RayBio® C-Series Human Protein Tyrosine Phosphorylation Antibody Array C1

For the semi-quantitative detection of 507 Tyrosine-phosphorylated human proteins in cell and tissue lysates

Patent Pending Technology

User Manual
(Revised July 8th, 2022)

Cat# AAH-PTYR-1-2 (2 Sample Kit)
Cat# AAH-PTYR-1-4 (4 Sample Kit)

Please read manual carefully
before starting experiment

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C-Series Antibody Arrays

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I. INTRODUCTION

Protein phosphorylation plays an unusually prominent role in cell signaling, development and growth. The RayBio® Human Protein Tyrosine Phosphorylation Antibody Array C1 is a very rapid, convenient and sensitive assay to simultaneous detect multiple protein phosphorylations and can be used to monitor activation or function of important biological pathways.

RayBiotech is committed to developing a series of phosphorylation antibody arrays. RayBio® Human Protein Tyrosine Phosphorylation Antibody Array C1 is specifically designed for simultaneously identifying the relative levels of Tyrosine phosphorylation of 507 different human proteins in cell lysates. By monitoring the changes in protein tyrosine phosphorylation in your experimental model system, you can verify pathway activation in your cell lines without spending excess time and effort in performing an analysis of immunoprecipitation and/or Western Blot.

By using RayBio® Human Protein Tyrosine Phosphorylation Antibody Array C1, treated or untreated cell lysate is added into antibody array membranes. The antibody array membranes are washed and biotinylated anti-phosphotyrosine antibody is used to detect phosphorylated tyrosines on target protein. After incubation with HRP-streptavidin, the signals are visualized by chemiluminescence.

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

1. More Data, Same or Less Sample: Antibody arrays provide high-content screening using about the same sample volume as traditional ELISA.

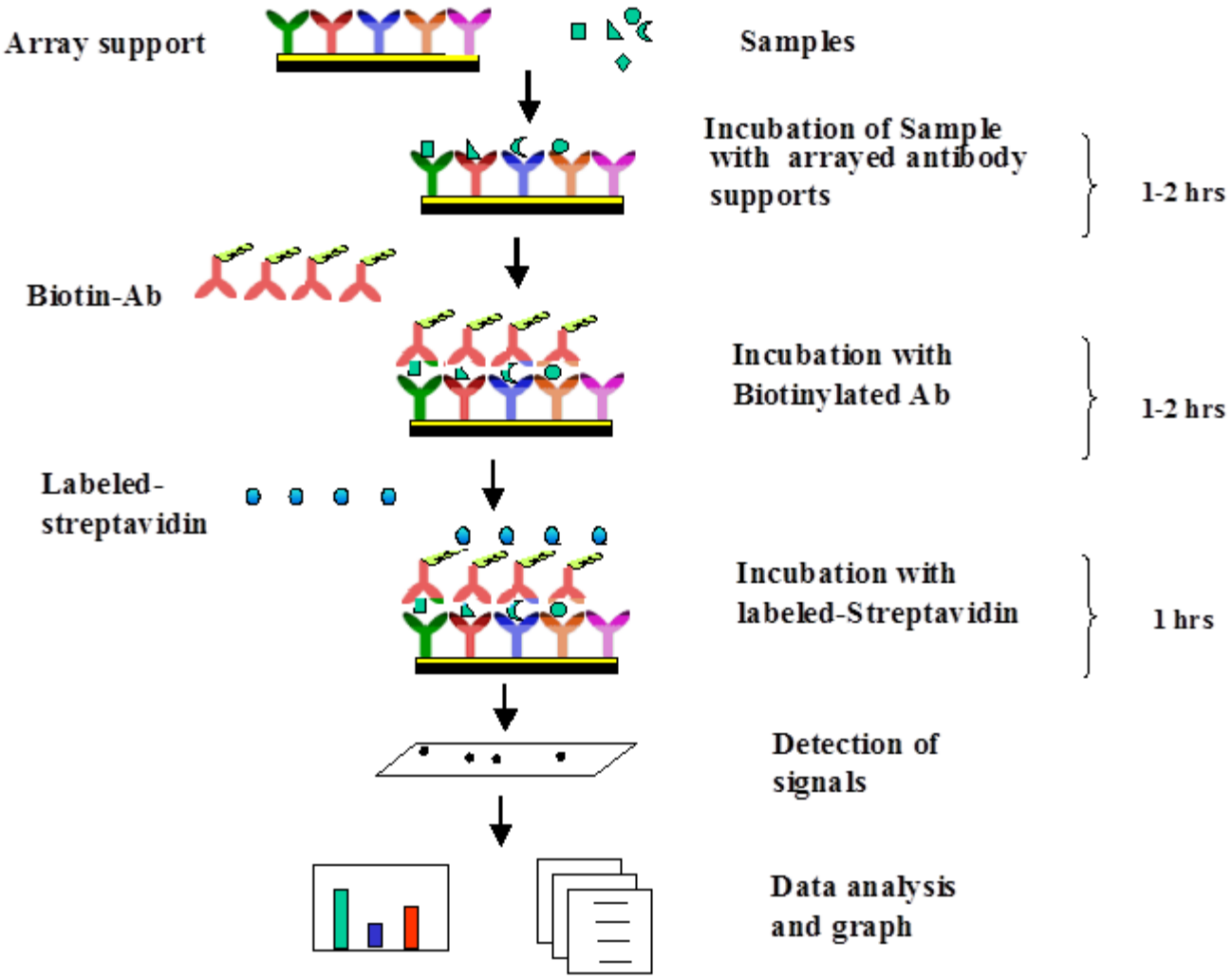
2. Global View of Cytokine Expression: Antibody array screening improves the chances for discovering key factors, disease mechanisms, or biomarkers related to cytokine signaling.

3. Similar (sometimes better) 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%).

II. HOW IT WORKS



III. COMPONENTS AND STORAGE

Store kit at ≤ -20 °C immediately upon arrival. Kit must use within the 6 months expiration date.

ITEM	COMPONENT	AAH-PTYR-1-2	AAH-PTYR-1-4	STORAGE TEMPERATURE AFTER THAWING**
1	Antibody Arrays	2 membranes	4 membranes	≤-20 °C
2	Blocking Buffer	3 vials (25ml/ea)	5 vials (25ml/ea)	
3	Biotinylated Anti-Phosphotyrosine Antibody	1 vial	2 vials	2-8 °C (for up to 3 days after dilution)
4	1,000X HRP-Streptavidin Concentrate	1 vial (50 µl)		2-8 °C
5	20X Wash Buffer I Concentrate	1 vial (30 ml)		2-8 °C
6	20X Wash Buffer II Concentrate	1 vial (30 ml)		
7	2X Cell Lysis Buffer Concentrate	1 vial (16 ml)		
8	Detection Buffer C	1 vial (10 ml)	2 vials (10 ml/ea)	
9	Detection Buffer D	1 vial (10 ml)	2 vials (10 ml/ea)	
10	Incubation Tray w/ Lid	2 trays	4 trays	Room Temperature
11	Protease Inhibitor Cocktail	1 vial		≤-20 °C
12	Phosphatase Inhibitor Cocktail II	1 vial		
Other Kit Components: Plastic Sheets, Array Map Template, User Manual				

*Each package contains 2 or 4 membranes
**For up to 3 months (unless stated otherwise) or until expiration date

IV. ADDITIONAL MATERIALS REQUIRED

- Pipettors, pipet tips and other common lab consumables
- Orbital shaker or oscillating rocker
- Tissue paper, blotting paper or chromatography paper
- Adhesive tape or plastic wrap
- Distilled or de-ionized water
- A chemiluminescent blot documentation system:
  - CCD Camera
  - X-Ray Film and a suitable film processor
  - Gel documentation system
  - Or another chemiluminescent detection system capable of imaging a western blot

V. SAMPLE TIPS AND GENERAL CONSIDERATIONS

A. Sample Collection, Preparation, and Storage

NOTE: *Optimal methods will need to be determined by each experimenter empirically based on researched literature and knowledge of the samples.*

- If not using fresh samples, freeze samples as soon as possible after collection.
- Avoid multiple freeze-thaw cycles. If possible, sub-aliquot samples prior to initial storage.
- It is strongly recommended to add a protease inhibitor cocktail to cell and tissue lysate samples.
- Avoid sonication of 1 ml or less as this can quickly heat and denature proteins
- Most samples will not need to be concentrated. If concentration is required, a spin column concentrator with a chilled centrifuge is recommended.
- Always centrifuge the samples hard after thawing (~10,000 RPM for 2-5 minutes) in order to remove any particulates that could interfere with detection.
- **The Cell Lysate can be prepared as follows:**
  - For attached cells, remove supernatant from cell culture, wash cells twice with cold 1X PBS (for suspension cells, pellet the cells by spinning down the cells at 1500 rpm for 10 min) making sure to remove any remaining PBS before adding Lysis Buffer. Solubilize the cells at $2x10^7$ cells/ml in 1X Lysis Buffer containing Protease Inhibitor Cocktail and Phosphatase Inhibitor Cocktail Set II (see preparation note shown on page 7 under Component Preparation Section). Pipette up and down to resuspend cells and rock the lysates gently at 2–8 °C for 30 minutes. Transfer extracts to microfuge tubes and centrifuge at 14,000 x g for 10 min.

    It is recommended that sample protein concentrations be determined using a total protein assay. For incubation with the Phosphorylation Antibody Array I, use at a protein concentration of 50-1000 µg/ml for cell lysates.

    Lysates should be used immediately or aliquot and stored at -70 °C. Thawed lysates should be kept on ice prior to use.

    If you experience high background, you may further dilute your samples. If signals are too weak, the cell lysates can be pretreated by immunoprecipitations before incubation with array membranes. Immunoprecipitations can be done using anti-phosphotyrosine and protein A.

General tips for preparing lysate samples can be viewed on the online Resources page of the website.

B. Sample Types and Recommended Dilutions/Amounts

NOTE: *Optimal sample dilutions and amounts will need to be determined by each experimenter empirically but the below recommendations may be used as a starting point. Blocking Buffer (ITEM 2) should be used to dilute samples. Normalize by loading equal amounts of protein per sample.*

- **Cell and Tissue Lysates**: load **50 to 1000 µg** of total protein (after at least a 5-fold dilution to minimize the effect of any detergent(s)). Therefore the original lysate concentration should be **250 µg to 5 mg/ml**.

C. Handling Membranes

- The antibody printed side of each membrane is marked by a dash (-) or number (#) in the upper left corner.
- Do not allow membranes to dry out during the experiment or they may become fragile and break OR high and/or uneven background may occur.
- Grasp membranes by the corners or edges only using forceps. DO NOT touch printed antibody spots.

D. Incubations and Washes

- Perform **ALL** incubation and wash steps under gentle rotation or rocking motion (~0.5 to 1 cycle/sec) using an orbital shaker or oscillating rocker to ensure complete and even reagent/sample coverage. Rocking/rotating too vigorously may cause foaming or bubbles to appear on the membrane surface which should be avoided.
- All washes and incubations should be performed in the Incubation Tray (ITEM 10) provided in the kit.
- Cover the Incubation Tray with the lid provided during all incubation steps to avoid evaporation and outside debris contamination.
- Ensure the membranes are completely covered with sufficient sample or reagent volume during each incubation.
- Avoid forceful pipetting directly onto the membrane; instead, gently pipette samples and reagents into a corner of each well.
- Aspirate samples and reagents completely after each step by suctioning off excess liquid with a pipette. Tilting the tray so the liquid moves to a corner and then pipetting is an effective method.
- Optional overnight incubations may be performed for the following steps to increase overall spot signal intensities:
    - Sample Incubation
    - Biotinylated Antibody Cocktail Incubation
    - HRP-Streptavidin Incubation

NOTE: *Overnight incubations should be performed at 4 °C (also with gentle rocking/shaking). Be aware that longer incubations can also increase the background response so complete liquid removal and washing is critical.*

VI. CHEMILUMINESCENCE DETECTION TIPS

- Beginning with adding the detection buffers and ending with exposing the membranes should take no more than 10-15 minutes as the chemiluminescent signals may start to fade at this point.
- Trying multiple exposure times is recommended to obtain optimum results.
- A few seconds to a few minutes is the recommended exposure time range, with 30 seconds to 1 minute being suitable for most samples.

VII. COMPONENT PREPARATION

NOTE: *Thaw all reagents to room temperature immediately before use. If wash buffers contain visible crystals, warm to room temperature and mix gently until dissolved.*

NOTE: *The Biotinylated Antibody Cocktail (ITEM 3) and the HRP-Streptavidin Concentrate (ITEM 4) vials should be briefly centrifuged (~1000 g) before opening to ensure maximum recovery and mixed well as precipitates may form during storage.*

ITEM	COMPONENT	PREPARATION	EXAMPLE
1	Antibody Arrays	No Preparation	N/A
2	Blocking Buffer		
3	Biotinylated Antibody Cocktail*	Pipette 1 ml of Blocking Buffer into each vial. Mix gently with a pipette. Transfer the entire contents into a tube containing 11 ml of the Blocking Buffer.	N/A
4	1,000X HRP-Streptavidin Concentrate	Dilute 1,000-fold with Blocking Buffer. Mix gently with a pipette.	10 µl of 1,000X concentrate + 9990 µl of Blocking Buffer = 10 ml of 1X working solution
5	20X Wash Buffer I Concentrate	Dilute **each** 20-fold with distilled or deionized water.	10 ml of 20X concentrate + 190 ml of water = 200 ml of 1X working solution
6	20X Wash Buffer II Concentrate		
7	2X Cell Lysis Buffer Concentrate**	Dilute 2-fold with distilled or deionized water.	10 ml of 2X concentrate + 10 ml of water = 20 ml of 1X working solution
8	Detection Buffer C	No Preparation	N/A
9	Detection Buffer D		
10	Incubation Tray w/ Lid		
11	Protease Inhibitor Cocktail	Pipette 60 µl of **1X** Cell Lysis Buffer into the vial to prepare 100X Protease Inhibitor Cocktail concentrate.	
12	Phosphatase Inhibitor Cocktail II	Add 180 µl of 1X Lysis Buffer into the vial to prepare 25X Phosphatase Inhibitor Cocktail Set II Concentrate. Dissolve the powder thoroughly by gentle mixing.	

*1 vial is enough to test 2 membranes

**Only for use for preparing cell or tissue lysates. General tips for preparing lysates and other common sample types can be found on the online Resources Page

Note: Prior to preparing cell or tissue lysates: Add 20 µl Protease Inhibitor Cocktail Concentrate (100X) and 80 µl Phosphatase Inhibitor Cocktail Set II Concentrate (25X) into 1.9 ml 1X Lysis Buffer immediately before use. Mix well.

VIII. PROTOCOL

NOTE: *Prepare all reagents and samples immediately prior to use. See Sections V and VII.* ***ALL*** *incubations and washes must be performed under gentle rotation/rocking (~0.5-1 cycle/sec)*

1) Remove the kit from storage and allow the components to equilibrate to room temperature (RT).

2) Carefully remove the Antibody Arrays (ITEM 1) from the plastic packaging and place each membrane (printed side up) into a well of the Incubation Tray (ITEM 10). One membrane per well.

NOTE: *The antibody printed side is marked by a dash (-) or number (#) in the upper left corner.*

A. Blocking

3) Pipette 6 ml of Blocking Buffer (ITEM 2) into each well and incubate for 1 hour at RT.

4) Aspirate blocking buffer from each well with a pipette.

B. Sample Incubation

5) Pipette 6 ml of diluted or undiluted sample into each well and incubate for 1.5 to 5 hours at RT OR overnight at 4 °C.

NOTE: *Longer incubations can help maximize the spot signal intensities. However, doing so can also increase the background response so complete liquid removal and washing is critical.*

6) Aspirate samples from each well with a pipette.

C. First Wash

NOTE: *The 20X Wash Buffer Concentrates I and II (ITEM 5 and 6) must be diluted 20-fold before use. See Section VII for details.*

7) Wash Buffer I Wash: Pipette 20 ml of **1X** Wash Buffer I into each well and incubate for 5 minutes at RT. Repeat this 2 more times for a total of 3 washes using fresh buffer and aspirating out the buffer completely each time.

8) Wash Buffer II Wash: Pipette 20 ml of **1X** Wash Buffer II into each well and incubate for 5 minutes at RT. Repeat this 1 more time for a total of 2 washes using fresh buffer and aspirating out the buffer completely each time.

D. Biotinylated Antibody Cocktail Incubation

NOTE: *The Biotinylated Antibody Cocktail (ITEM 3) must be prepared before use. See Section VII for details.*

9) Pipette 6 ml of the **prepared** Biotinylated Antibody Cocktail into each well and incubate for 1.5 to 2 hours at RT OR overnight at 4°C.
10) Aspirate biotinylated antibody cocktail from each well.

E. Second Wash

11) Wash membranes as directed in Steps 7 and 8.

F. HRP-Streptavidin Incubation

NOTE: *The 1,000X HRP-Streptavidin Concentrate (ITEM 4) must be diluted before use. See Section VII for details.*

12) Pipette 6 ml of **1X** HRP-Streptavidin into each well and incubate for 2 hours at RT OR overnight at 4°C.

13) Aspirate HRP-Streptavidin from each well.

G. Third Wash

14) Wash membranes as directed in Steps 7 and 8.

H. Chemiluminescence Detection

NOTE: *Do not allow membranes to dry out during detection.*

15) Transfer the membranes, printed side up, onto a sheet of chromatography paper, tissue paper, or blotting paper lying on a flat surface (such as a benchtop).

16) Remove any excess wash buffer by blotting the membrane edges with another piece of paper.

17) Transfer and place the membranes, printed side up, onto a plastic sheet (provided) lying on a flat surface.

NOTE: *Multiple membranes can be placed next to each other and fit onto a single plastic sheet. Use additional plastics sheets if necessary.*

18) Into a single clean tube, pipette equal volumes (1:1) of Detection Buffer C (ITEM 8) and Detection Buffer D (ITEM 9). Mix well with a pipette.

EXAMPLE*: 4.2 ml of Detection Buffer C + 4.2 ml of Detection Buffer D = 8.4 ml (enough for 2 membrane)*

19) Gently pipette 4 ml of the Detection Buffer mixture onto each membrane and incubate for 2 minutes at RT (DO NOT ROCK OR SHAKE). Immediately afterwards, proceed to Step 20.

NOTE: *Exposure should ideally start within 5 minutes after finishing Step 19 and completed within 10-15 minutes as chemiluminescence signals will fade over time. If necessary, the signals can usually be restored by repeating washing, HRP-Streptavidin and Detection Buffers incubations (Steps 11-19)*

20) Place another plastic sheet on top of the membranes by starting at one end and gently "rolling" the flexible plastic sheet across the surface to the opposite end to smooth out any air bubbles. The membranes should now be "sandwiched" between two plastic sheets.

NOTE: *Avoid "sliding" the top plastic sheet along the membranes' printed surface.*

21) Transfer the sandwiched membranes to the chemiluminescence imaging system such as a CCD camera (recommended) and expose.

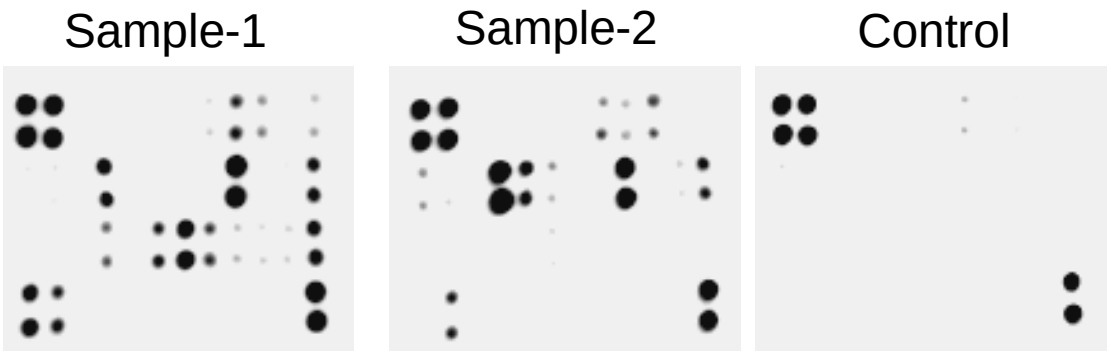
NOTE: *Optimal exposure times will vary so performing multiple exposure times is strongly recommended. See Section VI for additional details.*

I. <u>Storage</u>

22) To store, without direct pressure, gently sandwich the membranes between 2 plastic sheets (if not already), tape the sheets together or use plastic wrap to secure them, and store at ≤ -20 °C for future reference.

IX. TYPICAL RESULTS

Typical results obtained with RayBio® C-Series Antibody Arrays



The preceding figures present typical images obtained with RayBio® C-Series Antibody Arrays. These membranes were probed with conditioned media from two different cell lines. Membranes were exposed with Kodak X-Omat® film at room temperature for 1 minute.

Note the strong signals of the Positive Control spots in the upper left and lower right corners. (See below for further details on the control spots.)

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.

X. INTERPRETING THE RESULTS

A. Control Spots

Positive Control Spots (POS) – controlled amount of biotinylated antibody printed onto the array. Used for normalization and to orientate the arrays.

Blank Spots (BLANK) – nothing is printed here. Used to measure the background response.

B. Data Extraction

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.

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 website along with an array plug-in.

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 a high signal to noise ratio (strong spot signals and low background response). Strong Positive Control Spot signals but not too strong that that they are "bleeding" into one another is ideal. The 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 extraction circle dimensions (area, size, 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)

C. Data Analysis

NOTE: *RayBiotech offers Microsoft® Excel-based Analysis Software Tools for each array kit for automatic analysis. Please visit the website at www.raybiotech.com or contact us for ordering information.*

Once the raw numerical densitometry data is extracted, the background must be subtracted and the data normalized to the Positive Control signals to analyze.

Background Subtraction: Select values which you believe best represent the background. If the background is fairly even throughout the membrane, the Negative Control Spots (NEG) and/or Blank Spots (BLANK) should be similar and are accurate for this purpose.

Positive Control Normalization: The amount of biotinylated antibody 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 Array" to which the other arrays are normalized to. The choice of the Reference Array is arbitrary.

NOTE: *The RayBio® Analysis Software Tools always designate Array 1/Sample 1 as the Reference Array.*

Next, the simple algorithm below can be used to calculate and determine the signal fold expression between like analytes.

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"

For example:

Let's determine the relative expression for IL-6 on two different arrays (Arrays 1 and 2). Let's assume that the duplicate signals for the IL-6 spots on each array are identical (or that the signal intensity used in the following calculation is the mean of the two duplicates spots). Also assume the following:

P1 = 2500
P2 = 2700
IL-6 (1) = 300
IL-6 (2) = 455

Then IL-6(N2) = 455 *2500/2700 = 421.30

The fold increase of IL-6(N2) vs IL-6(1) = 421.3/300 = 1.40-fold increase or a 40% increase in the signal intensity of IL-6 in Array 2 vs. Array 1.

XI. ARRAY MAP

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1	POS1	POS2	POS3	Blank	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
2	POS1	POS2	POS3	Blank	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
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4	Blank	Blank	Blank	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53
5	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83
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7	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113
8	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113
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11	144	145	146	147	148	149	150	151	152	153	154	155	156	157	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173
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35	Blank	Blank	Blank	Blank	486	487	488	489	490	491	492	493	494	495	496	497	498	499	500	501	502	503	504	505	506	507	Blank	POS3	POS2	POS1
36	Blank	Blank	Blank	Blank	486	487	488	489	490	491	492	493	494	495	496	497	498	499	500	501	502	503	504	505	506	507	Blank	POS3	POS2	POS1

POS = Positive Control Spot
BLANK = Blank Spot

NOTE: *Protein alternative names, accession numbers, and official symbols can be accessed on www.raybiotech.com via the Resources Page.*

XII. ARRAY TARGET LIST

Number	Name	Number	Name	Number	Name	Number	Name	Number	Name	Number	Name	Number	Name
1	6Ckine	74	CNTF R alpha	147	FGF-19	220	IGFBP-4	293	IL-22 BP	366	MMP-24	439	Shh-N
2	Activin A	75	F3	148	FGF-20	221	IGFBP-6	294	IL-22 R	367	MMP-25	440	SPARC
3	Activin B	76	CRIM 1	149	FGF-21	222	IGFBP-rp1	295	IL-23	368	Musk	441	Spinesin
4	Activin C	77	Cripto-1	150	FGF-23	223	IGF-I	296	IL-23 R	369	MSPa	442	TACI
5	Activin RIA	78	CRTH-2	151	FLRG	224	IGF-I R	297	IL-24	370	MICA	443	Tarc
6	Activin RIB	79	Cryptic	152	Flt-3 Ligand	225	IGF-II	298	IL-26	371	NAP-2	444	TCCR
7	EYA2	80	CTACK	153	Follistatin	226	IGF-II R	299	IL-27	372	NCAM-1	445	TECK
8	Activin RIIA	81	CTGF	154	Follistatin-like 1	227	IL-1 alpha	300	IL-28A	373	Neuritin	446	TFPI
9	Adiponectin	82	CTLA-4	155	Fractalkine	228	IL-1 beta	301	IL-29	374	NeuroD1	447	TGF-alpha
10	AgRP	83	CV-2	156	Frizzled-1	229	IL-1 F5	302	IL-31	375	Neuropilin-2	448	TGF-beta 1
11	ALCAM	84	CXCL14	157	Frizzled-3	230	IL-1 F6	303	IL-31 RA	376	Neurturin	449	TGF-beta 2
12	Angiogenin	85	CXCL16	158	Frizzled-4	231	IL-1 F7	304	BACE-1	377	NGF R	450	TGF-beta 3
13	Angiopoietin-1	86	CXCR1	159	Frizzled-5	232	IL-1 F8	305	FACX	378	NOV	451	ATP2B1
14	Angiopoietin-2	87	CXCR2	160	Frizzled-6	233	IL-1 F9	306	Insulin	379	GGF2	452	TGF-beta RI
15	Angiopoietin-4	88	CXCR3	161	Frizzled-7	234	IL-1 F10	307	Insulin R	380	Nidogen-1	453	TGF-beta RII
16	ANGPTL1	89	CXCR4	162	Galectin-3	235	IL-1 R3	308	Insulysin	381	NrCam	454	Grb2
17	ANGPTL2	90	CXCR5	163	GASP-1	236	IL-1 R4	309	IP-10	382	NRG2	455	TGF-beta RIII
18	ANGPTL7	91	CXCR6	164	GASP-2	237	IL-1 R6	310	I-TAC	383	NRG3	456	Thrombopoietin
19	Angiostatin	92	D6	165	GCP-2	238	IL-1 R8	311	Kininostatin	384	NT-3	457	Thyroid Peroxidase
20	APJ	93	DAN	166	GCSF	239	IL-1 R9	312	Kremen-1	385	NT-4	458	Thrombospondin-1
21	Amphiregulin	94	DANCE	167	G-CSF R	240	IL-1 ra	313	Kremen-2	386	Orexin A	459	Thrombospondin-2
22	APRIL	95	DcR3	168	GDF1	241	IL-1 RI	314	Lck	387	Orexin B	460	Thrombospondin-4
23	Artemin	96	Decorin	169	GDF3	242	IL-1 RII	315	LTBP1	388	OSM	461	Thymopoietin
24	Axl	97	Dkk-1	170	GDF5	243	IL-2	316	LBP	389	Osteoactivin	462	Tie-1
25	B7-1	98	Dkk-3	171	GDF8	244	IL-2 R alpha	317	LECT2	390	Osteocrin	463	Tie-2
26	BAFF R	99	Dkk-4	172	GDF9	245	IL-2 R beta	318	Lefty-A	391	Osteoprotegerin	464	TIMP-1
27	BCMA	100	DR3	173	GDF11	246	IL-2 R gamma	319	Leptin R	392	OX40 Ligand	465	TIMP-2
28	BD-1	101	DR6	174	GDF-15	247	IL-3	320	Leptin	393	PARC	466	TIMP-3
29	BDNF	102	Dtk	175	GDNF	248	IL-3 R alpha	321	LFA-1 alpha	394	PD-ECGF	467	TIMP-4
30	beta-Catenin	103	EDA-A2	176	GFR alpha-1	249	IL-4	322	LIF	395	PDGF R alpha	468	DEFA5
31	Bax	104	EDAR	177	GFR alpha-2	250	IL-4 R	323	LIF R alpha	396	PDGF R beta	469	TLR1
32	beta-NGF	105	EDG-1	178	GFR alpha-3	251	IL-5	324	LIGHT	397	PDGF-AA	470	TLR2
33	BIK	106	EGF	179	GFR alpha-4	252	IL-5 R alpha	325	Lipocalin-1	398	PDGF-AB	471	TLR3
34	BLC	107	EGF R	180	GITR	253	IL-6	326	LRP-1	399	PDGF-BB	472	TLR4
35	BMP-2	108	EG-VEGF	181	GITR Ligand	254	IL-6 R	327	LRP-6	400	PDGF-C	473	TMEFF1
36	BMP-3	109	EMAP-II	182	CBR1	255	IL-7	328	L-Selectin	401	PDGF-D	474	TMEFF2
37	BMP-3b	110	ENA-78	183	Glut1	256	IL-7 R alpha	329	Lipocalin-2	402	PECAM-1	475	TNF-alpha
38	BMP-4	111	Endocan	184	Glut2	257	IL-8	330	Lymphotactin	403	Pentraxin3	476	TNF-beta
39	BMP-5	112	Endoglin	185	Glut3	258	IL-9	331	LTB	404	Persephin	477	TNF RI
40	BMP-6	113	Endostatin	186	Glut5	259	IL-10	332	LTBR	405	PF4	478	TNF RII
41	BMP-7	114	EN-RAGE	187	Glypican 3	260	IL-10 R alpha	333	MAC-1	406	PlGF	479	TRADD
42	BMP-8	115	Eotaxin	188	Glypican 5	261	IL-10 R beta	334	MCP-1	407	PLUNC	480	TRAIL
43	BMP-15	116	Eotaxin-2	189	GM-CSF	262	IL-11	335	MCP-2	408	Pref-1	481	TRAIL R1
44	BMPR-IA	117	Eotaxin-3	190	GM-CSF R alpha	263	IL-12 p40	336	MCP-3	409	Progranulin	482	TRAIL R2
45	BMPR-IB	118	Epiregulin	191	Granzyme A	264	IL-12 p70	337	MCP-4	410	Prolactin	483	TRAIL R3
46	BMPR-II	119	ErbB2	192	GREMLIN	265	IL-12 R beta 1	338	M-CSF	411	P-selectin	484	TRAIL R4
47	BTC	120	ErbB3	193	GRO	266	IL-12 R beta 2	339	M-CSF R	412	RAGE	485	TRANCE
48	Cardiotrophin-1	121	ErbB4	194	GRO-a	267	IL-13	340	MDC	413	RANK	486	TREM-1
49	CCL14	122	Erythropoietin	195	GH	268	IL-13 R alpha 1	341	MFG-E8	414	RANTES	487	TROY
50	CCL28	123	E-Selectin	196	GHR	269	IL-13 R alpha 2	342	MFRP	415	RELM beta	488	TSG-6
51	CCR1	124	Endothelin	197	HB-EGF	270	IL-15	343	MIF	416	RELT	489	TSLP R
52	CCR2	125	FADD	198	HCC-4	271	IL-15 R alpha	344	MIG	417	ROBO4	490	TWEAK
53	CCR3	126	FAM3B	199	HCR	272	IL-16	345	MIP-1a	418	S100 A8/A9	491	TWEAK R
54	CCR4	127	Fas	200	Hepassocin	273	IL-17	346	MIP-1b	419	S100A10	492	Ubiquitin+1
55	CCR5	128	Fas Ligand	201	GLO-1	274	IL-17B	347	MIP-1d	420	SAA	493	uPA
56	CCR6	129	FGF Basic	202	HGF	275	IL-17B R	348	MIP 2	421	SCF	494	uPAR
57	CCR7	130	FGF-BP	203	HGFR	276	IL-17C	349	MIP-3 alpha	422	SCF R	495	Vasorin
58	CCR8	131	FGF R3	204	HRG-alpha	277	IL-17D	350	MIP-3 beta	423	SDF-1	496	VCAM-1
59	CCR9	132	FGF R4	205	HRG-beta 1	278	IL-17E	351	MMP-1	424	sFRP-1	497	VE-Cadherin
60	CD14	133	FGF R5	206	HVEM	279	IL-17F	352	MMP-2	425	sFRP-3	498	VEGF
61	CD27	134	FGF-4	207	I-309	280	IL-17R	353	MMP-3	426	sFRP-4	499	VEGF R2
62	CD30	135	FGF-5	208	ICAM-1	281	IL-17RC	354	MMP-7	427	sgp130	500	VEGF R3
63	CD30 Ligand	136	FGF-6	209	ICAM-2	282	IL-17RD	355	MMP-8	428	SIGIRR	501	VEGF-B
64	CD40	137	FGF-7	210	ICAM-3	283	IL-18 BPa	356	MMP-9	429	Siglec-5	502	VEGF-C
65	CD40 Ligand	138	FGF-8	211	ICAM-5	284	IL-18 R alpha	357	MMP-10	430	Siglec-9	503	VEGF-D
66	CD 163	139	FGF-9	212	IFN-alpha/beta R1	285	IL-18 R beta	358	MMP-11	431	SLPI	504	VEGI
67	Cerberus 1	140	FGF-10	213	IFN-alpha/beta R2	286	IL-19	359	MMP-12	432	Smad 1	505	WIF-1
68	Chem R23	141	FGF-11	214	IFN-beta	287	IL-20	360	MMP-13	433	Smad 4	506	WISP-1
69	Chordin-Like 1	142	FGF-12	215	IFN-gamma	288	IL-20 R alpha	361	MMP-14	434	Smad 5	507	XEDAR
70	Chordin-Like 2	143	FGF-13 1B	216	IFN-gamma R1	289	IL-20 R beta	362	MMP-15	435	Smad 7		
71	Csk	144	FGF-16	217	IGFBP-1	290	IL-21	363	MMP-16	436	Smad 8		
72	CLC	145	FGF-17	218	IGFBP-2	291	IL-21 R	364	MMP-19	437	Prdx6		
73	CNTF	146	FGF-18	219	IGFBP-3	292	IL-22	365	MMP-20	438	Soggy-1		

XIII. TROUBLESHOOTING GUIDE

PROBLEM	CAUSE	RECOMMENDATION
No signals (not even the positive controls spots)	Chemiluminescent imager is not working properly	Contact image manufacturer
	Too Short Exposure	Expose the membranes longer
	Degradation of components due to improper storage	Store entire kit at ≤ - 20°C. Do not use kit after expiration date. See storage guidelines.
	Improper preparation or dilution of the HRP-Streptavidin	Centrifuge vial briefly before use, mix well, and do not dilute more than 1000-fold
	Waiting too long before exposing	The entire detection process should be completed in 10-15 minutes
Positive controls spots signals visible but no other spots	Low sample protein levels	Decrease sample dilution, concentrate samples, or load more protein initially
	Skipped Sample Incubation Step	Samples must be loaded after the blocking step
	Too Short of Incubations	Ensure the incubations are performed for the appropriate time or try the optional overnight incubation(s)
Uneven signals and/or background	Bubbles present on or below membrane	Don't rock/rotate the tray too vigorously or pipette the sample or reagent with excessive force
	Insufficient sample or reagent volume	Load enough sample and reagent to completely cover the membrane
	Insufficient mixing of reagents	Gently mix all reagents before loading onto the membrane, especially the HRP-Streptavidin and Biotin Antibody Cocktail
	Rocking/Rotating on an uneven surface while incubating	Rock/rotate on a flat surface or the sample or reagent can "pool" to one side
High background signals or all spots visible	Too much HRP-Streptavidin or Biotinylated Antibody Cocktail	Prepare these signal enhancing components precisely as instructed
	Membranes dried out	Do not let the membranes dry out during the experiment. Cover the incubation tray with the lid to minimize evaporation
	Too High of Sample Protein Concentration	Increase dilution of the sample or load less protein
	Exposed Too Long	Decrease exposure time
	Insufficient Washing	Ensure all the wash steps are carried out and the wash buffer is removed completely after each wash step
	Non-specific binding	Ensure the blocking buffer is stored and used properly.

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