RayBio® C-Series Human Protein Serine/Threonine Phosphorylation Antibody Array C2

For the semi-quantitative detection of 493 Serine/Threonine -phosphorylated human proteins in cell and tissue lysates

Patent Pending Technology

User Manual
(Revised Sept. 19th, 2022)

Cat# AAH-PST-2-2 (2 Sample Kit)
Cat# AAH-PST-2-4 (4 Sample Kit)

Please read manual carefully
before starting experiment

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the protein array pioneer company

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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 Serine/Threonine Phosphorylation Antibody Array C2 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 Serine/Threonine Phosphorylation Antibody Array C2 is specifically designed for simultaneously identifying the relative levels of Serine/Threonine phosphorylation of 493 different human proteins in cell lysates. By monitoring the changes in protein Serine/Threonine 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 Serine/Threonine Phosphorylation Antibody Array C2, treated or untreated cell lysate is added into antibody array membranes. The antibody array membranes are washed and biotinylated anti-phosphoSerine/Threonine antibody is used to detect phosphorylated Serine/Threonine s 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.

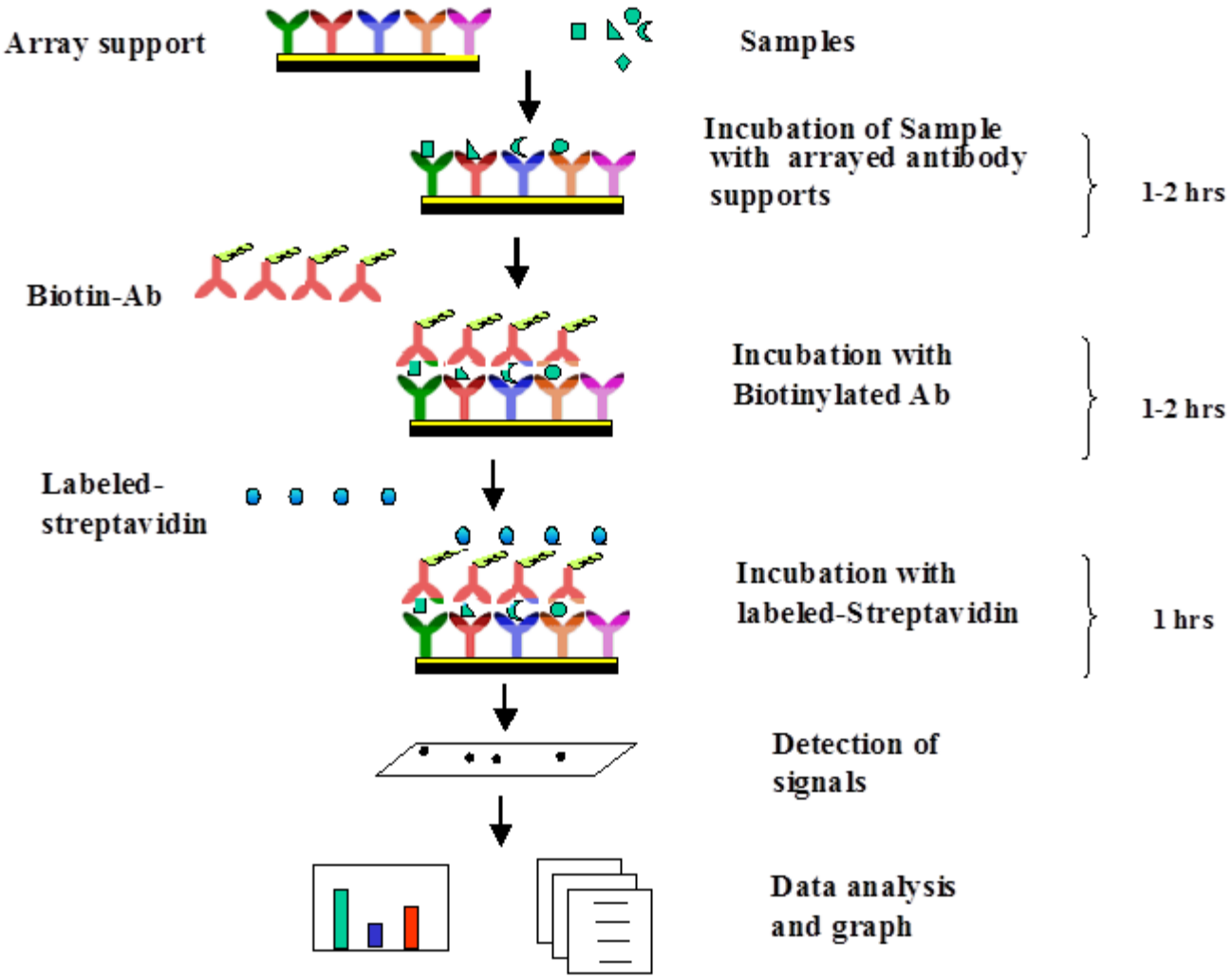
2. Global View of Cytokine Expression: Antibody array screening improves the chances for discovering key factors, disease mechanisms, or biomarkers related to cytokine signaling.

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-PST-1-2	AAH-PST-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-PhosphoSerine/Threonine 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	100x Phosphatase Inhibitor Cocktail I	1 vial		
13	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-phosphoSerine/Threonine 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.	N/A
12	100x Phosphatase Inhibitor Cocktail II	No Preparation	N/A
13	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.	N/A

*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), 20ul Phosphatase Inhibitor Cocktail Set I (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.

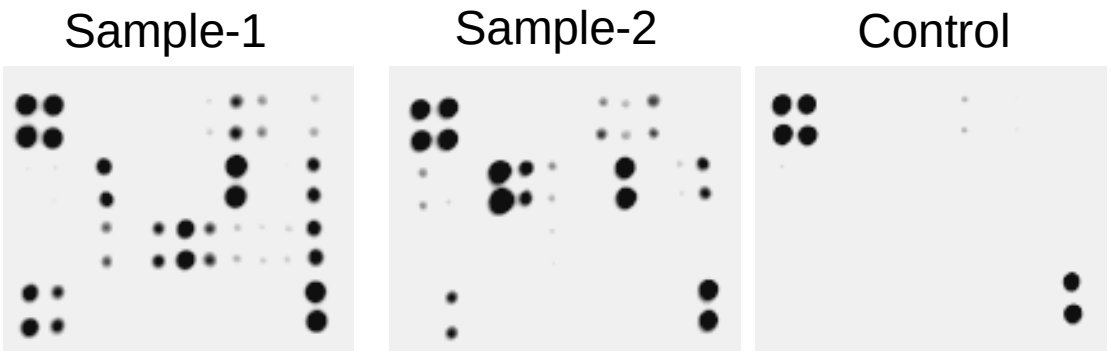
NOTE: *Optimal exposure times will vary so performing multiple exposure times is strongly recommended. See Section VI for additional details.*

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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34	Blank	Blank	Blank	462	463	464	465	466	467	468	469	470	471	472	473	474	475	476	477	478	479	480	481	482	483	484	485	Blank	Blank	Blank
35	Blank	Blank	Blank	Blank	486	487	488	489	490	491	492	493	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	POS3	POS2	POS1
36	Blank	Blank	Blank	Blank	486	487	488	489	490	491	492	493	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	11b-HSD1	73	BMX	145	CRTAM	217	FRK	289	KLF4	361	PI 16	433	SCGF
2	2B4	74	BNIP2	146	CSH1	218	ARB1	290	LAG-3	362	PIK3R1	434	SOST
3	4-1BB	75	BNP	147	Troponin T	219	Furin	291	Layilin	363	PIM2	435	SOX17
4	A1BG	76	Btk	148	CutA	220	Fyn	292	LDL R	364	PKM2	436	SOX2
5	A2M	77	C2	149	Cyclin D1	221	GADD45A	293	Legumain	365	Plasminogen	437	SPARCL1
6	ABL1	78	C3a	150	Cystatin A	222	Galanin	294	LH	366	Podocalyxin	438	SPINK1
7	ACE	79	C5a	151	Cystatin B	223	Galectin-1	295	LIMPII	367	POMC	439	SRMS
8	ACE-2	80	C7	152	Cystatin C	224	Galectin-3BP	296	LIN41	368	PON1	440	SSEA-1
9	ACK1	81	C8b	153	Cytochrome C	225	Galectin-7	297	Livin	369	PON2	441	SSEA-4
10	ACPP	82	C9	154	Cytokeratin 8	226	gamma-Thrombin	298	LOX-1	370	PPARg2	442	SSTR2
11	ACTH	83	CA9	155	Cytokeratin 18	227	Gas1	299	LPS	371	PPP2R5C	443	SSTR5
12	ADAM-9	84	CA15-3	156	Cytokeratin 19	228	Gastrin	300	LRG1	372	Presenilin 1	444	Survivin
13	ADAMTS1	85	CA19-9	157	DBI	229	GATA-3	301	LTF	373	Presenilin 2	445	SYK
14	ADAMTS10	86	CA125	158	DCBLD2	230	GATA-4	302	LTK	374	Pro-BDNF	446	Syndecan-1
15	ADAMTS13	87	Cadherin-13	159	D-Dimer	231	Gelsolin	303	Lumican	375	Procalcitonin	447	Syndecan-3
16	ADAMTS15	88	CLEC14A	160	DEFA1/3	232	Ghrelin	304	Lyn	376	Pro-Cathepsin B	448	TACE
17	ADAMTS17	89	Calbindin D	161	CPA1	233	GLP-1	305	LYRIC	377	Progesterone	449	TAF4
18	ADAMTS18	90	Calcitonin	162	Desmin	234	GMNN	306	LYVE-1	378	pro-Glucagon	450	Tec
19	ADAMTS19	91	Calreticulin	163	DLL1	235	GPBB	307	LZTS1	379	Prohibitin	451	TFF1
20	ADAMTS4	92	Calsyntenin-1	164	DLL4	236	GPI	308	Mammaglobin A	380	Pro-MMP-7	452	TFF3
21	ADAMTS5	93	CART	165	DMP-1	237	GPR-39	309	Marapsin	381	Pro-MMP-9	453	Thrombin
22	ADAMTSL2	94	Caspase-3	166	DPPIV	238	GPX1	310	MATK	382	Pro-MMP-13	454	Thrombomodulin
23	Adipsin	95	Caspase-8	167	E-Cadherin	239	GPX3	311	MBL	383	ProSAAS	455	TK1
24	Afamin	96	Cathepsin B	168	Endorphin Beta	240	GRP	312	C1qTNF1	384	Prostasin	456	Thyroglobulin
25	AFP	97	Cathepsin D	169	EDNRA	241	GRP75	313	Mer	385	Protein p65	457	TIM-1
26	ALBUMIN	98	Cathepsin L	170	Enolase 2	242	GRP78	314	Mesothelin	386	PSA-Free	458	TNK1
27	Aldolase A	99	Cathepsin S	171	ENPP2	243	GSR	315	MICB	387	PSA-total	459	TOPORS
28	Aldolase B	100	CBP	172	EpCAM	244	GST	316	Midkine	388	PSP	460	TPA
29	Aldolase C	101	CCK	173	EphA1	245	HADHA	317	MINA	389	PTH	461	TPM1
30	ALK	102	CD23	174	EphA2	246	HAI-1	318	MSHa	390	PTHLP	462	TRA-1-60
31	Alpha 1 AG	103	CD24	175	EphA3	247	HAI-2	319	MTUS1	391	PTN	463	TRA-1-81
32	A1M	104	CD36	176	EphA4	248	Haptoglobin	320	Myoglobin	392	PTPRD	464	Transferrin
33	Alpha Lactalbumin	105	CD38	177	EphA5	249	hCG alpha	321	NAIP	393	PYK2	465	Trappin-2
34	ALPP	106	CD44	178	EphA6	250	hCGb	322	Nanog	394	PYY	466	TRKB
35	AMICA	107	CD45	179	EphA7	251	Hck	323	NELL2	395	Ras	467	Troponin I
36	AMPKa1	108	CD46	180	EphA8	252	HE4	324	Neprilysin	396	RBP4	468	Troponin C
37	Amylin	109	CD47	181	EphB1	253	Hemopexin	325	Nesfatin	397	RECK	469	TRPC1
38	ANGPTL3	110	CD55	182	EphB2	254	Hepcidin	326	Nestin	398	RELM alpha	470	TRPC6
39	ANGPTL4	111	CD59	183	EphB3	255	HOXA10	327	NET1	399	Resistin	471	TRPM7
40	Annexin A7	112	CD61	184	EphB4	256	HSP10	328	Netrin G2	400	RET	472	Trypsin 1
41	APC	113	CD71	185	EphB6	257	HSP20	329	Netrin-4	401	RIP1	473	TSH
42	APCS	114	CD74	186	ERRa	258	HSP27	330	Neurokinin A	402	ROCK1	474	TSLP
43	Apelin	115	CD79 alpha	187	Erythropoietin R	259	HSP32	331	Neuropeptide Y	403	ROCK2	475	TXK
44	Apex1	116	CD90	188	ESAM	260	HSP40	332	NF1	404	ROR1	476	Tyk2
45	APN	117	CD97	189	EV15L	261	HSP60	333	NM23-H1/H2	405	ROR2	477	TYRO10
46	ApoA1	118	CD200	190	EXTL2	262	HSP70	334	Notch-1	406	ROS	478	Uromodulin
47	ApoA2	119	CEA	191	FABP1	263	HSP90	335	NPTX1	407	RYK	479	Vasopressin
48	ApoA4	120	CEACAM-1	192	FABP2	264	HSPA8	336	NPTXR	408	S100A4	480	VDUP-1
49	ApoB	121	Ceruloplasmin	193	FABP3	265	HTRA2	337	NR3C3	409	S100A6	481	VEGF R1
50	ApoB100	122	CFHR2	194	FABP4	266	IBSP	338	Ntn1	410	S100A8	482	VGF
51	ApoC1	123	Chemerin	195	Fc gamma RIIIB	267	IGF2BP1	339	OCT3/4	411	S-100b	483	VIPR2
52	ApoC2	124	CHI3L1	196	Factor XIII B	268	IGFBP-5	340	Omentin	412	SART1	484	Visfatin
53	ApoC3	125	Chromogranin A	197	FAK	269	IDUA	341	Osteocalcin	413	SART3	485	VDR
54	ApoD	126	Chymase	198	FAP	270	IL-33	342	Osteopontin	414	SCG3	486	VDB
55	ApoE	127	cIAP-2	199	Fcg RIIB/C	271	IL-34	343	OX40	415	Selenoprotein P	487	PROS1
56	ApoE3	128	Ck beta 8-1	200	Fen-1	272	IL-28B	344	p21	416	SEMA3A	488	Vitronectin
57	ApoH	129	CKMB	201	FER	273	INSL3	345	p27	417	Serotonin	489	VWF
58	ApoM	130	Claudin-3	202	Ferritin	274	INSRR	346	p53	418	Serpin G1	490	WT1
59	APP	131	Claudin-4	203	Fetuin A	275	ITGAV	347	PAI-1	419	Serpin A1	491	XIAP
60	ASPH	132	CLEC3B	204	Fetuin B	276	Itk	348	PAK7	420	Serpin A3	492	ZAG
61	Attractin	133	Clusterin	205	FGFR1	277	ITM2B	349	Pancreastatin	421	Serpin A4	493	ZAP70
62	B3GNT1	134	CNDP1	206	FGFR1 alpha	278	Kallikrein 2	350	PP	422	Serpin A5		
63	BAF57	135	COCO	207	FGFR2	279	Kallikrein 5	351	Pappalysin-1	423	Serpin A8		
64	BAFF	136	CFH	208	Fibrinogen	280	Kallikrein 6	352	PARK7	424	Serpin A9		
65	BAI-1	137	Contactin-1	209	Fibrinopeptide A	281	Kallikrein 7	353	P-Cadherin	425	Serpin A12		
66	BCAM	138	Contactin-2	210	Fibronectin	282	Kallikrein 8	354	PCAF	426	Serpin B5		
67	B2M	139	CBG	211	Ficolin-3	283	Kallikrein 10	355	PD-1	427	Serpin D1		
68	Beta Defensin 4	140	COX-2	212	FIH	284	Kallikrein 11	356	PDX-1	428	Serpin I1		
69	Beta IG-H3	141	C-peptide	213	FOLR1	285	Kallikrein 14	357	PEDF	429	SERTAD2		
70	Biglycan	142	CPN2	214	FOXN3	286	KCC3	358	PEPSINOGEN I	430	SHBG		
71	BLAME	143	Creatinine	215	FoxO1	287	KCTD10	359	PEPSINOGEN II	431	SMAC		
72	BMP-9	144	CRP	216	FoxP3	288	KIF3B	360	PGRP-S	432	SNCG		

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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