RayBio® C-Series Human Protein Tyrosine Phosphorylation Antibody Array C3

For the semi-quantitative detection of 500 Tyrosine-phosphorylated human proteins in cell and tissue lysates

Patent Pending Technology

User Manual
(Revised Aug. 15th, 2022)

Cat# AAH-PTYR-3-2 (2 Sample Kit)
Cat# AAH-PTYR-3-4 (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 C3 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 C3 is specifically designed for simultaneously identifying the relative levels of Tyrosine phosphorylation of 500 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 C3, 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.

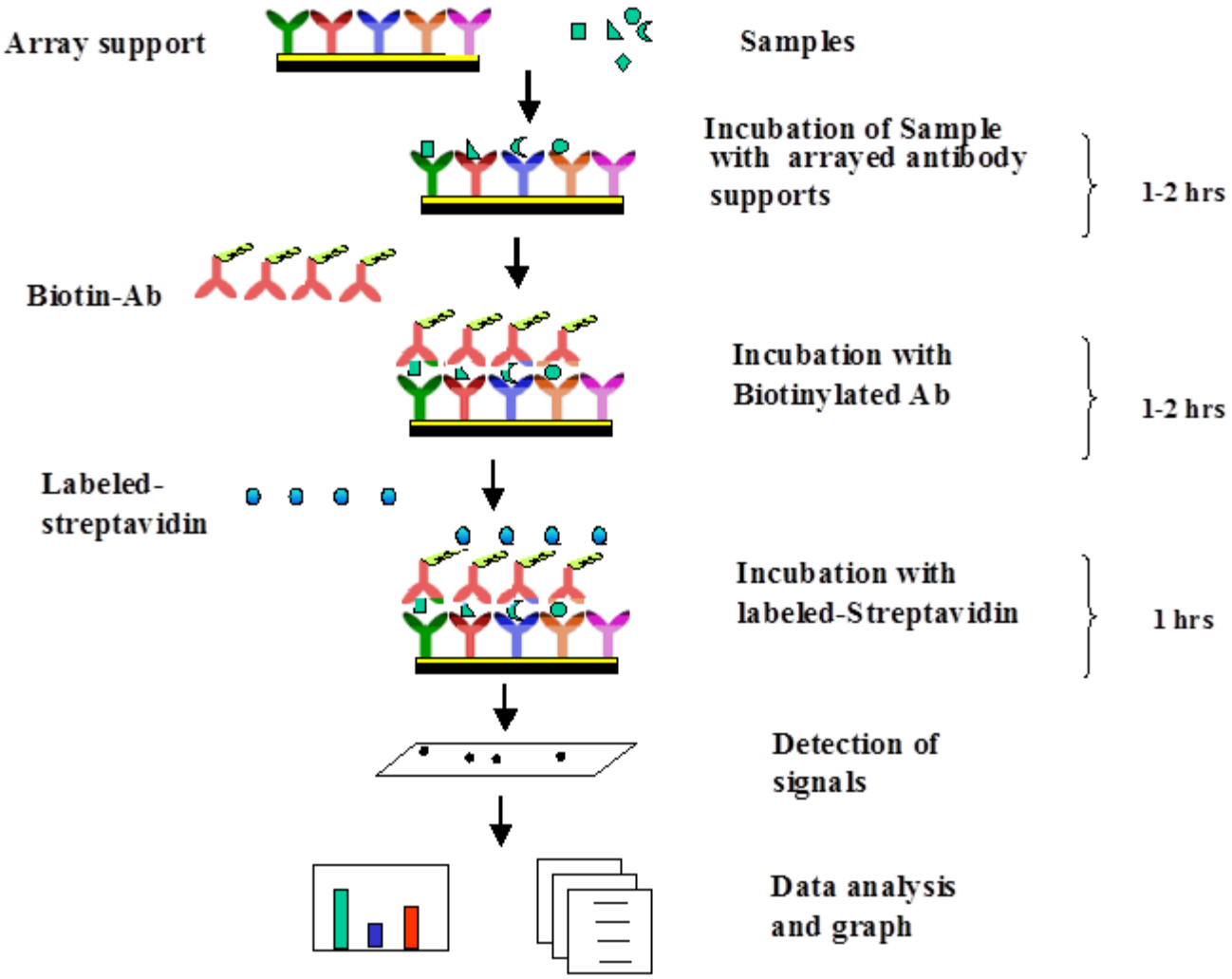
2. Global View of Cytokine Expression: Antibody array screening improves the chances for discovering key factors, disease mechanisms, or biomarkers related to cytokine signaling.

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 used within the 6 month expiration date.

ITEM	COMPONENT	AAH-PTYR-3-2	AAH-PTYR-3-4	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.

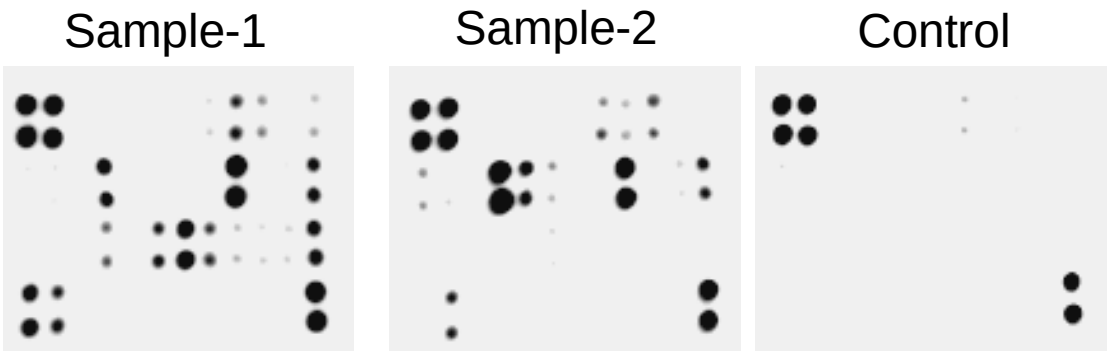
NOTE: *Optimal exposure times will vary so performing multiple exposure times is strongly recommended. See Section VI for additional details.*

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
3	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
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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34	474	475	476	477	478	479	480	481	482	483	484	485	486	487	488	489	490	491	492	493	494	495	496	497	498	499	500	Blank	Blank	Blank
35	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	POS3	POS2	POS1
36	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	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	14-3-3 beta	73	Antithrombin III	145	C4BPA	217	CHREBP	289	Cytokeratin 9	361	EVC2	433	Glyoxalase II
2	14-3-3 epsilon	74	APA	146	C5b-9	218	Chromogranin B	290	D4 GDI	362	Ezrin	434	GM2A
3	14-3-3 eta	75	APLP-1	147	C6	219	Chromogranin C	291	DAK	363	F11	435	GMF beta
4	14-3-3 gamma	76	APM2	148	C8G	220	CIP29	292	Contactin-4	364	FABP5	436	GNB1
5	14-3-3 sigma	77	Apo (a)	149	C9orf40	221	CKB	293	DARS2	365	Factor IX	437	GNPTG
6	14-3-3 theta	78	APOA1BP	150	CA1	222	CLIC1	294	DCI	366	Factor V	438	GOLPH2
7	14-3-3 zeta	79	ApoF	151	CA150	223	CLIC4	295	DCXR	367	Factor XII	439	GOLPH4
8	53BP1	80	ApoL1	152	CA2	224	CLIP170	296	DDAH1	368	Factor XIII	440	GOT2
9	67LR	81	ApoL2	153	CA3	225	CL-P1	297	DDT	369	FAM20C	441	GPR116
10	ABAT	82	ARFBP1	154	CACNB4	226	CLPS	298	DDX3Y	370	FAM3C	442	GPLD1
11	ABCF1	83	ARFGEF3	155	CAD	227	CLTA	299	DEFA6	371	Fascin	443	GRHL1
12	ABI3BP	84	ASL	156	Cadherin 22	228	CNN2	300	DEP-1	372	FASN	444	Granzyme M
13	ACAA1	85	ArgRS	157	Cadherin-6	229	CNOT1	301	DNER	373	fast skeletal Myosin	445	GRHPR
14	ACAA2	86	ARP19	158	CALD1	230	CO4A2	302	Dermcidin	374	FASTKD5	446	GRP
15	ACACA	87	Arp2	159	CALML5	231	COG4	303	Desmocollin 1	375	FBP38	447	GSTM1
16	ACAA	88	ARP2/3	160	Calmodulin	232	COL19A1	304	Desmocollin-2	376	FBP2	448	GSTP1
17	ACLP	89	Arp3	161	Calpain 1	233	COL4A3	305	Desmocollin-3	377	FBPase 1	449	Guanylin
18	ACLY	90	ARPC2	162	Calpain S1	234	Col6A2	306	Desmoglein-1	378	FCGBP	450	GULP1
19	Aconitase 1	91	ARPC3	163	Calpastatin	235	COL9A3	307	Desmoglein-2	379	FDPS	451	H6PD
20	ACTBL2	92	ART3	164	Calretinin	236	COLEC10	308	Desmoplakin	380	FH	452	HABP2
21	ACTC1	93	ARTS1	165	Calumenin	237	Collagen I a1	309	Desmuslin	381	Fibrillin 1	453	HBZ
22	Actinin alpha 1	94	ARX	166	CAP1	238	Collagen III	310	Destrin	382	FGG	454	HCFC1
23	ADAMDEC1	95	ASH2L	167	CapG	239	Collagen IVa6	311	DGK	383	Fibrinogen-like 2	455	HDGF
24	ADAS	96	ASGR2	168	CAPZA1	240	Collagen IX	312	DISC 1	384	Fibrinopeptide B	456	HEG1
25	ADH1B	97	ASK1	169	CPB2	241	Collagen V	313	DMGDH	385	Fibulin 3	457	Hemoglobin
26	ADH1C	98	AST	170	CARHSP1	242	Collagen VI	314	DMRN9	386	Ficolin-2	458	Hemoglobin A1c
27	ADH4	99	DNPEP	171	Caspase-14	243	Collagen X	315	DBH	387	Filamin A	459	HBB
28	ADH5	100	ASXL1	172	Catalase	244	COL15A1	316	DOT1L	388	Filamin B	460	HBD
29	ADM	101	ATBF1	173	Cathelicidin	245	COMP	317	DPEP2	389	Filamin C	461	HBG2
30	Advillin	102	ATP5A	174	Cathepsin A	246	CFB	318	DPP3	390	FKBP12	462	HEXB
31	AFG3L2	103	ATP5O	175	Cathepsin G	247	Contactin-3	319	DPPI	391	FKBP25	463	HGFA
32	AGA	104	ATPB	176	Cathepsin H	248	COPS8	320	DRIL1	392	FKBP51	464	hGH
33	Aggrecan	105	B3GNT2	177	Cathepsin Z	249	Corneodesmosin	321	DSCAM	393	FLG2	465	hHR23b
34	AGXT	106	B4GalT1	178	CBS	250	Coronin 3	322	DSPG3	394	FOLR3	466	HIBADH
35	AHNAK	107	B7-H2	179	CCDC126	251	Cortactin	323	Dystroglycan	395	Frizzled 8	467	HINT1
36	Ahsp	108	B7-H3	180	CCDC25	252	COTL1	324	UBA1	396	FRY	468	HIP1R
37	AIF	109	BAD	181	CCT3	253	CPE	325	ECHS1	397	FSH	469	Histone H1.2
38	AK2	110	Band 3	182	CD109	254	CPEB3	326	ECM-1	398	Azurocidin	470	Histone H1.3
39	AKAP9	111	BASP1	183	CD133	255	CPM	327	EEF1G	399	FUCA1	471	Histone H2A
40	AKR1B1	112	Bassoon	184	CD155	256	CPN1	328	EEF2	400	FUCA2	472	Histone H2A.Z
41	AKR1C3	113	BAZ2B	185	CD157	257	CPNE3	329	EFEMP2	401	FAH	473	Histone H2B K
42	AKR7A2	114	BCHE	186	CD16	258	CPS1	330	EFTUD2	402	G0/G1switch 2	474	Histone H3.3
43	ALAD	115	Bcl-w	187	CD21	259	CKMM	331	EHD1	403	G3BP	475	Histone H4
44	ALT	116	BCOR	188	CD32	260	CRF21	332	EHD3	404	GALNT2	476	HLA-C
45	ADH	117	beta 1 Spectrin	189	CD35	261	CRHBP	333	EIF3S2	405	gamma Catenin	477	HMGB1
46	AOX1	118	CRYBB1	190	CD39L4	262	CrkL	334	eIF4A1	406	GAPDH	478	HMGB2
47	ALDH16A1	119	beta 1 Tubulin	191	CD41	263	CRMP2	335	eIF5A	407	GARNL1	479	HMGB3
48	ALDH1A1	120	CUBB3	192	CD42b	264	CRTAC1	336	ELAVL1	408	GART	480	HMGN2
49	ALDH9A1	121	BID	193	CD48	265	CS	337	EMILIN1	409	Gastrokine 1	481	HN1
50	ALKP	122	BIN2	194	CD5L	266	Ctip2	338	EMSY	410	GATM	482	FoxA1
51	ALP	123	BIRC6	195	CD9	267	Cux2	339	EN2	411	GBE1	483	hnRNP A1
52	MAN1A1	124	BLMH	196	CD98	268	Cyclophilin A	340	Endorepellin	412	GCDFP 15	484	hnRNP A2B1
53	alpha Actinin 4	125	BLVRB	197	CDA	269	Cyclophilin B	341	ENO1	413	GCLC	485	hnRNP C1+C2
54	Alpha Fodrin	126	BMP-1	198	CDC5L	270	Cystatin D	342	ENO1+ENO2+ENO3	414	GCSH	486	hnRNP G
55	alpha Glucosidase II	127	BPGM	199	CDK2	271	Cystatin E	343	ENSA	415	GDA	487	hnRNP L
56	alpha-Synuclein	128	BPIFB1	200	CEACAM-8	272	Cystatin S	344	Envoplakin	416	GDF7	488	hnRNP M1-M4
57	alpha Tubulin	129	BPIL1	201	CECR1	273	Cystatin SN	345	EDN	417	GDI1	489	hnRNP U
58	CRYAA	130	BRCA 2	202	CENPF	274	CSRP1	346	EPB41	418	GDI2	490	Hornerin
59	ALS	131	BRD2	203	CEP57	275	CYTL1	347	EPCR	419	Gephyrin	491	Hoxb3
60	Als2	132	Brevican	204	CES1	276	Cytochrome b5	348	Ephrin B1	420	GFAP	492	HOXD11
61	ALS2CR1	133	Brg1	205	CETP	277	Cytochrome c (n)	349	Ephrin B2	421	GHRF	493	HP1BP3
62	Aminoacylase	134	BRSK1	206	Cezanne	278	Cytokeratin 1	350	EPHX2	422	GIP	494	HPD
63	Androgen Receptor	135	BTD	207	CFHR1	279	Cytokeratin 10	351	EPPK1	423	GLIPR2	495	HPR
64	ANGPTL6	136	BTF3	208	CFHR4	280	Cytokeratin 13	352	Eps15	424	GLRX1	496	HPRT
65	ANGPTL8	137	C1q	209	CFHR5	281	Cytokeratin 14	353	ERAB	425	G6PD	497	HRG
66	ANK	138	C1qA	210	CFI	282	Cytokeratin 15	354	ERAP2	426	PRKCSH	498	HRSP12
67	Ankrd26	139	C1qB	211	CFL1	283	Cytokeratin 16	355	ERp29	427	GLUD1	499	HSC70
68	Annexin A1	140	C1qR1	212	CFVII	284	Cytokeratin 17	356	ERp57	428	CGH	500	HSP47
69	Annexin A2	141	C1RL	213	CHC17	285	Cytokeratin 20	357	ERp72	429	GSTO1		
70	Annexin A6	142	C1s	214	Chitobiase	286	Cytokeratin 3	358	ESD	430	GSS		
71	Annexin V	143	ELP6	215	Chitotriosidase	287	Cytokeratin 4	359	ESR1	431	GPD1		
72	ANP	144	C4.4A	216	CHORDC1	288	Cytokeratin 5	360	ETL	432	Glycoprotein V		

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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