retain full activity even if stored for up to 24 hours at room temperature.

D. 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 with each sample.
- If spot intensities are weak, increase sample concentration in subsequent experiments.
- If background or spot intensities are too strong, decrease sample concentration in subsequent experiments.
- Most samples will not need to be concentrated. If concentration is required, we recommend using a spin-column concentrator with a chilled centrifuge.
- Unless otherwise noted, dilute all samples using the same dilution factor in 1X Blocking Buffer.

2. Recommended Sample Volumes and Dilution Factors

NOTE: All sample dilutions should be made using the 1X Blocking Buffer provided in this kit. For all sample types, final sample volume = 1.0-1.2 ml per membrane

- **Cell Cultured Media**: Neat (no dilution needed)
- **Serum & Plasma**: 2-fold to 5-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 lysate. **Optimal sample concentration of cell and tissue lysates should be determined empirically.**

- *For more details, please see Section I.A.4 (Cell and Tissue Lysates/Homogenates).*

- **Other Liquid Sample Types**: Most often Neat or 2-fold to 5-fold. However, optimal dilutions should be determined empirically. *For more details, please see Section I.A.6 (Other Liquid Samples), below.*

3. Preparing Serum/Plasma:
   - Prepare samples according to established protocols or collection tube manufacturer's instructions. Sub-aliquot into plastic tubes. Store at -20°C or -80°C.
   - We do not recommend comparing results between serum and plasma samples or between plasma prepared using different anticoagulants.
   - For most applications, you may test plasma samples prepared using any anticoagulant (ie, Heparin, EDTA or Citrate). However, EDTA-prepared plasma may interfere with optimal detection of MMPs and other metal-binding proteins.
   - If possible, avoid testing hemolyzed Serum or Plasma, as these samples may generate anomalous cytokine expression patterns and/or high background signals.

4. Preparing Cell-Cultured Media:
   - Expression of proteins in cell culture may depend on many variables, including cell type, starting cell number, media composition and growth conditions.
   - To start, we recommend seeding ≥1 x 10^6 cells in a 100 mm culture dish. However, you should consult the scientific literature for tips on how to cultivate your particular cell type.
   - Cell type and experimental protocols can profoundly influence cytokine expression. Please consult the scientific literature for details on the effectiveness of various treatments to elicit a desired response, optimal timeframes for growing cells prior to treatment, optimal concentrations and exposure times for treatments and the timing of sample collection.

- If possible, use media that is free of recombinant or purified growth factors. If you must add them, we strongly recommend testing an uncultured media aliquot as a sample "blank" to assess baseline signal response for comparison with cultured media samples.
- Serum-containing media rarely produce a baseline signal response with this product. Nevertheless, an ideal experimental design would be to test uncultured media as a sample "blank" to assess baseline signal responses.

5. Preparing Cell and Tissue Lysates/Homogenates:
   - IMPORTANT: Lysate sample volumes required must be determined empirically and will depend upon the total protein concentration of each lysate and the intensity of background signals for each sample.
     - You must determine the total protein concentration of each lysate/homogenate. We recommend using the BCA method (available from Pierce), as it is insensitive to detergents commonly found in lysis buffers.
     - Minimum Recommended Total Lysate Protein Concentration (prior to sample dilution) = 1.0 µg/µl
     - 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 1 ml final volume.
     - For your first experiment, we recommend using 200-250 µg of total protein in 1 ml of 1X Blocking Buffer (final volume) for each array membrane.
     - Optimal amounts of total lysate protein may range from 50-1000 µg per array membrane. Based upon the signal intensities of background and spots obtained with each sample, you may need to increase or decrease the volume of lysate used in subsequent experiments.
   - We recommend preparing cell or tissue lysates using 2X RayBio® Cell Lysis Buffer (Cat# 0103004-L), which is provided in all C Series Antibody Array Kits. Be sure to

properly dilute RayBio® Cell Lysis Buffer (1:1 with deionized H_2O) before use.

- Other lysis buffers can be used if they are non-denaturing, non-reducing, total salt concentration ≤700 mM), contain ≤2% total detergent (v/v) and are free of sodium azide (NaN_3).
- *We strongly recommend adding protease inhibitors to your cell lysis buffer.* Any broad-spectrum protease inhibitor cocktail intended for preparation of mammalian cell/tissue lysates should be sufficient, but please consult the scientific literature before deciding upon the exact composition of cocktail to use.
- Optimal protocols for mechanical disruption vary for different cell and tissue types. Please consult the scientific literature for examples of successful detection of proteins using ELISA or multiplex ELISA techniques in lysates made from cell or tissue samples similar to yours.
- BEWARE! Sonication can quickly heat volumes of 1 ml or less and denature proteins in your samples!
- After extraction, clarify your lysates by centrifugation and save the supernatant for your experiment. Preparing sub-aliquots is strongly recommended.

6. Other Liquid Samples:
   - RayBio® Cytokine Antibody Arrays are compatible with most liquid samples, including extracts, perfusates and lavages, as well as body fluids, such as CSF, Sputum, Saliva, Tears, and Urine.
   - Be sure to measure the total protein concentrations of these samples prior to sample incubation.
   - For samples that have fairly consistent concentrations of total protein between samples (CV ≤20%), dilute samples using equal volumes for each sample (ie, use the same dilution factor, v/v).
   - For samples exhibiting wider ranges of total protein content, dilute to equal concentrations of total protein (eg, 200 µg of total protein) in 1X Blocking Buffer to a final volume of 1 ml.

B. Handling Array Membranes

- Array membranes are fragile when dry. Handle with care.
- Wet or dry, grasp membranes by the edges using forceps.
- Do not allow membranes to dry out during experiments.
- The printed side of each array membrane is denoted by a dash mark (-) or Array number in the upper left corner.

C. Incubations and Washes

- All washes and incubations in the standard protocol can be performed using the 8-well tray provided in the kit.
- Place the cover on 8-well trays with lid to avoid drying, particularly during extended incubation or wash steps.
- Be sure to completely cover the membranes with sample or reagent during each incubation
- During incubation steps, avoid foaming and be sure to remove all bubbles from the membrane surface.
- 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.
- If you perform overnight Sample incubations, we recommend adding the optional "Large Volume Wash" described in Step 4 to minimize background signals.
- Overnight Blocking and Wash steps are useful for reducing background signal intensities even with completed membranes. Wash with Wash Buffer II, followed by repeating incubation with Streptavidin-HRP and chemiluminescent detection may greatly improve signal-to-noise ratios in your developed array images.

D. Chemiluminescence Detection

- We strongly recommend using multiple exposures to obtain optimum results. Begin by exposing the membranes for 40 Seconds. Then re-expose the film accordingly.
- If the signals are too strong (or background is too high), reduce exposure time (e.g. 5-30 seconds).
- If the signals are weak, increase exposure time (up to overnight).

- Blot documentation systems that use CCD cameras to detect chemiluminescence are ideal for imaging RayBio® array membranes. They can easily be programmed to take multiple exposures, and the dynamic range of these detectors tends to be 2-3 orders of magnitude greater than that of X-ray film or and much more sensitive to chemiluminescence than phosphorimaging systems.

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 reconstitution or dilution is required. Store at -20°C or -80°C when not in use.

2. Wash Buffers I and II are supplied at 20X concentration.
   a). For each membrane to be used in the experiment, dilute 1 ml of Wash Buffer I to a final volume of 20 ml deionized water.
   b). For each membrane to be used in the experiment, dilute 1 ml of Wash Buffer II to a final volume of 20 ml deionized water.
   c). 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 2000X concentration as a small liquid bead (typically ~2-5 µl). *Spin down the tube prior to reconstitution, as the concentrated liquid bead may have moved to the top of the tube during handling.*
   a). Prepare stock reagent by adding 100 µl 1X Blocking Buffer to the tube containing 2000X Biotin-Conjugated Anti-Cytokines.
   b). Mix well and quantitatively transfer stock reagent to larger tube containing 1900 µl of 1X Blocking Buffer.
   c). 1X Biotin-Conjugated Anti-Cytokines may be stored for 2-3 days at 4°C.

4. Streptavidin-HRP is supplied at 1000x concentration.
   a). Mix the tube containing 1,000X Streptavidin-HRP well before use, as precipitants may form during storage.
   b). Add 2 µl of 1000X Streptavidin-HRP to 1998 µl of 1X Blocking Buffer.
   c). This working dilution can be stored for 3-5 days at 4°C.

5. Detection Buffers C & D are supplied as 1X solutions that are intended to be mixed in a 1:1 ratio *immediately prior use*. Detection Buffers C & D may be stored at 4°C for up to 3 months.

B. Blocking and Incubations

NOTE: Please prepare all reagents immediately prior to use as described above (Section IV.A) and carefully read tips on Sample Preparation (Section III.A) and Incubations and Washes (Section III.C) before proceeding.

1) Place each membrane printed side up (see Section III.B) into the 8-well tray provided in the kit.

2) Block membranes by incubating with 2 ml 1X Blocking Buffer at room temperature (RT) for 30 min.

3) Decant Blocking Buffer, and incubate membranes with 1 ml of sample at RT for 1-2 h.

4) Aspirate samples from membranes, and wash 3 x 5 min with 2 ml Wash Buffer I at RT. Use fresh buffer for each wash.

   OPTIONAL Large Volume Wash: After Step 4 and before Step 5, place membranes into clean container(s). Add 20-30 ml of Wash Buffer I per membrane, and wash at RT with gentle shaking or rocking for 30-45 min. Return membranes to the 8-well tray.

5) Wash 2 x 5 min with 2 ml of 1X Wash Buffer II each at RT. Decant and use fresh wash buffer each time.

6) Add 1 ml of 1X Biotin-conjugated Anti-Cytokines to each membrane. Incubate at room temperature for 1-2 hours.

7) Decant or aspirate Anti-Cytokine reagent and repeat washes as described in steps 4 and 5 above.

8) Incubate at room temperature for 2 hours with 1 ml of 1X Streptavidin-HRP.

9) Wash membranes as directed in steps 4 and 5.

10) Proceed with Detection protocol (IV.B, below) or store membrane as directed in Section III.B (Handling Array Membranes)

C. Chemiluminescence Detection

NOTE: Do not allow membranes to dry out during detection. Detection of chemiluminescence should be started within 5 minutes after removing Detection Buffers and must be completed within 20 minutes.

11) Place a plastic sheet (provided in the kit) on your benchtop.

12) Place one or more array membranes protein side up (see Section III.B) on the plastic sheet. Drain excess liquid by touching one edge to blotting paper or tissue paper.

13) Into a single, clean tube, add equal volumes of Detection Buffer *C* and Detection Buffer *D* immediately prior to detection. *Mix well.* Add 250 µl of each buffer per membrane to be detected, eg, for 4 membranes, combine 1 ml of each detection buffer.

14) Pipette the mixed Detection Buffers on to each membrane. Place another plastic sheet on top, starting at one end and "rolling" the flexible plastic across the surface to the opposite end. During this process, ensure that the detection mixture completely covers each membrane, and gently smooth out any air bubbles. Avoid sliding the plastic sheet along the membranes' printed surfaces.

15) Incubate at room temperature for 2 minutes.

16) Remove top plastic sheet and remove excess liquid (see Step 12).

17) Gently replace the membranes (protein side up) on the bottom plastic sheet and replace the top plastic sheet (see Step 14). Gently smooth out any air bubbles on the membrane surfaces.

18) Detect signals using a chemiluminescence imaging system or expose the array membranes to x-ray film (we recommend Kodak's X-Omat AR film) and detect the signal using a film developer (See tips for obtaining array images in Section III.D).

19) For each array, try multiple exposures to obtain an image with low background and strong Positive Control signals that do not bleed into one another. Typical exposure times are 10 seconds to 2 minutes.

20) When you finish your last exposure, remove the top plastic sheet. Gently rinse membranes and plastic sheets with Wash Buffer II. Remove excess wash buffer as described in Step 14, and replace the membranes between the plastic sheets.

21) Wrap the sheets in Saran Wrap, and store the membranes at -20°C to -80°C. (Alternatively, you may store membranes for up to 5 days at 4°C in Wash Buffer II. Be sure to cover the container to avoid evaporation.)

V. Interpretation of Results:

Typical results obtained with RayBio® C Series Antibody Arrays

Sample-1 Sample-2 Control

The preceding figure presents typical images obtained with RayBio® Human Cytokine Antibody Array. These membranes were probed with conditioned media from two different cell lines. Membranes were exposed to Kodak X-Omat® film at room temperature for 1 minute.

Note the strong signals of the Positive Control spots, provided by biotin-conjugated IgG printed directly onto the array membrane in the upper-left and lower-right corners. These Positive Control spots are useful for proper orientation of the array image.

The signal intensity for each antigen-specific antibody spot is proportional to the relative concentration of the antigen in that sample. Comparison of signal intensities for individual antigen-specific antibody spots between and among array images can be used to determine relative differences in expression levels of each analyte sample-to-sample or group-to-group.

Obtaining Densitometry Data:

Visual comparison of array images may be sufficient to see differences in relative protein expression. However, most researchers will want to perform numerical comparisons of the signal intensities (or more precisely, signal *densities*), using 2-D densitometry. Gel/Blot documentation systems and other chemiluminescent or phosphorescent detection systems are usually sold as a package with compatible densitometry software.

To obtain densitometry data from an X-ray film, one must first scan the film to obtain a digitized image using an ordinary office scanner with resolution of 300 dpi or greater. Any densitometry software should be sufficient to obtain spot signal densities from your scanned images. One such software program, ImageJ, is available for free from the NIH (for more info, visit http://rsbweb.nih.gov/ij/).

We suggest using the following guidelines when extracting densitometry data from our array images:

- For each array membrane, identify a single exposure that the exhibits reasonably low background signal intensity and strong

Positive Control signals that do not "bleed" into one another. Exposure time does not need to be identical for each array, but Positive Control signals on each array image should have similar intensities.

- Measure the density of each spot using a circle that is roughly the size of one of the largest spots. Be sure to use the same circle (area and shape) for measuring the signal densities on every array for which you wish to compare the results.
- For each spot, use the summed signal density across the entire circle (ie, total signal density per unit area)

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

Background Subtraction:

On each array, several "Negative Control" and/or "Blank" spots will be included. Blank spots are literally blank; nothing has been printed there. Negative Control spots are printed with the same buffer we use to dilute antibodies printed on the array. Thus, the signal intensities of the Negative Controls represent the background plus non-specific binding to the printed spots. We recommend subtracting the mean of 4 or more Negative Control spots for background correction.

Normalization of Array Data:

The amount of biotin-conjugated IgG protein printed for each Positive Control spot is consistent from array to array. As such the intensity of these Positive Control signals can be used to normalize signal responses for comparison of results across multiple arrays, much like housekeeping genes and proteins are used to normalize results of PCR gels and Western Blots, respectively.

To normalize array data, one 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 first column on the left of 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 density of Positive Control spots on reference array
P(y) = mean signal density of Positive Control spots on Array "y"
X(y) = mean signal density for spot "X" on Array for sample "y"
X(Ny)= normalized signal intensity for spot "X" on Array "y"

After normalization to Positive Control signal intensities, yo.u can compare the relative expression levels, analyte-by-analyte, among or between your samples or groups. By comparing these signal intensities, one can determine relative differences in cytokine expression in each sample.

The RayBio® Analysis Tool software is available for use with data obtained using RayBio® Cytokine Antibody Arrays. Copy and paste your signal intensity data into the "Aligning Data" worksheet, and it will assist in compiling and organizing your data, as well as automatically subtracting background signals and normalizing to the Positive Controls. To order the Analysis Tool, please contact us at +1-770-729-2992 or info@raybiotech.com for more information.

VI. RayBio® C Series Human Th1/Th2/TH17 Antibody Array Maps:

	A	B	C	D	E	F	G	H	I	J	K	L
1	POS	POS	NEG	NEG	CD30	CD40 Ligand	CD40	GCSF	GITR	GM-CSF	IFN-gamma	IL-1 sRI
2	POS	POS	NEG	NEG	CD30	CD40 Ligand	CD40	GCSF	GITR	GM-CSF	IFN-gamma	IL-1 sRI
3	IL-1 sRII	IL-10	IL-12 p40	IL-12 p70	IL-13	IL-17	IL-17F	IL-17R	IL-1 β	IL-2	IL-21	IL-21 R
4	IL-1 sRII	IL-10	IL-12 p40	IL-12 p70	IL-13	IL-17	IL-17F	IL-17R	IL-1 β	IL-2	IL-21	IL-21 R
5	IL-22	IL-23 p19	IL-28A	IL-4	IL-5	IL-6	IL-6 sR	MIP-3α	sgp130	TGF-β1	TGF-β3	TNF-α
6	IL-22	IL-23 p19	IL-28A	IL-4	IL-5	IL-6	IL-6 sR	MIP-3α	sgp130	TGF-β1	TGF-β3	TNF-α
7	TNF-β	TRANCE	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	POS
8	TNF-β	TRANCE	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	POS

POS	1, 2, 13, 14, 84, 96
CD30	5, 17
CD40 Ligand	6, 18
CD40	7, 19
G-CSF	8, 20
GITR	9, 21
GM-CSF	10, 22
IFN-gamma	11, 23
IL-1 sRII	12, 24
IL-10	25, 37
IL-1 sRIII	26, 38
IL-12 p40	27, 39
IL-12 p70	28, 40
IL-13	29, 41
IL-17	30, 42
IL-17F	31, 43
IL-17 R	32, 44
IL-1 beta	33, 45

NEG	3, 4, 15, 16, 75-83, 88-95
IL-2	34, 46
IL-21	35, 47
IL-21R	36, 48
IL-22	49, 61
IL-23 (p19)	50, 62
IL-28A	51, 63
IL-4	52, 64
IL-5	53, 65
IL-6	54, 66
IL-6 R	55, 67
MIP-3 alpha	56, 68
spg130	57, 69
TGF beta 1	58, 70
TGF beta 3	59, 71
TNF alpha	60, 72
TNF beta	61, 73
TRANCE	62, 74

VII. Troubleshooting guide

Problem	Cause	Recommendation
No signal for any spots, including Positive Controls	Global detection failure	Repeat incubation with HRP-Streptavidin and Detection Buffers
Weak or no signals antigen-specific spots	Sample is too dilute	Repeat experiment using higher sample concentration
	Improper dilution of HRP-Streptavidin	Tube may contain precipitants. Repeat detection, mix 1000X HRP-Streptavidin well before diluting reagent
	Waiting too long to detect chemiluminescent signals	Repeat detection, making sure to complete this process within 20 min.
	Other Tips	Incubate with sample O/N at 4°
		Increase concentration of HRP-Streptavidin
		Increase concentration of Biotin-conjugated Anti-Cytokine
		Extend exposure time (may go overnight)
Uneven signal or background	Bubbles present on membrane during incubations	Be sure to completely remove all bubbles from membrane surface
	Membranes were not evenly covered during washes/incubations or allowed to dry out	Completely cover membranes with solution, use a rocker or shaker during washes and incubations
High background signals	Overexposure	Decrease exposure time
	Sample is too concentrated	Repeat experiment using more dilute sample
	NOTE: To reduce background on completed membrane, wash O/N @ 4°C in Wash Buffer II, then re-incubate with HRP-Streptavidin and repeat detection.	

VIII. Selected References Featuring RayBio® C-Series Arrays

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Customized RayBio® Cytokine Antibody Arrays. Select your cytokines of interest from the following list, and we will produce the customized array for you. For more information, please visit our website, www.raybiotech.com.

4-1BB
ACE-2
Acrp30
Activin A
Adiposin
Adipsin
AgRP
ALCAM
α-Fetoprotein
Amphiregulin
Angiogenin
Angiopoietin-1
Angiopoietin-2
Angiostatin
ANGPTL4
Axl
B7-1
BCAM
BCMA
BDNF
β2M
β IG-H3
bFGF
BLC
BMP-4
BMP-5
BMP-6
BMP-7
β-NGF
BTC
CA125
CA15-3
CA19-9
CA IX
Cardiotrophin-1
Cathepsin S
CCL14a
CCL21
CCL-28
CD14
CD23
CD30
CD40
CD40 Ligand
CD80
CEA
CEACAM-1
CK b 8-1
CNTF
Cripto
CRP
CTACK
CXCL16
DAN
Decorin
Dkk-1
Dkk-3
Dkk-4
DPPIV
DR6
Dtk
E-Cadherin
EDA-A2
EGF
EGFR
EG-VEGF
ENA-78
Endoglin
Eotaxin
Eotaxin-2
Eotaxin-3
Ep CAM
ErbB2
ErbB3
EPO R
E-Selectin
Fas
Fas Ligand
Fcr RIIB/C
Ferritin
FGF-4
FGF-6
FGF-6
FGF-7
FGF-9
Fit-3 Ligand
FLRG
Follistatin
Fractalkine
FSH
Furin
Galectin-7
GCP-2
G-CSF
GDF-15
GDNF
GITR
GITR Ligand
GM-CSF
GRO
GROα
GH
HB-EGF
HCC-4
hCG (intact)
HGF
HVEM
I-309
ICAM-1
ICAM-2
ICAM-3
IFNγ
IGF-1 SR
IGFBG-1
IGFBP-2
IGFBP-3
IGFBP-4
IGFBP-6
IGF-I
IGF-I SR
IGF-II
IL-1α
IL-1β
IL-1 R II
IL-1 R4/ST2
IL-1 RI
IL-1 sRI
IL-10
IL-10 Rα
IL-10 Rβ
IL-11
IL-12
IL-12 p40
IL-12 p70
IL-13
IL-13 Rα-2
IL-13 RI
IL-15
IL-16
IL-17
IL-17B
IL-17C
IL-17F
IL-17R
IL-18 BPα
IL-18 Rβ
IL-1ra
IL-2
IL-2 Rβ
IL-2 Rγ
IL-2 Ra
IL-21R
IL-22
IL-28A
IL29
IL-3
IL-31
IL-4
IL-5
IL-5 Rα
IL-6
IL-6 sR
IL-7
IL-8
IL-9
Insulin
IP-10
I-TAC
LAP
Leptin
Leptin R
LIF
LIGHT
LIMPII
L-Selectin
LH
Lymphotactin
LYVE-1
Marapsin
MCP-1
MCP-2
MCP-3
MCP-4
M-CSF
M-CSF R
MDC
MICA
MICB
MIF
MIG
MIP-1α
MIP-1β
MIP-1δ
MIP-3α
MIP-3β
MMP-1
MMP-10
MMP-13
MMP-2
MMP-3
MMP-7
MMP-8
MMP-9
MPIF-1
MSPα
NAP-2
NCAM-1
NGF R
Nidogen-1
NrCAM
NRG1-β1
NT-3
NT-4
Oncostatin M
Osteopontin
OPG
PAI-I
PARC
PDGF Rα
PDGF Rβ
PDGF-AA
PDGF-AB
PDGF-BB
PECAM-1
PlGF
PF4
Procalcitonin
Prolactin
PSA-free
PSA-total
RAGE
RANK
RANTES
Resistin
S-100b
SAA
SCF
SCF R
SDF-1
SDF-1β
SAA
sgp130
Shh N
Siglec-5
Siglec-9
ST2
sTNF RI
sTNF RII
TACE
TARC
TECK
TGFα
TGFβ1
TGFβ2
TGFβ3
TPO
Thyroglobulin
Tie-1
Tie-2
TIM-1
TIMP-1
TIMP-2
TIMP-4
TNFα
TNFβ
TNFRSF21
TNFRSF6
TRAIL R2
TRAIL R3
TRAIL R4
Trappin-2
TREM-1
TSH
TSLP
Ubiquitin
uPAR
VCAM-1
VE-Cadherin
VEGF
VEGF R2
VEGF R3
VEGF-C
VEGF-D
XEDAR

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