

RayBio[®] Label-Based (L-Series) Human PI3K-Akt Signaling Pathway Array, Glass Slide

**Patent Pending Technology
User Manual (Oct 13, 2022)**

For the simultaneous detection of the relative expression of 307 Human proteins in serum, plasma, cell culture supernatants, cell/tissue lysates or other body fluids.

**AAH-BLG-AKT-4 (4 Sample Kit)
AAH-BLG-AKT-8 (8 Sample Kit)**

Please read manual carefully before starting experiment



Your Provider of Excellent Protein Array Systems and Services

**Tel: +1-770-729-2992 or 1-888-494-8555 (Toll Free); Fax: +1-770-206-2393;
Website: www.raybiotech.com Email: info@raybiotech.com**

TABLE OF CONTENTS

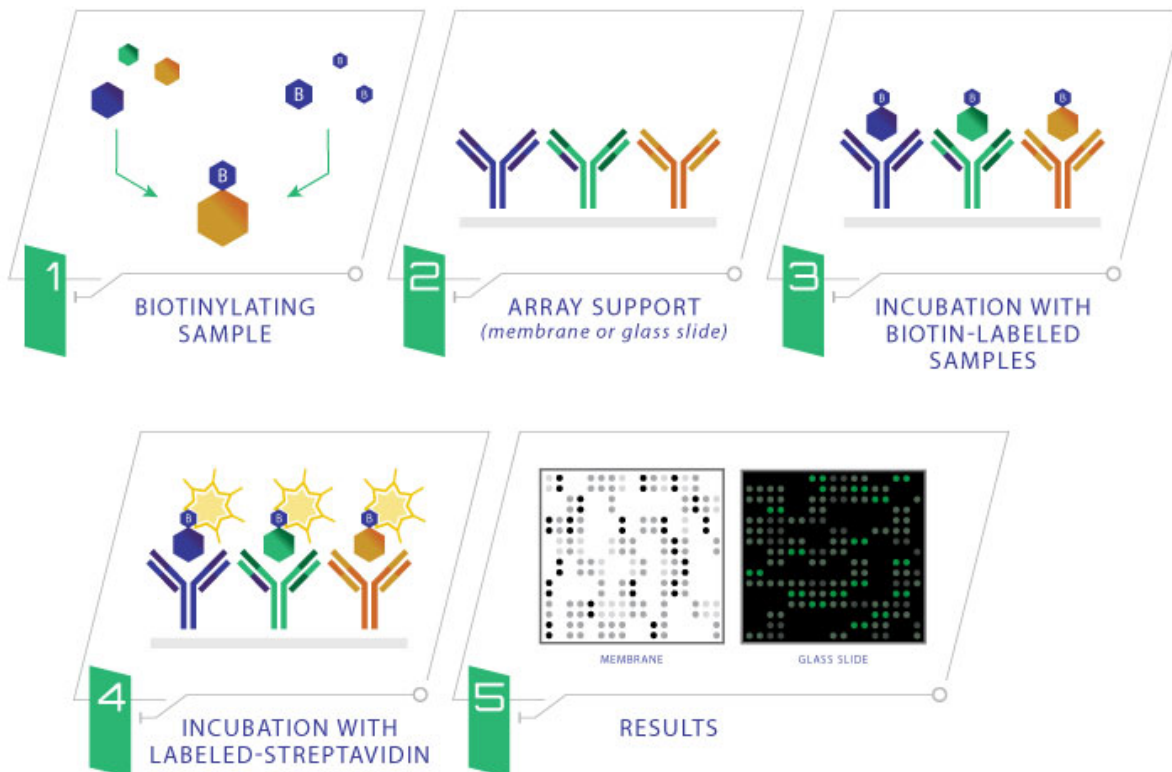
I.	Introduction and How It Works.....	3
II.	Materials Provided.....	4
	A. Storage Recommendations.....	4
	B. Additional Materials Required.....	5
III.	Overview and General Considerations.....	5
	A. Preparation and Storage of Samples.....	5
	B. Handling the Glass Slides.....	7
	C. Layout of Array Slide.....	8
	D. Incubation and Washes.....	9
IV.	Protocol.....	10
	A. Sample Purification.....	10
	B. Biotin Labeling of Sample.....	11
	C. Drying of the Glass Slide.....	12
	D. Blocking and Incubations.....	12
	E. Fluorescence Detection.....	15
V.	Antibody Array Map.....	16
VI.	Antibody Array Target Lists.....	17
VII.	Interpretation of Results.....	18
	A. Explanation of Controls Spots.....	18
	B. Typical Results.....	18
	C. Background Subtraction.....	19
	D. Normalization of Array Data.....	19
	E. Threshold of Significant Difference.....	20
VIII.	Troubleshooting Guide.....	21

IX. Selected References.....	22
------------------------------	----

I. Introduction

Combining direct antigen-labeling technology with our vast library of array-validated antibodies, RayBiotech has created the largest commercially available antibody array to date. With the L-Series high density array platform, researchers can now detect thousands of proteins simultaneously, obtaining a broad, panoramic view of protein expression. Our newly expanded panel includes a wide variety of metabolic enzymes, structural proteins, epigenetic markers, neuroregulatory factors, in addition to our popular list of cytokines, growth factors, receptors, adipokines, proteases, and signaling proteins. Available on both glass slide and membrane formats, this array is ideally suited for biomarker discovery studies and exploratory screens.

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 1×10^6 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 ddH₂O). Solubilize the cells at 2×10^7 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 ddH₂O).

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).

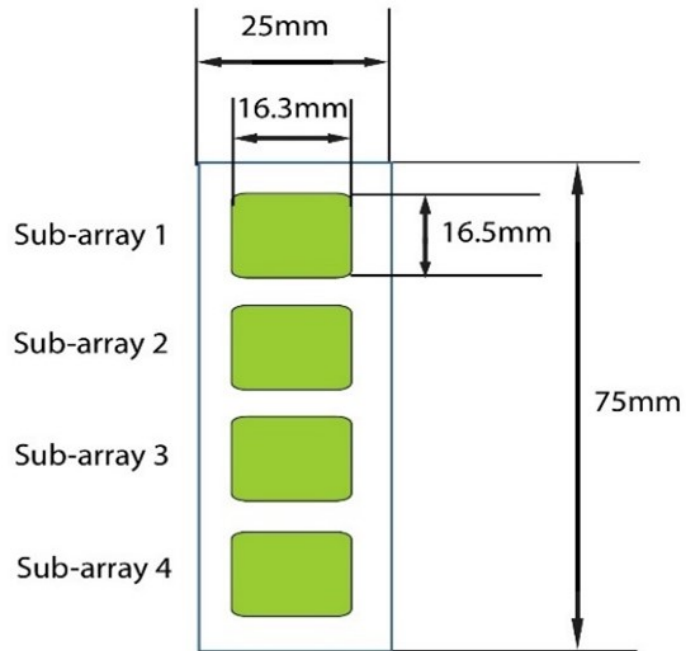
B. Handling the Glass Slides

- 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

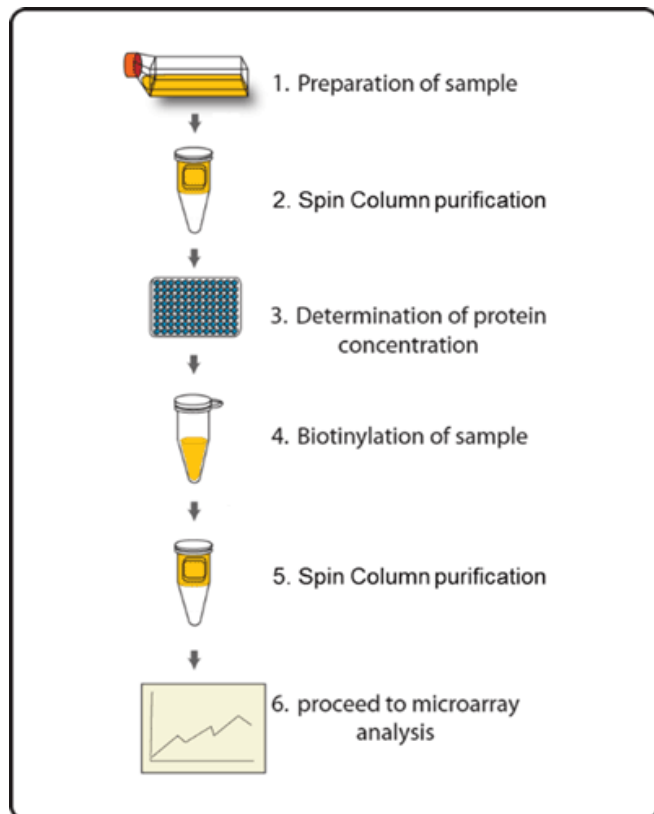
D. Incubations and Washes

- 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.

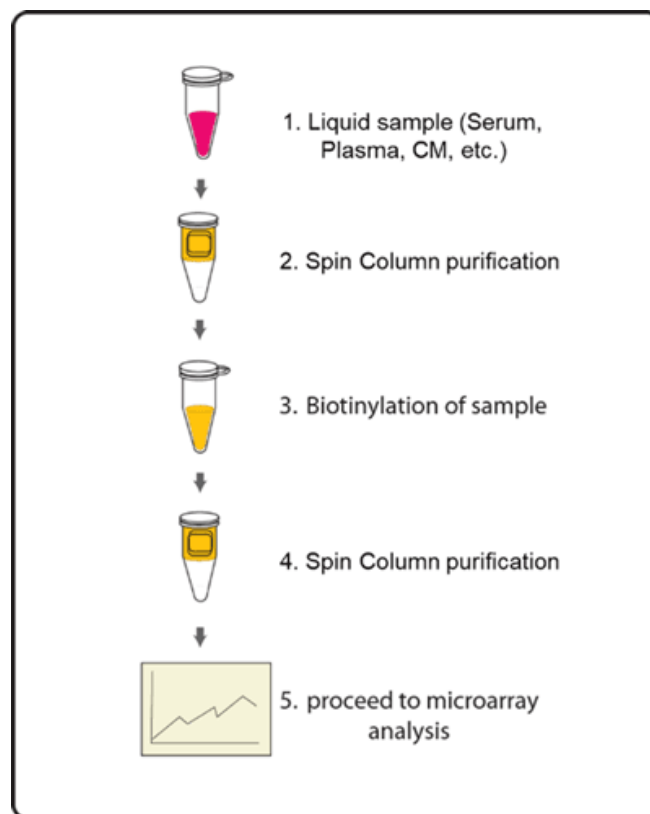
IV. Protocol

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 $1,500 \times g$ for 1 minute to remove the storage buffer. Discard the flow-through.
3. Wash the Spin Column three times with $300 \mu\text{l}$ Labeling Buffer each, centrifuge at $1,500 \times g$ 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 μ l 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 μ l 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 ddH₂O
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 ddH₂O 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.

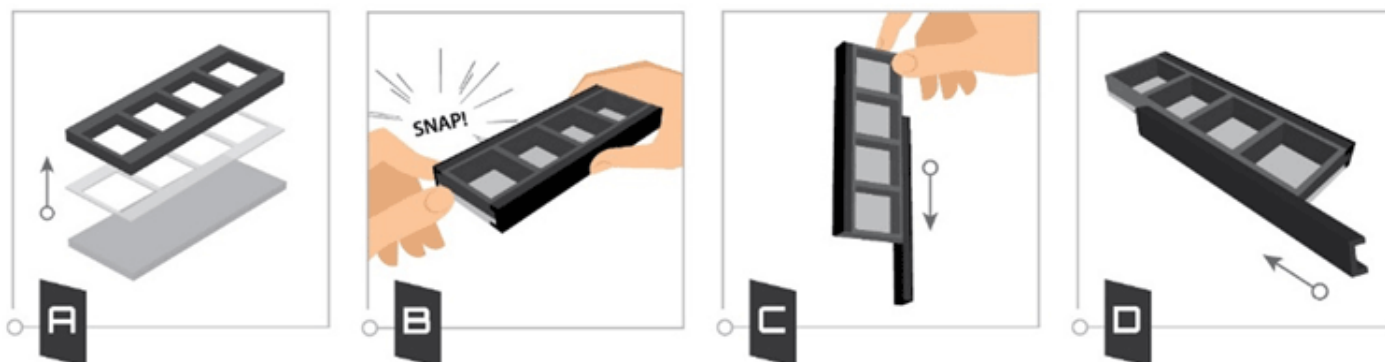
E. Fluorescence Detection

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

PI3K-Akt signaling pathway 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	POS1	POS2	POS2	POS3	POS3	Neg	Neg	1	1	2	2	3	3	4	4	5	5	6	6	7	7	8	8	9	9	10	10	11	11
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
3	27	27	28	28	29	29	30	30	31	31	32	32	33	33	34	34	35	35	36	36	37	37	38	38	39	39	40	40	41	41
4	42	42	43	43	44	44	45	45	46	46	47	47	48	48	49	49	50	50	51	51	52	52	53	53	54	54	55	55	56	56
5	57	57	58	58	59	59	60	60	61	61	62	62	63	63	64	64	65	65	66	66	67	67	68	68	69	69	70	70	71	71
6	72	72	73	73	74	74	75	75	76	76	77	77	78	78	79	79	80	80	81	81	82	82	83	83	84	84	85	85	86	86
7	87	87	88	88	89	89	90	90	91	91	92	92	93	93	94	94	95	95	96	96	97	97	98	98	99	99	100	100	101	101
8	102	102	103	103	104	104	105	105	106	106	107	107	108	108	109	109	110	110	111	111	112	112	113	113	114	114	115	115	116	116
9	117	117	118	118	119	119	120	120	121	121	122	122	123	123	124	124	125	125	126	126	127	127	128	128	129	129	130	130	131	131
10	132	132	133	133	134	134	135	135	136	136	137	137	138	138	139	139	140	140	141	141	142	142	143	143	144	144	145	145	146	146
11	147	147	148	148	149	149	150	150	151	151	152	152	153	153	154	154	155	155	156	156	157	157	158	158	159	159	160	160	161	161
12	162	162	163	163	164	164	165	165	166	166	167	167	168	168	169	169	170	170	171	171	172	172	173	173	174	174	175	175	176	176
13	177	177	178	178	179	179	180	180	181	181	182	182	183	183	184	184	185	185	186	186	187	187	188	188	189	189	190	190	191	191
14	192	192	193	193	194	194	195	195	196	196	197	197	198	198	199	199	200	200	201	201	202	202	203	203	204	204	205	205	206	206
15	207	207	208	208	209	209	210	210	211	211	212	212	213	213	214	214	215	215	216	216	217	217	218	218	219	219	220	220	221	221
16	222	222	223	223	224	224	225	225	226	226	227	227	228	228	229	229	230	230	231	231	232	232	233	233	234	234	235	235	236	236
17	237	237	238	238	239	239	240	240	241	241	242	242	243	243	244	244	245	245	246	246	247	247	248	248	249	249	250	250	251	251
18	252	252	253	253	254	254	255	255	256	256	257	257	258	258	259	259	260	260	261	261	262	262	263	263	264	264	265	265	266	266
19	267	267	268	268	269	269	270	270	271	271	272	272	273	273	274	274	275	275	276	276	277	277	278	278	279	279	280	280	281	281
20	282	282	283	283	284	284	285	285	286	286	287	287	288	288	289	289	290	290	291	291	292	292	293	293	294	294	295	295	296	296
21	297	297	298	298	299	299	300	300	301	301	302	302	303	303	304	304	305	305	306	306	307	307	Neg	Neg	POS3	POS3	POS2	POS2	POS1	POS1

VI. Antibody Array Target List

PI3K-Akt signaling pathway array													
Number	Name	Number	Name	Number	Name	Number	Name	Number	Name	Number	Name	Number	Name
1	AKT1	46	COL9A3	91	FGF7	136	IFNA4	181	ITGB7	226	PCK2	271	RPS6KB1
2	AKT2	47	COMP	92	FGF8	137	IFNA5	182	ITGB8	227	PDGFA	272	RXRA
3	AKT3	48	CREB1	93	FGF9	138	IFNA6	183	JAK1	228	PDGFB	273	SCFR
4	ANGPT1	49	CREB3L1	94	FGFR1	139	IFNA7	184	JAK3	229	PDGFC	274	SGK1
5	ANGPT2	50	CREB3L2	95	FGFR2	140	IFNA8	185	KDR	230	PDGFD	275	SOS2
6	ANGPT4	51	CRTC2	96	FGFR3	141	IFNAR1	186	KIAA1303	231	PDGFRA	276	SPP1
7	AREG	52	CSF1	97	FGFR4	142	IFNAR2	187	KITLG	232	PDGFRB	277	STK11
8	ATF2	53	CSF1R	98	FLT1	143	IFNB1	188	KRAS	233	PDPK1	278	SYK
9	ATF4	54	CSF3	99	FLT3	144	IGF1	189	LAMA1	234	PGF	279	TCL1A
10	BAD	55	CSF3R	100	FLT3L	145	IGF1R	190	LAMA2	235	PIK3AP1	280	TCL1B
11	BCL2	56	CSH1	101	FLT4	146	IGF2	191	LAMA3	236	PIK3CA	281	TGFA
12	BCL2L1	57	CSH2	102	FN1	147	IKBKB	192	LAMA4	237	PIK3CB	282	THBS1
13	BCL2L11	58	EFNA1	103	G6PC	148	IKBKG	193	LAMB1	238	PIK3CD	283	THBS2
14	BDNF	59	EFNA3	104	GH1	149	IL2	194	LAMB2	239	PIK3CG	284	THBS3
15	BRCA1	60	EFNA4	105	GH2	150	IL2RA	195	LAMC1	240	PIK3R1	285	THBS4
16	CASP9	61	EFNA5	106	GHR	151	IL2RB	196	LPAR1	241	PIK3R2	286	TIE2
17	CCND1	62	EGF	107	GNB1	152	IL2RG	197	LPAR2	242	PIK3R3	287	TLR2
18	CCND2	63	EGFR	108	GNB2	153	IL3	198	LPAR4	243	PIK3R5	288	TLR4
19	CCND3	64	EIF4B	109	GNB3	154	IL3RA	199	LPAR5	244	PIK3R6	289	TNC
20	CCNE1	65	EIF4E	110	GNB4	155	IL4	200	MAGI2	245	PKN1	290	TNN
21	CCNE2	66	EIF4EBP1	111	GNB5	156	IL4R	201	MAP2K1	246	PKN2	291	TNR
22	CD19	67	EPHA2	112	GNG11	157	IL6	202	MAP2K2	247	PKN3	292	TNXB
23	CDC37	68	EPO	113	GNG12	158	IL6R	203	MAPK1	248	PPP2CA	293	TP53
24	CDK2	69	EPOR	114	GNG13	159	IL7	204	MAPK3	249	PPP2CB	294	TSC1
25	CDK4	70	ERBB2	115	GNG4	160	IL7R	205	MCL1	250	PPP2R1A	295	TSC2
26	CDK6	71	ERBB3	116	GNG5	161	INS	206	MDM2	251	PPP2R1B	296	VEGFA
27	CDKN1A	72	ERBB4	117	GNG8	162	INSR	207	MET	252	PPP2R2A	297	VEGFB
28	CDKN1B	73	EREG	118	GNGT1	163	IRS1	208	MLST8	253	PPP2R2B	298	VEGFC
29	CHAD	74	F2R	119	GNGT2	164	ITGA1	209	MTCP1	254	PPP2R2C	299	VEGFD
30	CHRM1	75	FASLG	120	GRB2	165	ITGA11	210	MTOR	255	PPP2R5C	300	VTN
31	CHRM2	76	FGF1	121	GSK3B	166	ITGA2	211	MYB	256	PPP2R5E	301	VWF
32	CHUK	77	FGF10	122	GYS1	167	ITGA2B	212	MYC	257	PRKAA1	302	YWHAB
33	COL1A1	78	FGF16	123	GYS2	168	ITGA3	213	NFKB1	258	PRKAA2	303	YWHAE
34	COL1A2	79	FGF17	124	HGF	169	ITGA4	214	NGF	259	PRKCA	304	YWHAG
35	COL2A1	80	FGF18	125	HRAS	170	ITGA5	215	NGFR	260	PRL	305	YWHAH
36	COL4A1	81	FGF19	126	HSP90AA1	171	ITGA6	216	NOS3	261	PRLR	306	YWHAQ
37	COL4A2	82	FGF2	127	HSP90AB1	172	ITGA7	217	NR4A1	262	PTEN	307	YWHAZ
38	COL4A3	83	FGF20	128	HSP90B1	173	ITGA8	218	NRAS	263	PTK2	308	
39	COL4A4	84	FGF21	129	IBSP	174	ITGA9	219	NTF3	264	RAC1	309	
40	COL4A5	85	FGF22	130	IFNA10	175	ITGAV	220	NTF4	265	RAF1	310	
41	COL4A6	86	FGF23	131	IFNA14	176	ITGB1	221	NTRK1	266	RBL2	311	
42	COL6A1	87	FGF3	132	IFNA16	177	ITGB3	222	NTRK2	267	RELA	312	
43	COL6A2	88	FGF4	133	IFNA17	178	ITGB4	223	OSM	268	RELN	313	
44	COL6A3	89	FGF5	134	IFNA2	179	ITGB5	224	OSMR	269	RHEB	314	
45	COL9A1	90	FGF6	135	IFNA21	180	ITGB6	225	PCK1	270	RPS6	315	

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.

Sample image
Image not found or unknown

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 completely 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 drying out during experiment

IX. Selected References

- Christina Scheel et al., *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 hepatocellular 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 al., *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 and LV 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 endometriosis*. 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.

RayBio® L-series Antibody Arrays are patent-pending technology developed by RayBiotech.

This product is intended for research only and is not to be used for clinical diagnosis. Our products may not be resold, modified for resale, or used to manufacture commercial products without written approval by RayBiotech, Inc.

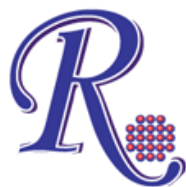
Under no circumstances shall RayBiotech be liable for any damages arising out of the use of the materials.

Products are guaranteed for six months from the date of shipment when handled and stored properly. In the event of any defect in quality or merchantability, RayBiotech's liability to buyer for any claim relating to products shall be limited to replacement or refund of the purchase price.

RayBio® is a registered trademark of RayBiotech, Inc.

GenePix® is a registered trademark of Molecular Devices, Inc.

This product is for research use only.



©2022 RayBiotech, Inc