RayBio® Label-Based (L-Series) Human Phosphorylation Screening Array

Patent Pending Technology
User Manual (Mar 13, 2023)

For the simultaneous detection of the relative expression of 500 Human proteins in serum, plasma, cell culture supernatants, cell/tissue lysates or other body fluids.

AAH-BLG-PHO1-4 (4 Sample Kit)
AAH-BLG-PHO1-8 (8 Sample Kit)

Please read manual carefully before starting experiment

RayBiotech
Empowering your proteomics

Your Provider of Excellent Protein Array Systems and Services

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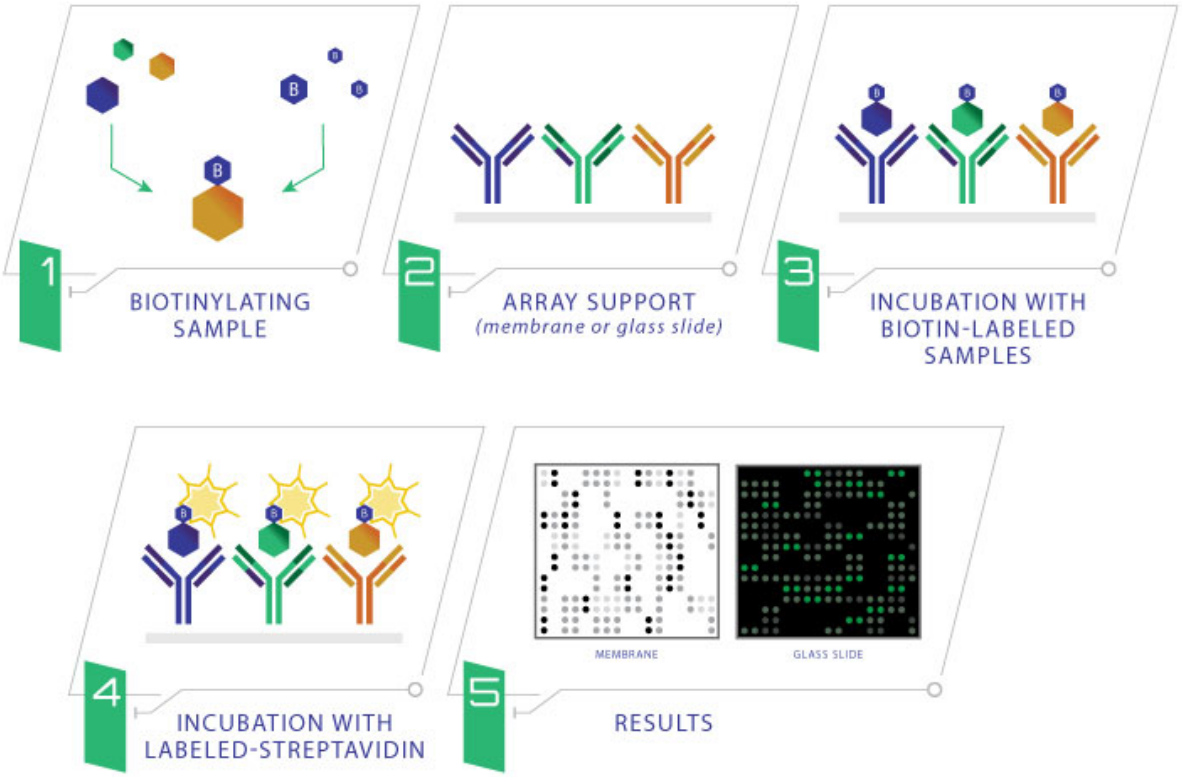
I. Introduction

The first step in using the RayBio® L-Series Antibody Array is to biotinylate the primary amine groups of the proteins in your sample (sera or plasma, cell culture supernatants, cell lysates or tissue lysates). The glass slide arrays are then blocked, just like a western blot, and the biotin-labeled sample is added onto the glass slide, which is pre-printed with capture antibodies. The slide is incubated to allow binding of target proteins. Streptavidin-conjugated fluorescent dye (Cy3 equivalent) is then applied to the array. Finally, the glass slide is dried, and laser fluorescence scanning is used to visualize the signals.



II. Materials Provided

A. Storage Recommendations

Upon receipt, the kit should be stored at -20°C until needed. It is recommended to use the kit within 6 months of the date of shipment. After initial use, remaining reagents should be stored at 4°C and may be stored for up to 3 months. Labeling Reagent (Item B) should be prepared fresh each time before use. Unused glass slides should be kept at -20°C and repeated freeze-thaw cycles should be avoided (slides may be stored for 6 months).

ITEM	DESCRIPTION	4 SAMPLE KIT	8 SAMPLE KIT
A	Spin Columns (0.5ml)	8 columns	16 columns
B	Labeling Reagent	1 vial	2 vials
D	Stop Solution	1 vial (50 µl)	1 vial (50 µl)
E	RayBio® L-Series Glass Slide*	1 slide	2 slides
F	Blocking Buffer	1 bottle (8 ml)	2 bottles (8 ml)
G	20X Wash Buffer I	1 bottle (30 ml)	1 bottle (30 ml)
H	20X Wash Buffer II	1 bottle (30 ml)	1 bottle (30 ml)
I	Cy3 equivalent-Conjugated Streptavidin	1 vial	2 vials
J	Adhesive Plastic Strips		
K	Labeling Buffer	1 bottle (30 ml)	1 bottle (30 ml)
n/a	2X Cell Lysis Buffer**	1 bottle (10 ml)	1 bottle (10 ml)
M	30 ml Centrifuge Tube	1 tube	1 tube

*Each slide contains 4 identical subarrays
**Only needed if testing cell or tissue lysates

B. Additional Materials Required

- 1 ml tube, small plastic or glass containers
- Orbital shaker or oscillating rocker
- Pipettors, pipette tips and other common lab consumables
- Laser scanner for fluorescence detection
- Aluminum foil

III. Overview and General Considerations

A. Preparation and Storage of Samples

1. Preparation of Cell Culture Supernatants
    1. Seed cells at a density of $1x10^6$ cells in 100 mm tissue culture dishes.*
    2. Culture cells in complete culture medium for ~24-48 hours.**
    3. Replenish with serum-free or low-serum medium such as 0.2% FCS/FBS serum, and then incubate cells again for ~48 hours.**,+ The membrane-based array is recommended if high serum medium such as 10% FCS/FBS is used, as high background can occur on glass slide arrays with high serum containing media samples.
    4. To collect supernatants, centrifuge at 1,000 x g for 10 minutes and store as less than or equal 1 ml aliquots at -80°C until needed.
    5. If you want to use cell mass for inter-sample normalization, measure the total wet weight of cultured cells in the pellet and/or culture dish. You may then normalize between arrays by dividing fluorescent signals by total cell mass (i.e., express results as the relative amount of protein expressed/mg total cell mass). Or you can normalize between arrays by determining cell lysate concentration using a total protein assay (BCA Protein Assay Kit, Pierce, Prod #: 23227).

    **The density of cells per dish used is dependent on the cell type. More or less cells may be required.*

    ***Optimal culture time may vary and will depend on the cell line, treatment conditions and other factors.*

    +Bovine serum proteins produce detectable signals on the RayBio® L-Series Array in media containing serum concentrations as low as 0.2%. When testing serum-containing media, we strongly recommend testing an uncultured media blank for comparison with sample results.

2. Extracting Protein from Cells
    1. Centrifuging Cells
        a. Adherent Cells:
            i. Remove supernatant from cell culture and wash cells gently twice with cold 1X PBS taking care not to disturb cell layer.
            ii. Add enough cold 1X PBS to cover cell layer and use cell scraper to detach cells.
        b. Cells in Suspension: Pellet the cells by centrifuging using a microcentrifuge at 1500 rpm for 10 minutes.
    2. Make sure to remove any remaining PBS before adding 1X Cell Lysis Buffer (2X Cell Lysis Buffer should be diluted 2-fold with ddH_2O). Solubilize the cells at $2x10^7$ cells/ml in 1X Cell Lysis Buffer.

3. 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 13,000 rpm for 10 minutes at 2-8°C.

   Note: If the lysates appear to be cloudy, transfer the lysates to a clean tube, centrifuge again at 13,000 rpm for 20 minutes at 2-8°C. If the lysates are still not clear, store them at -20°C for 20 minutes. Remove from the freezer and immediately centrifuge at 13,000 rpm for 20 minutes at 2-8°C.

4. Transfer lysates to a clean tube. Determining cell lysate concentrations using a total protein assay (BCA Protein Assay Kit, Pierce, Prod# 23227). Aliquot the lysates and store at -80°C.

3. Extracting Protein from Crude Tissue

   1. Transfer approximate 100 mg crude tissue into a tube with 1 ml 1X Cell Lysis Buffer (2X Cell Lysis Buffer should be diluted 2-fold with ddH_2O).
   2. Homogenize the tissue according to homogenizer manufacturer instructions.
   3. Transfer extracts to microcentrifuge tubes and centrifuge for 20 minutes at 13,000 rpm (4°C).

      Note: If the supernatant appears to be cloudy, transfer the supernatants to a clean tube, centrifuge again at 13,000 rpm for 20 minutes at 2-8°C. If the supernatant is still not clear, store the lysate at -20°C for 20 minutes. Remove from the freezer, immediately centrifuge at 13,000 rpm for 20 minutes at 2-8°C.

   4. Transfer supernatant to a clean tube and store at -80°C.

4. Determine the total protein concentration

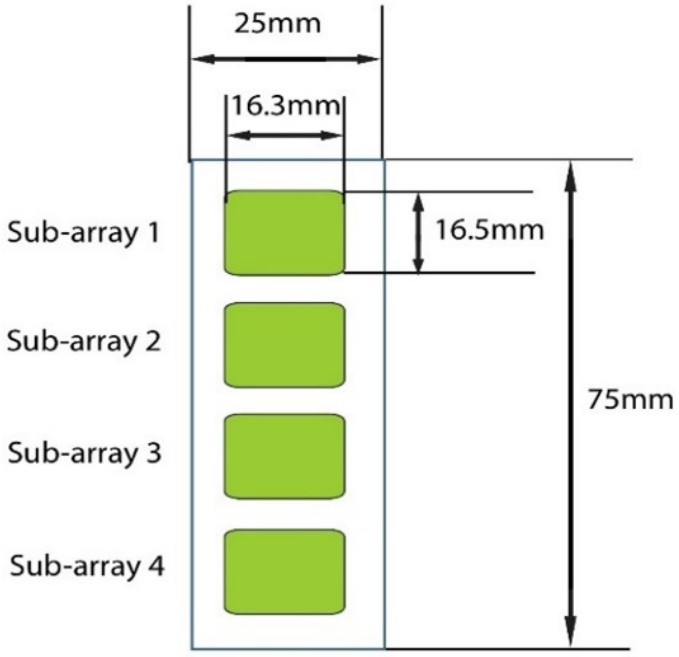
   For optimal biotin labeling, it is necessary to determine the protein concentration in the cell/tissue lysate. We recommended using a BCA total protein assay (e.g., Pierce, Catalog # 23227).

B. Handling the Glass Slides

- The microarray slides are delicate. Please do not touch the array surface with pipette tips, forceps or your fingers. Hold the slides by the edges only.
- Handle the slides with powder-free gloves and in a clean environment.
- Do not remove the glass slide from the chamber assembly until step 20, and take great care not to break the glass slide when doing so.
- Remove reagents/sample by gently applying suction with a pipette to corners of each chamber. Do not touch the printed area of the array, only the sides as seen in image below.

C. Layout of Array Slide

Four identical sub-arrays on one slide



4 printed sub-arrays per glass chip

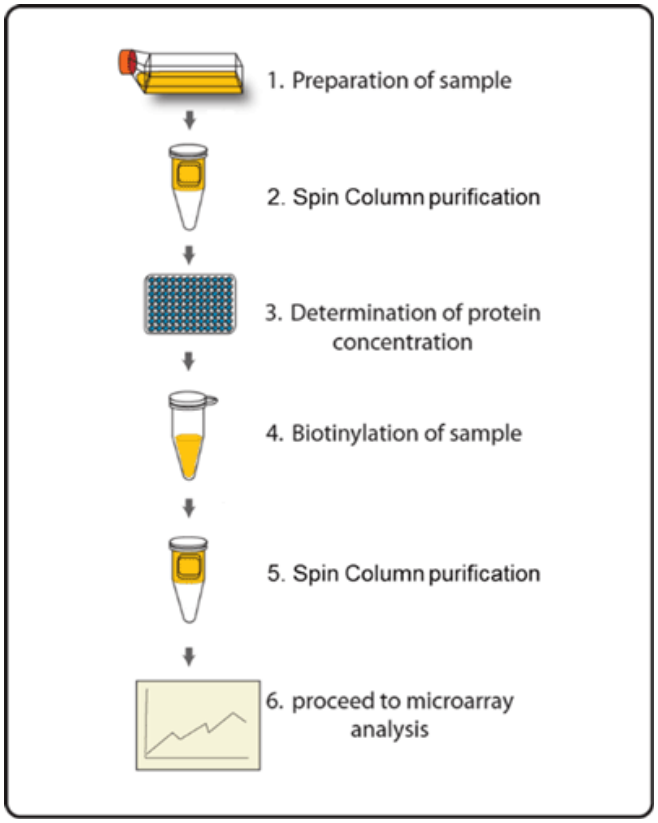
D. Incubations and Washes

- Cover incubation chamber with a Plastic Adhesive Strip (Item J) to prevent evaporation during incubation or wash steps, particularly those steps lasting 2 hours or longer.
- During incubation and wash steps avoid foaming and remove all bubbles from the sub-array surface.
- Perform all incubation and wash steps under gentle rotation or rocking motion (~0.5 to 1 cycle/sec).
- Wash steps in Wash Buffer II and all incubation steps may be performed overnight at 4°C
- Avoid cross-contamination of samples to neighboring wells. To remove Wash Buffers and other reagents from chamber wells, you may invert the Glass Slide Assembly to decant, and aspirate the remaining liquid.
- Unlike most Cy3 fluors, the streptavidin-conjugated fluor used in this kit is very stable at room temperature (RT) and resistant to photobleaching on the hybridized glass slides. However, please protect glass slides from direct, strong light and temperatures above RT.

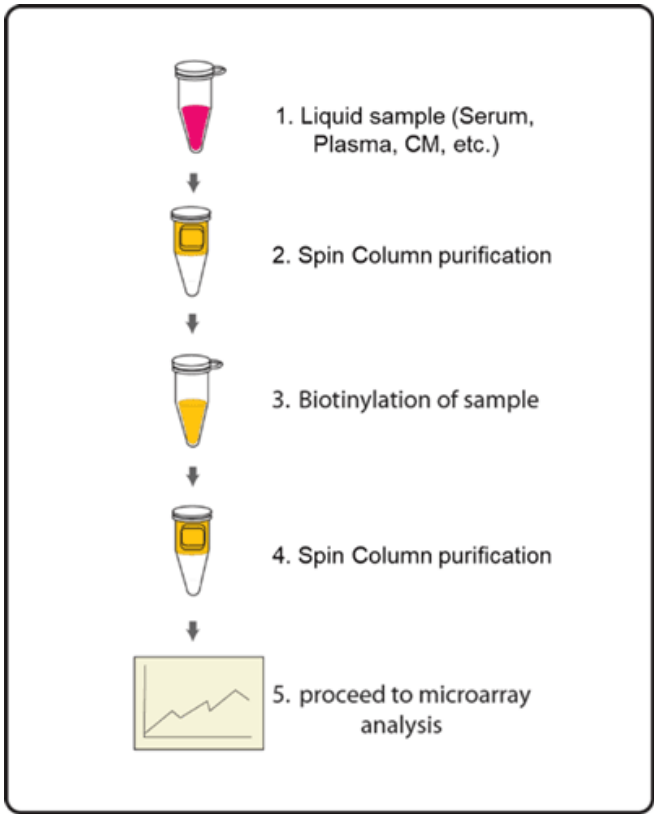
IV. Protocol

Assay Diagram

1. Cell/tissue lysates



2. Serum, plasma, or Cell culture supernatants



A. Sample Purification

Note: This step removes low molecular weight amine derivatives or unwanted buffer from samples to ensure quality biotinylation in Steps 5-7.

1. Twist to remove the bottom plug of the Spin Column and loosen the cap (do not remove).
2. Place the Spin Column into a collection tube and centrifuge at 1,500 x g for 1 minute to remove the storage buffer. Discard the flow-through.
3. Wash the Spin Column three times with 300 µl Labeling Buffer each, centrifuge at 1,500 x g for 1 minute to remove the flow-through. Discard the flow-through and blot the bottom of the column to remove excess liquid. Transfer the Spin Column to a new collection tube.
4. Apply sample on top of the resin within the next few minutes. Centrifuge at 1,500 x g for 2 minutes. Collect the flow-through that contains the sample. The recommended sample dilutions are as follows:

- *Cell culture supernatant: 120 µl neat supernatant*
- *Serum/Plasma: 2 µl serum/plasma in 100 µl Labeling Buffer*
- *Cell/tissue lysate: 20 µg lysate in 100 µl Labeling Buffer*

Note: Each labelled sample volume is enough for at least 3 arrays following the protocol below.

Note: The maximal sample volume is 130 µl for each Spin Column. Do not load over 130 µl of sample into a Spin Column.

B. Biotin Labeling the Sample

Note: Amines (e.g., Tris, glycine) and azides quench the biotinylation reaction. Avoid contaminating samples with these chemicals prior to biotinylation.

5. Immediately before use, prepare the Labeling Reagent. Briefly spin down the Labeling Reagent tube (Item B). Add 100 µl Labeling Buffer into the tube, then pipette up and down or vortex slightly to dissolve the lyophilized reagent.
6. Add Labeling Reagent to the sample tube. Incubate the reaction solution at RT with gentle rocking or shaking for 30 min. Mix the reaction solution by gently tapping the tube every 5 minutes.
   a. For labeling cell culture supernatants: Add 8 µl of Labeling Reagent into the sample tube (for 120 µl supernatant).
   b. For labeling serum or plasma: Add 8 µl of Labeling Reagent into the sample tube (for 2 µl serum/plasma *in 100 µl labeling buffer*).
   c. For labeling cell or tissue lysates: Add 4 µl of 1X Labeling Reagent into the sample tube (for 20 µg lysate *in 100 µl labeling buffer*).
   d. For all other body fluid: Add 2 µl of Labeling Reagent Solution per 100 µg sample to be labelled.

   Note: The addition of Labeling Reagent volume is based upon the sample amount used in Step 4. If the amount of sample being labelled differs from the example in Step 6, adjust this volume proportionally.
7. Add 3 µl Stop Solution (Item D) to each sample tube. Using a new spin column, repeat Steps 1-4 of section A. Sample Purification to remove the excess non-reacted biotin reagent from each sample.

   Note: Biotinylated samples can be stored at -20°C or -80°C until you are ready to proceed with the assay.

C. Drying the Glass Slide

8. Remove the package containing the Assembled Glass Slide (Item E) from the freezer. Place unopened package on the bench top for ~15 minutes, and allow the Assembled Glass Slide to equilibrate to RT.

9. Open package, and take the Assembled Glass Slide out of the sleeve. Do not disassemble the Glass Slide from the chamber assembly. Place glass slide assembly in laminar flow hood or similar clean environment for 1-2 hours at RT.

   Note: Protect the slide from dust or other contaminants.

D. Blocking and Incubations

Note: Glass slide should be completely dry before adding Blocking Buffer to wells.

10. Block sub-arrays by adding 400 µl of Blocking Buffer (Item F) into each well of Assembled Glass Slide and incubating at RT for 30 minutes. Ensure there are no bubbles on the array surfaces.
11. Dilute samples with Blocking Buffer. Recommended dilution of the biotin-labeled samples with Blocking Buffer is 10-fold for cell culture supernatants, 20-fold for serum/plasma and 100-fold for cell/tissue lysate. *Dilution for other body fluid needs to be determined by the end user. Generally, most samples can be 10-20x dilution, while tears and saliva samples may need 100x dilution.*

    Note: Optimal sample dilution factor will depend on the abundance of target proteins. If the background or antigen-specific antibody signals are too strong, the sample can be diluted further in subsequent experiments. If the signal is too weak, more concentrated samples can be used.
12. Completely remove the Blocking Buffer from each well. Add 400 µl of diluted sample into appropriate wells. Remove any bubbles on array surfaces. Incubate arrays with gentle rocking or shaking for 2 hours at RT or overnight at 4°C

    Note: Avoid the flow of sample into neighboring wells.
13. Based on number of samples and remaining protocol, calculate the amount of 1X Wash Buffer I and 1X Wash Buffer II needed to complete the experiment. Separately dilute the required amounts of 20X Wash Buffer I Concentrate (Item G) 20-fold and 20X Wash Buffer II Concentrate (Item H) with ddH_2O
14. Decant the samples from each well and wash 3 times with 800 µl of 1X Wash Buffer I at RT with gentle rocking or shaking for 5 minutes per wash.
15. Obtain a clean container (e.g., pipette tip box or slide-staining jar), place the Assembled Glass Slide into the container with enough volume of 1X Wash Buffer I to completely cover the entire assembly, and remove any bubbles in wells. Wash 2 times at RT with gentle rocking or shaking for 10 minutes per wash.
16. Decant the Wash Buffer I from each well, place the Assembled Glass Slide into the container with enough volume of 1X Wash Buffer II to completely cover the entire assembly, and remove any bubbles in wells. Wash 2 times at RT with gentle rocking or shaking for 5 minutes per wash.
17. Prepare 1X Cy3-Conjugated Streptavidin:
    a. Briefly spin down tube containing the Cy3-Conjugated Streptavidin (Item I) immediately before use.

b. Add 1000 µl of Blocking Buffer into the Cy3-Conjugated Streptavidin tube to prepare a concentrated Cy3-Conjugated Streptavidin stock solution. Pipette up and down to mix gently (do not store the stock solution for later use).

c. To prepare 1X Cy3-Conjugated Streptavidin, add 200 µl of the concentrated Cy3-Conjugated Streptavidin stock solution into a tube with 800 µl of Blocking Buffer. Mix gently.

18. Carefully remove Assembled Glass Slide from container. Remove all of Wash Buffer II from the wells. Add 400 µl of 1X Cy3-Conjugated Streptavidin to each sub-array. Cover the incubation chamber with the plastic adhesive strips.

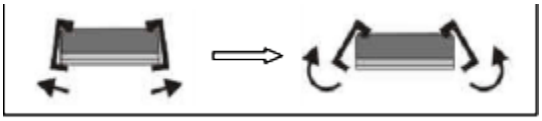
    Note: Avoid exposure to light in Steps 19-25 by covering the Glass Slide Assembly with aluminum foil or incubate in a dark room.

19. Incubate with 1X Cy3-Conjugated Streptavidin at RT for 1 hour with gentle rocking or shaking.

    Note: Incubation may be done overnight at 4°C

20. Decant the solution and disassemble the glass slide from the incubation frame and chamber. Disassemble the device by pushing clips outward from the side, as shown below. Carefully remove the glass slide from the gasket.

    Note: Be careful not to touch the printed surface of the glass slide, which is on the same side as the barcode.



21. Gently place the glass slide into 30 ml Centrifuge Tube (Item M). Add enough 1X Wash Buffer I to cover the entire glass slide (about 30 ml). Wash with gentle rocking or shaking for 10 min. Remove the wash buffer. Repeat 2 times for a total of 3 washes.

22. Add enough 1X Wash Buffer II to cover the entire glass slide (about 30 ml). Wash with gentle rocking or shaking for 5 minutes. Remove the wash buffer. Repeat one time for a total of two washes for 5 minutes per wash.

23. Finally, wash the glass slide with 30 ml of ddH_2O for 5 minutes. Remove glass slide and decant water from Centrifuge Tube.

24. Remove buffer droplets from the slide completely by one of the following ways:
    - Put the glass slide into the Slide Washer/Dryer, and dry the glass slide by centrifuge at 1,000 rpm for 3 minutes without cap.
    - Or dry the glass slide by a compressed N2 stream.
    - Or gently apply suction with a pipette to remove buffer droplets. Do not touch the array surface, only the sides.

    Note: Make sure the finished glass slide is completely dry before scanning or storage.

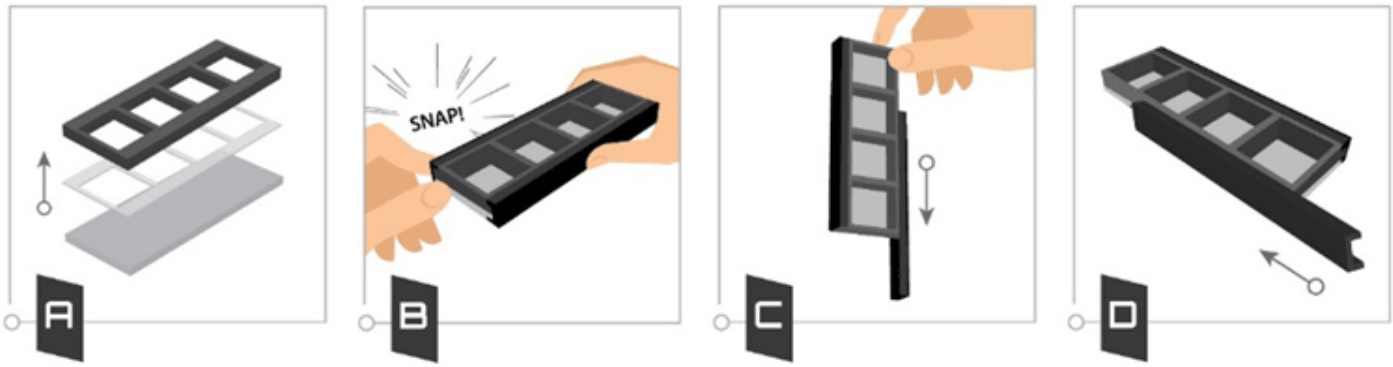
E. Fluorescence Detection

25. You may proceed immediately to scanning or you may store the slide at -20°C in the Centrifuge Tube provided or at RT to scan at a later time.

    Note: Please protect the finished glass slides from temperatures above RT and store them in the dark. Do not expose glass slide to strong light, such as sunlight or a UV lamp.

    Note: If you need to repeat any of the incubation steps after finishing the experiment, you must first re-assemble the glass slide into the incubation chamber by following the steps as described below. To avoid breaking the printed glass slide, you may first want to practice assembling the device with a blank glass slide.

    1. *Apply slide to incubation chamber barcode facing upward (image A).*
    2. *Gently snap one edge of a snap-on side (image B).*
    3. *Gently press other of side against lab bench and push in lengthwise direction (image C).*
    4. *Repeat with the other side (image D)*



V. Antibody Array Map

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2	12	12	13	13	14	14	15	15	16	16	17	17	18	18	19	19	20	20	21	21	22	22	23	23	24	24	25	25	26	26
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35	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	POS3	POS3	POS2	POS2	POS1	POS1

VI. Antibody Array Target List

Number	Name	Number	Name	Number	Name	Number	Name	Number	Name	Number	Name	Number	Name
1	Abl1	73	CDK1 (pT14)	145	ErbB2 (pY877)	217	InsR	289	MKK3 (pY230)	361	PKCa	433	SHIP1 (pY187)
2	Abl1 (pY139)	74	CDK1 (pT14/pY15)	146	ErbB3	218	InsR (pY1189)	290	MKK4	362	PKCa (pT497)	434	SHIP2
3	Abl1 (pY226)	75	CDK1 (pT161)	147	ErbB3 (pY1289)	219	IRAK1 (pT387)	291	MKK4 (pS257)	363	PKCa (pY195)	435	SHIP2 (pY886)
4	Abl1 (pY257)	76	CDK1 (pY19)	148	ErbB3 (pY1307)	220	IRAK4 (pT345/pS346)	292	MKK4 (pS80)	364	PKCb	436	SIK
5	Abl1 (pY264)	77	CDK10	149	ErbB3 (pY1328)	221	ITK	293	MKK7	365	PKCb (pS661)	437	SIK (pT182)
6	Abl1 (pY469)	78	CDK10 (pT196)	150	ErbB4	222	ITK (pY512)	294	MKK7 (pT275)	366	PKCb (pT500)	438	SIK2
7	Abl2	79	CDK11A (pT583)	151	ErbB4 (pY733)	223	ITSN2 (pY968)	295	MLK3 (pS281)	367	PKG1	439	SIK2 (pS358)
8	Abl2 (pY439)	80	CDK12 (pS383/pS385)	152	ErbB4 (pY875)	224	JAK2	296	MLK3 (pT277/pS281)	368	PKG1 (pT515/pT517)	440	SIK3
9	Abl2 (pY439/pT440)	81	CDK12 (pT893)	153	ERBIN (pY1104)	225	JAK2 (pY1007/pY1008)	297	MLTK (pT161/pT162)	369	PKM2 (pY390)	441	SIK3 (pT163)
10	ACK1 (pY284)	82	CDK16 (pY176)	154	ERK1	226	JAK2 (pY570)	298	MOK	370	PKR1 (pT446)	442	SIK3 (pT411)
11	ACK1 (pY518)	83	CDK2 (pT160)	155	ERK1 (pT202/pY204)	227	JAK3	299	MOK (pT159/pY161)	371	PLCG1 (pY783)	443	SIT1 (pY90)
12	ACK1 (pY859/pY860)	84	CDK4 (pT172)	156	ERK1 (pT207)	228	JAK3 (pY980/pY981)	300	MOK (pY167)	372	PLCG2 (pY759)	444	SIT1 (pY95)
13	ACTB (pY294)	85	CDK5 (pY15)	157	ERK1 (pY204)	229	JNK1	301	Mos	373	Plk1	445	SLK (pS189)
14	ACTB (pY53)	86	CDK6 (pY13)	158	ERK1 (pY204/pT207)	230	JNK1 (pY185)	302	Mos (pY263)	374	Plk1 (pT210)	446	SMG1
15	ACTN1 (pY246)	87	CDK6 (pY24)	159	ERK2	231	Jun (pS63)	303	MSK1 (pS212)	375	Plk1 (pY217)	447	SMG1 (pT3550)
16	Akt1	88	CDK7	160	ERK2 (pT185/pY187)	232	KHS1	304	MSK2 (pT194/pS196)	376	Plk4	448	snRNP70 (pY126)
17	Akt1 (pS473)	89	CDK7 (pT170)	161	ERK3 (pS189)	233	KHS1 (pS174)	305	MSK2 (pT687)	377	Plk4 (pT170)	449	Src (pY419)
18	Akt1 (pT308)	90	CDK9 (pS347)	162	ERK4 (pS186)	234	Kit	306	MST3 (pT184)	378	PPP1R11 (pY64)	450	SRPK1 (pS222)
19	Akt1 (pY315)	91	CDK9 (pT186)	163	ERK5	235	Kit (pY936)	307	MST3 (pT190)	379	PRAG1 (pY413)	451	STAM2 (pY374)
20	Akt1 (pY326)	92	CDKL5 (pY171)	164	ERK5 (pY221)	236	Ksr1 (pS406)	308	mTOR	380	PRK1 (pT774)	452	STK25 (pT174)
21	ALK	93	Chk1 (pS280)	165	ESYT1 (pY822)	237	KSR2 (pS490)	309	mTOR (pS2448)	381	PRKACA (pT196/pT198)	453	STK36 (pS159)
22	ALK (pY1092)	94	Chk1 (pS317)	166	FAK (pY397)	238	LATS1	310	mTOR (pS2478/pS2481)	382	PRKCD (pT507)	454	STK4 (pT183)
23	ALK (pY1096)	95	Chk1 (pS345)	167	FAK (pY576/pY577)	239	LATS1 (pS464)	311	MUSK (pY756)	383	PRKCD (pY313)	455	Syk (pY323)
24	ALK (pY1507)	96	Chk2 (pT383)	168	FAK (pY577)	240	LATS1 (pS909)	312	NDR1 (pS281/pT282)	384	PRKCD (pY334)	456	Syk (pY525/pY526)
25	AMPKa1	97	Chk2 (pT68)	169	FER (pY402)	241	Lck (pY192)	313	NEDD9 (pY166)	385	PRKCQ (pS695)	457	TAO1 (pS181)
26	AMPKa1 (pT183/pS184)	98	CK2a	170	FES (pY713)	242	Lck (pY263/pY264)	314	NEK2 (pS171)	386	PRKCQ (pY545)	458	TAO1 (pY309)
27	AMPKa2	99	CK2a (pY255)	171	FES (pY713/pS716)	243	LIMK1 (pT508)	315	NEK2 (pT170/pS171)	387	PRKCZ (pS262/pY263)	459	TBC1D7 (pY14)
28	AMPKa2 (pS377)	100	CLK1 (pS337)	172	FGFR1	244	LKB1	316	NEK6 (pS206)	388	PRKCZ (pT410)	460	TBK1 (pS172)
29	ANXA1-pY207	101	CLK1 (pS337/pT338)	173	FGFR1 (pY653/pY654)	245	LKB1 (pS31)	317	NEK7 (pT191/pS195)	389	PRKD1 (pS205)	461	TEC (pY519)
30	ANXA2-pY238	102	CREB (pS133)	174	FGFR2	246	LKB1 (pS428)	318	NLK (pT298)	390	PRKD1 (pS738/pS742)	462	TGM2 (pY369)
31	ARAF	103	CSF1R	175	FGFR2 (pY656/pY657)	247	LMTK2 (pS1450)	319	NUAK1 (pT211)	391	PRKD2 (pS197/pS198)	463	TIE2 (pY897)
32	ARAF (pY302)	104	CSF1R (pS807/pY809)	176	FGFR3	248	LOK (pS191)	320	OSR1 (pT185)	392	PRKDC	464	TIE2 (pY992)
33	ASK1 (pS1033)	105	CSF1R (pY699)	177	FGFR3 (pY647/pY648)	249	LOK (pT952)	321	p38b	393	PRKDC (pT2609)	465	TLN1 (pY70)
34	ASK1 (pT838)	106	CSF1R (pY809)	178	FGR	250	LTK (pY672)	322	p38b (pT180/pY182)	394	PRKDC (pY883)	466	TNK1 (pY277)
35	ATF2 (pT69/pT71)	107	Csk (pY184)	179	FGR (pY208/pY209)	251	Lyn	323	p38d	395	PRKX	467	TRIM28 (pY458)
36	ATM	108	DAPK1 (pS269)	180	FGR (pY412)	252	Lyn (pY508)	324	p38d (pY182)	396	PRKX (pT201/pT203)	468	TRIM28 (pY517)
37	ATM (pS1981)	109	DDR1	181	Flt3	253	MAK (pT157)	325	p70S6K	397	PRMT5 (pT634)	469	TRIM33 (pS1119)
38	ATM (pY2969)	110	DDR1 (pY796/pY797)	182	Flt3 (pY842)	254	MAP3K7	326	p70S6K (pT252)	398	PRP4K (pY849)	470	TrkA (pY680/pY681)
39	ATR	111	DDR1 (pY797)	183	Frk	255	MAP3K7 (pS439)	327	p70S6K (pT412)	399	PTK6 (pS446/pY447)	471	TrkB (pY516)
40	ATR (pS435/pS436)	112	DDR2 (pY736)	184	Frk (pY387)	256	MAP3K7 (pT184/pT187)	328	p70S6K (pT444/pS447)	400	PTK6 (pY342)	472	TrkB (pY702)
41	AurKA	113	DDR2 (pY740)	185	Frk (pY497)	257	MAP3K8	329	PAK1	401	PTRF (pY308)	473	TrkC (pY709/pY710)
42	AurKA (pT287/pT288)	114	DOK3 (pY398)	186	Fyn	258	MAP3K8 (pS334)	330	PAK1 (pS144)	402	PYK2 (pY402)	474	TSSK3 (pT168)
43	AurKB	115	DTK (pY681)	187	Fyn (pY213/pY214)	259	MAPKAPK2 (pT222)	331	PAK1 (pT423)	403	PYK2 (pY579/pY580)	475	TTK (pT676)
44	AurKB (pS227)	116	DTK (pY685/pY686)	188	Fyn (pY531)	260	MAPKAPK2 (pY225/pT226)	332	PAK2	404	Raf1	476	TTK (pY833/pY836)
45	AurKB (pT232)	117	DYRK1A (pY321)	189	G6PD (pY401)	261	MAPKAPK3 (pY76)	333	PAK2 (pS141)	405	Raf1 (pS259)	477	TWF1 (pY309)
46	AurKC	118	DYRK2	190	GAB1 (pY406)	262	MAPKAPK5 (pT186)	334	PAK2 (pY130)	406	Raf1 (pS296)	478	TWF2 (pY309)
47	AurKC (pS193)	119	DYRK2 (pY382)	191	GCK (pS170)	263	MARK1	335	PAK4 (pS474)	407	Raf1 (pS301/pT303)	479	TXK (pY420)
48	Axl	120	EEF1A1 (pY141)	192	GIT1 (pY545)	264	MARK1 (pT215)	336	PAK5	408	Ret	480	Tyk2
49	Axl (pY702/pY703)	121	EEF2 (pT57)	193	GRK2 (pS670)	265	MARK3	337	PAK5 (pS602)	409	Ret (pY905)	481	TYK2 (pY1054/pY1055)
50	Bcr (pY591)	122	EGFR	194	GRK2 (pY356)	266	MARK3 (pT507)	338	PBK (pY74)	410	RIOK1 (pY466)	482	VAV1 (pY826)
51	Bcr (pY644)	123	EGFR (pY1069)	195	GSK3a	267	MEK1	339	PCTK2	411	RIPK1 (pY384)	483	VEGFR1
52	BLK (pY187)	124	EGFR (pY1110)	196	GSK3a (pS278/pY279)	268	MEK1 (pS222)	340	PCTK2 (pS180)	412	RIPK2 (pS176)	484	VEGFR1 (pY1048)
53	BLK (pY188)	125	EGFR (pY1172)	197	GSK3a (pT19/pS21)	269	MEK5	341	PCYT1A (pS329/pS331)	413	RIPK2 (pY381)	485	VEGFR1 (pY1053)
54	BLK (pY389)	126	EGFR (pY869)	198	GSK3a (pY279)	270	MEK5 (pS311)	342	PCYT1A (pT342/pS343)	414	RIPK4 (pS438)	486	VEGFR2
55	BMPR2 (pS375)	127	EGFR (pY998)	199	GSK3a (pY284/pY285)	271	MEKK2	343	PCYT1A (pY359/pS362)	415	ROCK1 (pY913)	487	VEGFR2 (pY1054)
56	B-Raf	128	EIF2AK3 (pT982)	200	GTF2F1 (pS385/pT389)	272	MEKK2 (pS239)	344	PCYT1B (pS315/pS319)	416	ROCK2 (pY722)	488	VEGFR3
57	B-Raf (pS446/pS447)	129	EIF4EBP1 (pT37/pT46)	201	GUK1 (pY53)	273	MELK	345	PDGFRa	417	Ron	489	VEGFR3 (pY1068)
58	B-Raf (pS729)	130	EML4 (pY226)	202	HCA59 (pY147)	274	MELK (pY438)	346	PDGFRa (pS847/pY849)	418	Ron (pY1238 / pY1239)	490	VIM (pY117)
59	BRD2 (pS37)	131	ENO1 (pY44)	203	HGK	275	MERTK (pY749)	347	PDGFRa (pY762)	419	Ron (pY1238)	491	WAS (pY291)
60	BRSK1 (pT189)	132	ENO2 (pY25)	204	HGK (pT187)	276	MERTK (pY749/pY753)	348	PDGFRa (pY768)	420	ROR2 (pY645/pY646)	492	WEE1 (pS642)
61	Btk (pY223/pY225)	133	EphA1 (pY781)	205	HGS (pY216)	277	MERTK (pY753)	349	PDK1 (pS241)	421	Ros	493	WNK1
62	Btk (pY551)	134	EphA2 (pY588)	206	HIPK1 (pY352)	278	Met	350	PDLIM5 (pY251)	422	Ros (pY2114/pY2115)	494	WNK1 (pS382)
63	BUB1B (pS670)	135	EphA2 (pY772)	207	HSP90B	279	Met (pS1236)	351	PEAK1 (pY635)	423	RPS6 (pS235/pS236/pS240)	495	WNK1 (pT60)
64	CaMK1a (pT177)	136	EphA3 (pY779)	208	HSP90B (pY484)	280	Met (pT1241)	352	PECAM-1 (pY713)	424	RPS6KB2 (pS423)	496	YES1 (pY222/pY223)
65	CAMK1D (pT180)	137	EphB1 (pY594)	209	ICK (pY156/pT157)	281	Met (pT1355/pY1356)	353	PFN1 (pY129)	425	RSK1	497	ZAP70 (pY248)
66	CaMK2A (pT286)	138	EphB2	210	ICK (pY159)	282	Met (pY1003)	354	PGK1 (pY196)	426	RSK1 (pS221)	498	ZAP70 (pY292)
67	CaMK4	139	EphB2 (pY780)	211	IGF1R	283	Met (pY1230 (nopT pY1234)	355	PIK3R1 (pY467)	427	RSK1 (pS380)	499	ZAP70 (pY319)
68	CaMK4 (pT200)	140	EphB3 (pY600)	212	IGF1R (pY1161/pT1163)	284	Met (pY1234)	356	PIK3R1 (pY580)	428	RSK1 (pT573)	500	ZAP70 (pY492/pY493)
69	CaMKK1	141	EphB4 (pY596)	213	IKKa (pT179/pS180)	285	Met (pY1234/pY1235)	357	PIK3R2 (pY464)	429	RSK1 (pY220/pS221)		
70	CaMKK1 (pS74)	142	ErbB2	214	IKKE (pS172)	286	Met (pY1234/pY1235/pS1236)	358	Pim2	430	RSK3 (pY217/pS218)		
71	CDC7 (pT376)	143	ErbB2 (pY1248)	215	ILK1 (pS343)	287	MKK3	359	Pim2 (pT195)	431	SCYL1 (pS754)		
72	CDK1	144	ErbB2 (pY735)	216	ILK1 (pY351)	288	MKK3 (pS218)	360	PIP5K (pS307)	432	SHIP1		

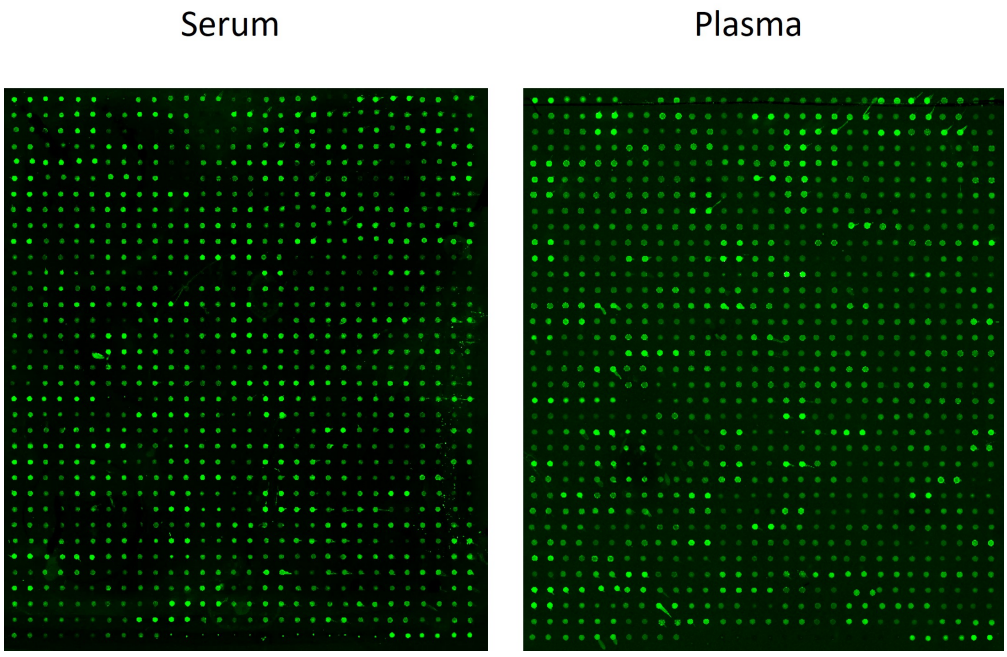
VII. Interpretation of Results

A. Explanation of Controls Spots

There are three Positive Controls (POS1, POS2, POS3) in each array. These are three levels of standardized biotinylated IgG. All other variables being equal, the Positive Control intensities will be the same for each sub-array. This allows for normalization based upon the relative fluorescence signal responses to a known control, much as "housekeeping" genes or proteins are used to normalize results in PCR or Western blots, respectively.

B. Typical Results

The following figure shows the typical result of this array probed with sample(s). The images were captured using an Axon GenePix laser scanner. The Positive control signals in the upper left and lower right corners of each array can be used to identify the orientation and help normalize the results between arrays.



Note: In the absence of an external standard curve for each protein detected, there is no means of assessing absolute or relative concentrations of different proteins in the same sample using immunoassays. If you wish to obtain quantitative data (i.e., concentrations of the various analytes in your samples), try using our Quantibody® Arrays as a targeted follow-up experiment.

C. Background Subtraction

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

Most laser fluorescence scanners' software has an option to automatically measure the local background around each spot. For best results, we recommend comparing signal intensities representing the MEAN signals minus local background. If your resulting fluorescence signal intensity reports do not include these values (e.g., a column labeled as "F532 Mean - B532"), you may need to subtract the background manually or change the default settings on your scanner's data report menu.

D. Normalization of Array Data

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

You can calculate the normalized values as follows:

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

Where:
P1 = mean signal intensity of POS spots on reference array
P(y) = mean signal intensity of POS spots on Array "y"
X(y) = mean signal intensity for spot "X" on Array "y"
X(Ny) = normalized signal intensity for spot "X" on Array "y"

The RayBio® Analysis Tool software is freely available for use with data obtained using RayBio® Biotin Label-based Antibody Arrays. You can copy and paste your signal intensity data (with and without background) into the Analysis Tool, and it will automatically normalize signal intensities to the Positive Controls. Analysis Tool software can be downloaded from the product page on the RayBiotech website.

E. Threshold of Significant Difference

After subtracting background signals and normalization to Positive Controls, comparison of signal intensities between and among array images can be used to determine relative differences in expression levels of each protein between samples or groups.

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

VIII. Troubleshooting Guide

Problem	Cause	Recommendation
Weak Signal	Inadequate detection	Increase laser power and PMT parameters
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
	Short incubation time	Ensure sufficient incubation time and change sample incubation step to overnight
	Too low protein concentration in sample	Dilute starting sample less or concentrate sample
	Improper storage of kit	Store kit as suggested temperature. Don't freeze/thaw the slide.
Uneven Signal	Bubble formed during incubation	Handle and pipette solutions more gently; De-gas solutions prior to use
	Arrays are not completed covered by reagent	Prepare more reagent and completely cover arrays with solution
	Reagent evaporation	Cover the incubation chamber with adhesive film during incubation
General	Cross-contamination from neighboring wells	Avoid overflowing wash buffer between wells
	Comet tail formation	Air dry the slide for at least 1 hour before usage
	Inadequate detection	Increase laser power so the highest standard concentration for each cytokine receives the highest possible reading yet remains unsaturated
High Background	Overexposure	Lower the laser power
	Dark spots	Completely remove wash buffer in each wash step
	Insufficient wash	Increase wash time and use more wash buffer
	Dust	Minimize dust in work environment before starting experiment
	Slide is allowed to dry out	Take additional precautions to prevent slides from dying out during experiment

IX. Selected References

- Christina Scheel et all., *Paracrine and Autocrine Signals Induce and Maintain Mesenchymal and Stem Cell States in the Breast.* Cell. 2011;145, 926-940.
- Lin Y, Huang R, Chen L, et al., *Profiling of cytokine expression by biotin-labeled-based protein arrays.* Proteomics. 2003, 3: 1750-1757.
- Huang R, Jiang W, Yang J, et al., *A Biotin Label-based Antibody Array for High-content Profiling of Protein Expression.* Cancer Genomics Proteomics. 2010; 7(3):129-141.
- Liu T, Xue R, Dong L, et al., *Rapid determination of serological cytokine biomarkers for hepatitis B-virus-related hepatocellulare carcinoma using antibody arrays.* Acta Biochim Biophys Sin. 2011; 43(1):45-51.
- Cui J, Chen Y, Chou W-C, et al., *An integrated transcriptomic and computational analysis for biomarker identification in gastric cancer.* Nucl Acids Res. 2011; 39(4):1197-1207.
- Jun Zhong et all., *Temporal Profiling of the Secretome during Adipogenesis in Humans.* Journal of Proteome Research. 2010, 9, 5228-5238.
- Chowdury UR, Madden BJ, Charlesworth MC, Fautsch MP., *Proteomic Analysis of Human Aqueous Humor.* Invest Ophthalmol Visual Sci. 2010; 51(10):4921-4931.
- Wei Y, Cui C, Lainscak M, et al., *Type-specific dysregulation of matrix metalloproteinases and their tissue inhibitors in end-stage heart failure patients: relationship between MMP-10 andLV remodeling.* J Cell Mol Med. 2011; 15(4):773-782.
- Kuranda K, Berthon C, Lepêtre F, et al., *Expression of CD34 in hematopoietic cancer cell lines reflects tightly regulated stem/progenitor-like state.* J Cell Biochem. 2011; 112(5):1277-1285.
- Toh HC, Wang W-W, Chia WK, et al., *Clinical Benefit of Allogenic Melanoma Cell Lysate-Pulsed Autologous Dendritic Cell Vaccine in MAGE-Positive Colorectal Cancer Patients.* Clin Chem Res. 2009; 15:7726-7736.
- Zhen Hou, *Cytokine array analysis of peritoneal fluid between women with endometriosis of different stages and those without endometriosi.* Biomarkers. 2009;14(8): 604-618.
- Yao Liang Tang, et al., *Hypoxic Preconditioning Enhances the Benefit of Cardiac Progenitor Cell Therapy for Treatment of Myocardial Infarction by Inducing CXCR4.* Circ Res. 2009;109:197723.

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