RayBio® Label-Based (L-Series) Human Endocrine screening array

Patent Pending Technology
User Manual (Apr 14, 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-END-4 (4 Sample Kit)
AAH-BLG-END-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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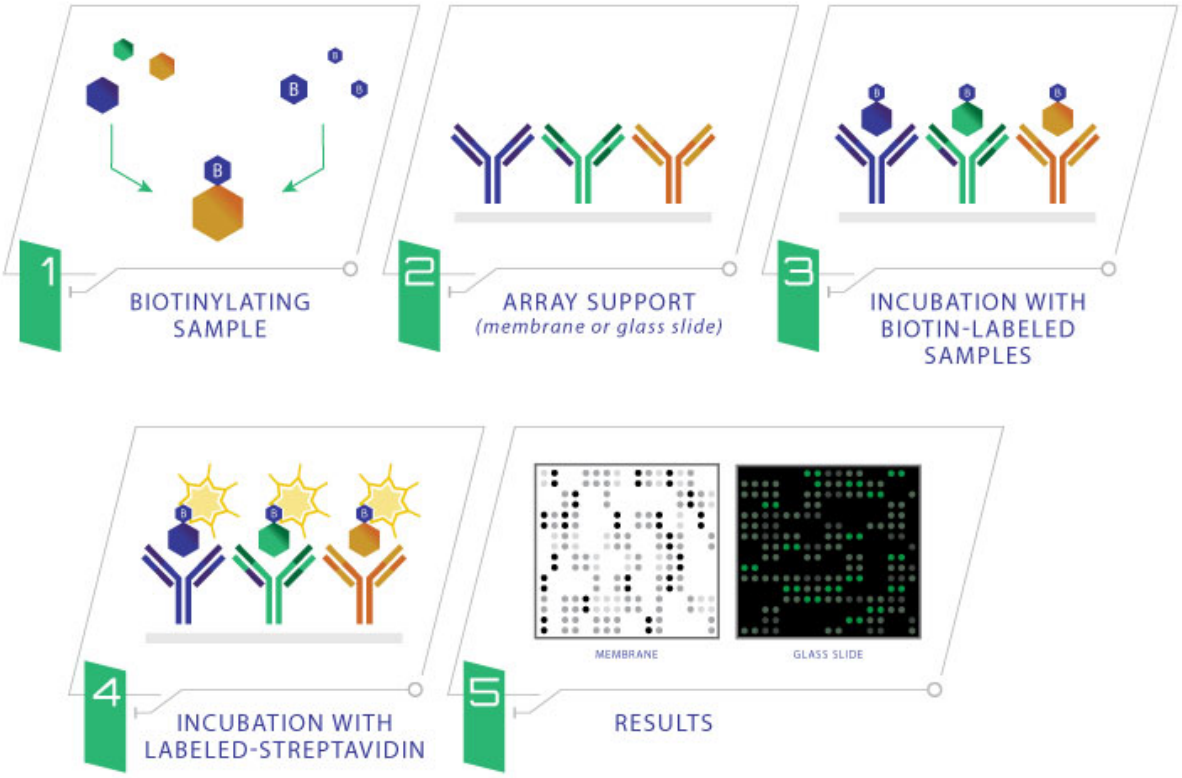
I. Introduction

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 $1x10^6$ 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

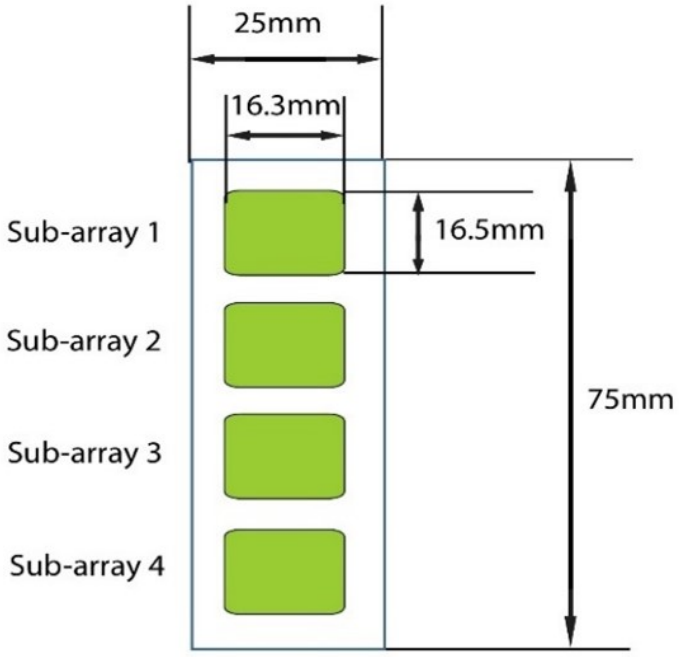
   For optimal biotin labeling, it is necessary to determine the protein concentration in the cell/tissue lysate. We recommended using a BCA total protein assay (e.g., Pierce, Catalog # 23227).

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

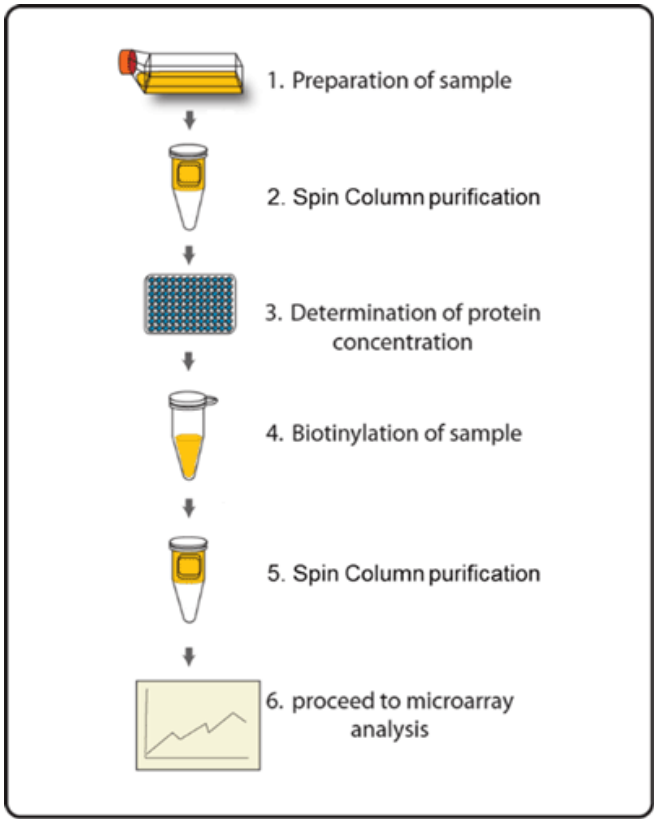
D. Incubations and Washes

- 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.

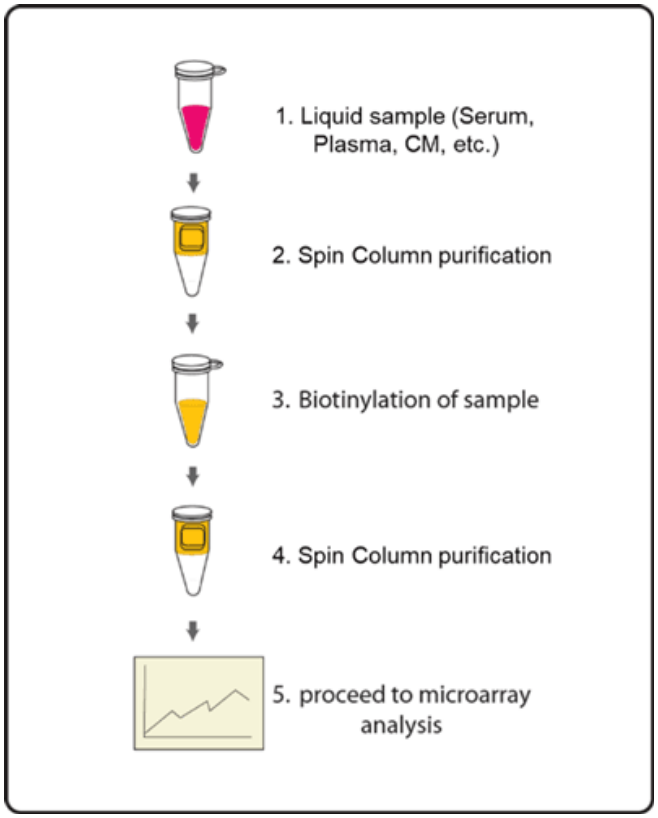
IV. Protocol

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.

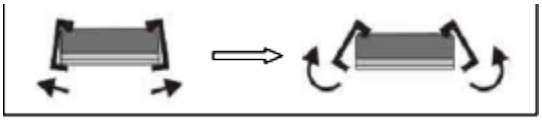
    Note: Avoid exposure to light in Steps 19-25 by covering the Glass Slide Assembly with aluminum foil or incubate in a dark room.

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.

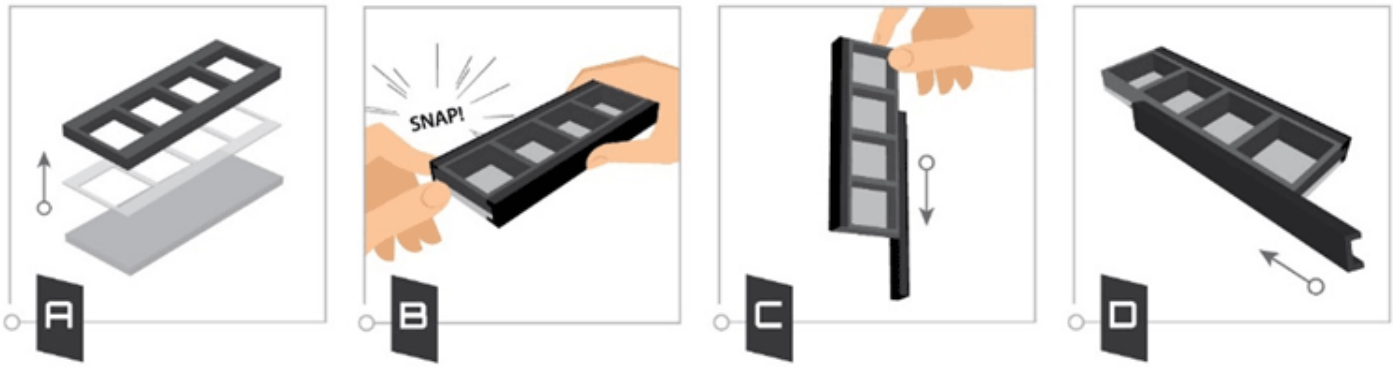
E. Fluorescence Detection

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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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
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VI. Antibody Array Target List

Number	Name	Number	Name	Number	Name	Number	Name	Number	Name	Number	Name	Number	Name
1	ABCC8	73	CACNG6	145	FASN	217	ITGB3	289	MMP2	361	PLK1	433	SHC1
2	ACACA	74	CACNG8	146	FBP1	218	JUN	290	MMP9	362	PLN	434	SHC2
3	ACE	75	CALM1	147	FBP2	219	KAT2B	291	MYC	363	PNPLA2	435	SIK2
4	ACOT1	76	CALM2	148	FKBP4	220	KCNJ11	292	MYH6	364	POMC	436	SIN3A
5	ACOT2	77	CALML3	149	FKBP5	221	KCNJ12	293	MYH7	365	PPARA	437	SIRT1
6	ACSBG1	78	CALML5	150	FLOT2	222	KCNJ2	294	MYL6	366	PPARGC1A	438	SLC2A1
7	ACSL1	79	CAMK1	151	FOS	223	KCNJ3	295	MYL6B	367	PPP1CA	439	SLC2A2
8	ACSL4	80	CAMK2A	152	FOXO1	224	KCNJ5	296	MYLK4	368	PPP1CC	440	SLC2A4
9	ACSL5	81	CAMK2B	153	FSHB	225	KCNJ6	297	NCOA2	369	PPP1R3A	441	SLC5A5
10	ADCY1	82	CAMK2D	154	FSHR	226	KCNJ9	298	NCOA3	370	PPP1R3B	442	SLC9A1
11	ADCY2	83	CAMK4	155	FXYD2	227	KCNMA1	299	NCOR1	371	PPP1R3C	443	SNAP25
12	ADCY3	84	CAMKK2	156	FZD1	228	KCNN4	300	NFATC1	372	PPP3CA	444	SOCS2
13	ADCY4	85	CANX	157	FZD2	229	KIAA1303	301	NFATC2	373	PPP3CB	445	SOCS3
14	ADCY5	86	CASP9	158	FZD3	230	KITLG	302	NFATC3	374	PPP3R1	446	SOCS4
15	ADCY6	87	CBL	159	FZD4	231	KRAS	303	NFATC4	375	PPP3R2	447	SOCS5
16	ADCY7	88	CCK	160	FZD5	232	KRT10	304	NFKB1	376	PPP4C	448	SOCS7
17	ADCY8	89	CCKAR	161	FZD6	233	KRT12	305	NFKBIA	377	PRKAA1	449	SOS2
18	ADCY9	90	CCNA1	162	FZD7	234	KRT13	306	NFKBIB	378	PRKAA2	450	SP1
19	ADCYAP1R1	91	CCNA2	163	G6PC	235	KRT14	307	NFKBIE	379	PRKAB1	451	SPDYC
20	ADIPOQ	92	CCNB2	164	GABBR1	236	KRT15	308	NOS3	380	PRKAB2	452	SRC
21	ADIPOR1	93	CCND1	165	GATA4	237	KRT16	309	NOTCH1	381	PRKACA	453	SREBF1
22	ADIPOR2	94	CCND2	166	GCG	238	KRT17	310	NOTCH2	382	PRKACB	454	STAT1
23	ADRB1	95	CD36	167	GCGR	239	KRT18	311	NOTCH3	383	PRKACG	455	STAT3
24	ADRB2	96	CD38	168	GCK	240	KRT19	312	NOTCH4	384	PRKAR1A	456	STAT5A
25	ADRB3	97	CDC16	169	GIP	241	KRT20	313	NPPA	385	PRKAR1B	457	STAT5B
26	AGRP	98	CDC23	170	GLP1R	242	KRT23	314	NPR1	386	PRKAR2A	458	STK11
27	AGT	99	CDC25A	171	GNAI1	243	KRT24	315	NPY	387	PRKCA	459	STX1A
28	AGTR1	100	CDC25B	172	GNAI2	244	KRT25	316	NPY1R	388	PRKCB	460	TBC1D4
29	AKR1C3	101	CDC25C	173	GNAI3	245	KRT27	317	NR4A1	389	PRKCD	461	TCF7
30	AKT1	102	CDC26	174	GNAO1	246	KRT31	318	NR4A2	390	PRKCE	462	TCF7L1
31	AKT2	103	CDC27	175	GPX1	247	KRT32	319	NRAS	391	PRKCG	463	TFF1
32	AKT3	104	CDC42	176	GPX2	248	KRT33B	320	OPRM1	392	PRKCI	464	TG
33	ALOX5	105	CDK2	177	GPX3	249	KRT34	321	ORAI1	393	PRKCQ	465	TGFA
34	ANAPC1	106	CDKN1A	178	GPX5	250	KRT36	322	PAX8	394	PRKCZ	466	TH
35	ANAPC13	107	CGA	179	GPX7	251	KRT37	323	PCK1	395	PRKD2	467	THRA
36	ANAPC2	108	CHRM3	180	GRB2	252	KRT38	324	PCK2	396	PRKD3	468	THRB
37	ANAPC4	109	CHUK	181	GRM1	253	KRT9	325	PCLO	397	PRKG2	469	TNFRSF1A
38	AQP1	110	CISH	182	GSK3B	254	LDHA	326	PDE1A	398	PRL	470	TNFRSF1B
39	ARAF	111	CLCA1	183	GSR	255	LDHB	327	PDE1C	399	PRLR	471	TNFSF11
40	ASGR1	112	CPEB3	184	GYS1	256	LDLR	328	PDE2A	400	PRMT1	472	TP53
41	ASIP	113	CPT1A	185	GYS2	257	LEF1	329	PDHA1	401	PTGER2	473	TRADD
42	ATF1	114	CPT1B	186	HBEGF	258	LEP	330	PDIA4	402	PTGER3	474	TRAF2
43	ATF2	115	CREB1	187	HDAC1	259	LEPR	331	PDPK1	403	PTGS1	475	TSC1
44	ATF4	116	CREB3L1	188	HDAC2	260	LHB	332	PDX1	404	PTGS2	476	TSC2
45	ATP1A1	117	CREB3L2	189	HDAC3	261	LIPE	333	PFKM	405	PTK2B	477	TSHB
46	ATP1A2	118	CREBBP	190	HIF1A	262	LRP2	334	PFKP	406	PTPN1	478	TSHR
47	ATP1A3	119	CRK	191	HK1	263	MAD1L1	335	PGR	407	PTPN11	479	TTF1
48	ATP1B1	120	CRKL	192	HK2	264	MAD2L1	336	PIK3CA	408	PTPRF	480	TTF2
49	ATP1B2	121	CRTC2	193	HKDC1	265	MAP2K1	337	PIK3CB	409	PYGB	481	TTR
50	ATP1B3	122	CTNNB1	194	HRAS	266	MAP2K2	338	PIK3CD	410	PYGL	482	TYRP1
51	ATP1B4	123	CTSB	195	HSD17B1	267	MAP2K3	339	PIK3CG	411	PYGM	483	VAMP2
52	ATP2A1	124	CTSD	196	HSD3B1	268	MAP2K4	340	PIK3R1	412	RAF1	484	WNT1
53	ATP2A3	125	CYP19A1	197	HSP90AA1	269	MAP2K5	341	PIK3R2	413	RARA	485	WNT10A
54	AURKA	126	CYP1B1	198	HSP90AB1	270	MAP2K7	342	PIK3R3	414	RCAN1	486	WNT10B
55	BAD	127	CYP21A2	199	HSP90B1	271	MAP3K1	343	PIK3R5	415	RELA	487	WNT11
56	BCL2	128	DVL1	200	HSPA1A	272	MAP3K2	344	PIK3R6	416	REN	488	WNT16
57	BMP15	129	DVL2	201	HSPA1L	273	MAP3K3	345	PKLR	417	RHEB	489	WNT2
58	BMP4	130	DVL3	202	HSPA2	274	MAPK1	346	PLA2G16	418	RHOA	490	WNT2B
59	BMP6	131	EBAG9	203	HSPA5	275	MAPK11	347	PLA2G4A	419	ROCK1	491	WNT3A
60	BRAF	132	EDN1	204	HSPA6	276	MAPK12	348	PLA2G4F	420	ROCK2	492	WNT4
61	BUB1	133	EDN3	205	HSPA8	277	MAPK13	349	PLCB1	421	RPS6	493	WNT5A
62	CACNA1C	134	EDNRA	206	IGF1	278	MAPK14	350	PLCB2	422	RPS6KA1	494	WNT5B
63	CACNA1G	135	EDNRB	207	IGF1R	279	MAPK3	351	PLCB3	423	RPS6KA2	495	WNT6
64	CACNA1S	136	EGFR	208	IKBKB	280	MAPK7	352	PLCB4	424	RPS6KA3	496	WNT7A
65	CACNA2D4	137	EGR1	209	IKBKG	281	MAPK8	353	PLCD1	425	RPS6KA6	497	WNT7B
66	CACNB1	138	EIF4E	210	INPPL1	282	MAPK9	354	PLCD3	426	RPS6KB1	498	WNT8B
67	CACNB2	139	EIF4EBP1	211	INS	283	MDM2	355	PLCD4	427	RXRA	499	WNT9A
68	CACNB3	140	ELK1	212	INSR	284	MED1	356	PLCG1	428	RXRB	500	WNT9B
69	CACNG1	141	EP300	213	IRF1	285	MED4	357	PLCG2	429	RXRG		
70	CACNG3	142	ESR1	214	IRS1	286	MEF2C	358	PLCZ1	430	RYR2		
71	CACNG4	143	ESR2	215	IRS2	287	MITF	359	PLD1	431	SCARB1		
72	CACNG5	144	FABP4	216	ITGAV	288	MMP14	360	PLD2	432	SERPINA7		

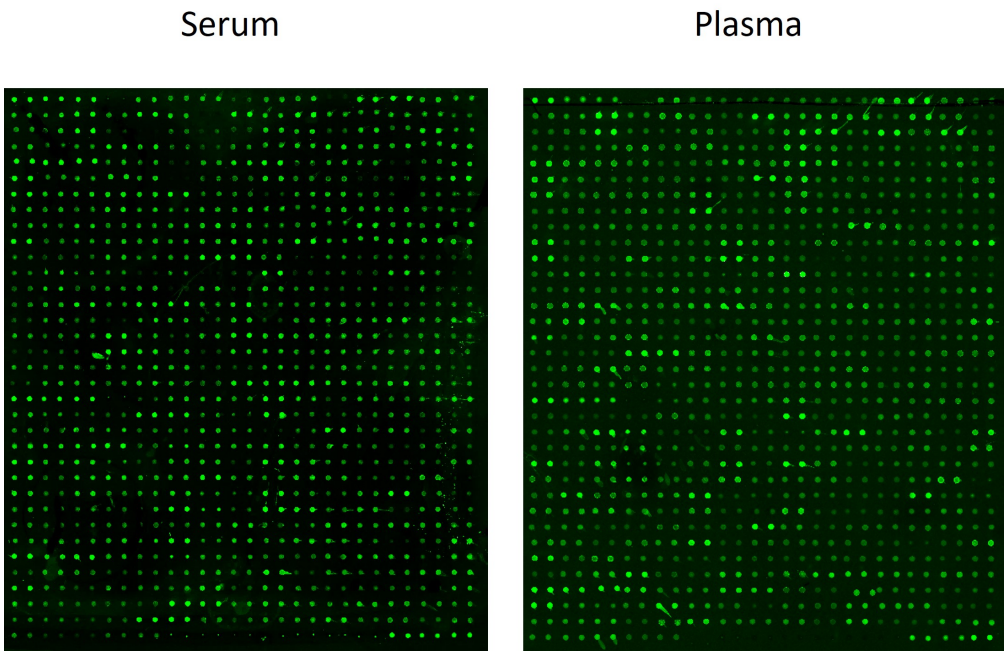
VII. Interpretation of Results

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

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