RayBio® Label-Based (L-Series) Human L3 Array Membrane Kit

Patent Pending Technology
User Manual (Jan 1, 2022)

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.

L-Series Human L3 Array, Membrane
AAH-BLM-3-2 (2 Sample Kit)
AAH-BLM-3-4 (4 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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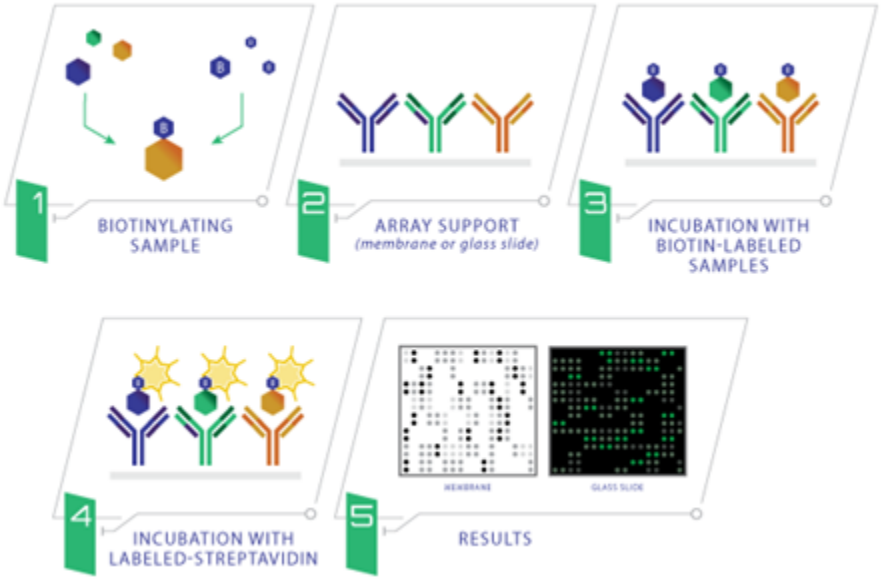
TABLE OF CONTENTS

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 membrane arrays are then blocked, similar as a Western blot, and the biotin-labeled sample is added onto the membrane array which is pre-printed with capture antibodies and incubated to allow for interaction of target proteins. After incubation with HRP-Conjugated Streptavidin, the signals can be visualized by chemiluminescence.



II. Materials Provided

A. Storage Recommendations

Upon receipt, Box 1 should be stored at -20°C and Box 2 should be stored at 4°C. The kit must be used within 6 months from the date of shipment. After initial use, Blocking Buffer, Stop Solution, HRP-Conjugated Streptavidin, Detection Buffers C and D should be stored at 4°C to avoid repeated freeze-thaw cycles (may be stored for up to 3 months, Labeling Reagent, Item B should be fresh preparation before use). The Array Membrane should be kept at -20°C and avoid repeated freeze-thaw cycles (may be stored for up to 6 months).

Box 1 (store at -20°C):

ITEM	DESCRIPTION	2 MEMBRANE KIT	4 MEMBRANE KIT
B	Labeling Reagent	1 vial	2 vials
D	Stop Solution	1 vial (50 µl)	1 vial (50 µl)
E	L-series Antibody Array Membranes	2 membranes	4 membranes
F	4X Blocking Buffer	1 bottle (30 ml)	1 bottle (30 ml)
I	500X HRP-Conjugated Streptavidin Concentrate	1 vial (100 µl)	1 vial (100 µl)
K	Detection Buffer C	1 bottle (10 ml)	2 bottles (10 ml)
L	Detection Buffer D	1 bottle (10 ml)	2 bottles (10 ml)
Other Kit Components: Plastic Sheets			

Box 2 (store at 4°C):

ITEM	DESCRIPTION	2 MEMBRANE KIT	4 MEMBRANE KIT
G	20X Wash Buffer 1 Concentrate	1 bottle (30 ml)	1 bottle (30 ml)
H	20X Wash Buffer 2 Concentrate	1 bottle (30 ml)	1 bottle (30 ml)
	Labeling Buffer	1 bottle (30 ml)	2 bottles (30 ml/ea)
J-2	Spin Columns	4 columns	8 columns
N/A	Plastic Incubation Trays (w/lid)	2 trays	4 trays
N/A	2X Lysis Buffer	1 bottle (10 ml)	1 bottle (10 ml)

B. Additional Materials Required

- 2-5 ml tube, small plastic or glass containers
- 15 ml conical collection tubes
- Orbital shaker or oscillating rocker
- Kodak X-Omat™ AR film (REF 165 1454) and film processor or Chemiluminescence imaging system
- Pipettors, pipette tips and other common lab consumables
- Eppendorf tube

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.**,+
    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 densitometry 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).

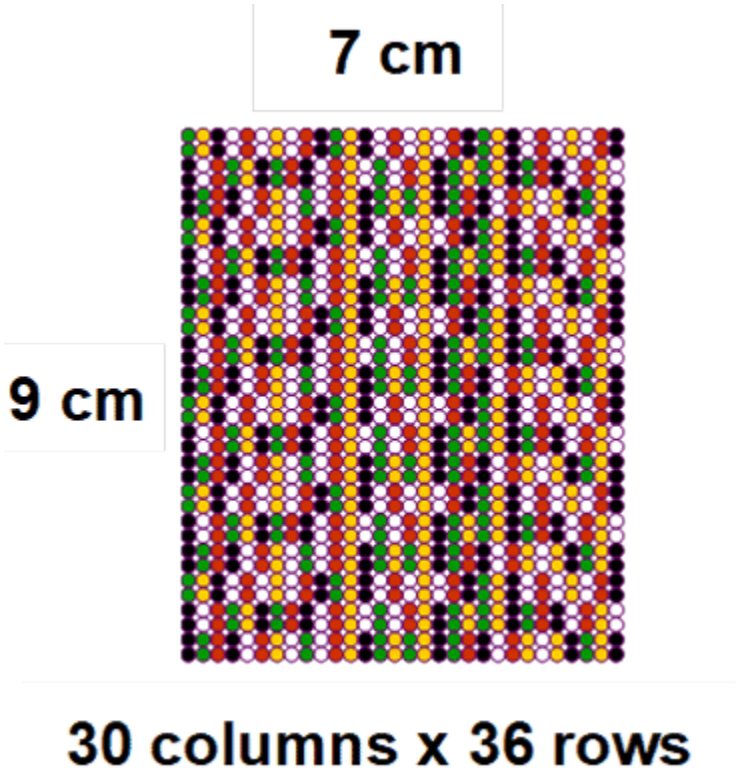
B. Handling the Array Membranes

- Always use forceps to handle membranes and grip the membranes by the edges only.
- Never allow membranes to dry during the experiment.
- Avoid touching membranes with hands or any sharp tools.

C. Incubations of Antibody Array

- Completely cover membranes with sample or buffer during incubation and cover the Plastic Incubation Tray with the lid to avoid drying.
- Avoid foaming during incubation steps.
- Perform all incubation and wash steps under gentle rotation.
- Several incubation steps such as step 3 (sample incubation) or step 7 (HRP-Conjugated Streptavidin incubation) may be done at 4°C overnight.

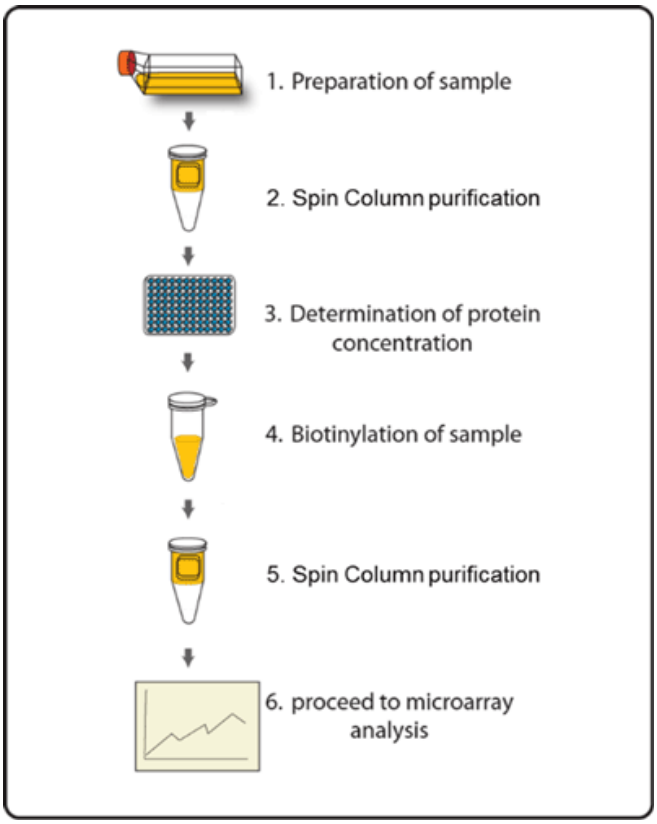
D. Layout of Array Membrane



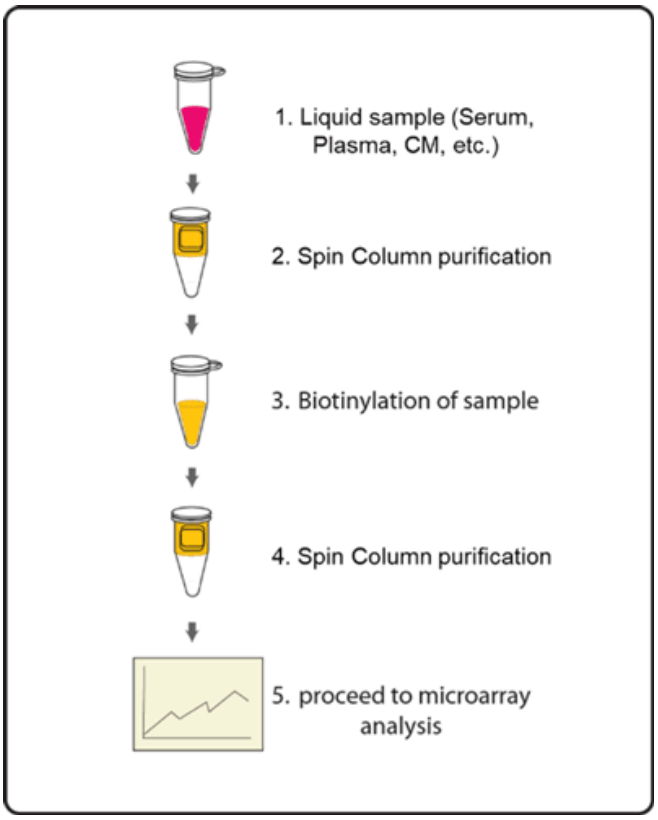
IV. Protocol

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 15 ml conical collection tube, centrifuge at 1,000 x g for 3 minutes to remove the storage buffer. Discard the flow-through.
3. Wash the column three times with 1 ml labeling buffer each, centrifuge 1,000 x g for 3 minutes to remove the flow-through. Blot the bottom of the column to remove excess liquid, and transfer device to a new collection tube.
4. Apply sample on top of the resin within the next few minutes. Centrifuge at

1,000 x g for 3 minutes to collect the flow-through that contains sample. The recommended sample dilution as following:

- *Cell culture supernatant: 600 µl neat supernatant*
- *Serum/Plasma: 10 µl serum/plasma in 600 µl Labeling Buffer*
- *Cell/tissue lysate: 100 µg lysate in 500 µl Labeling Buffer*

Note: The maximal sample volume is 700 µl for each Spin Column. Do not load over 700 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 10 µl of Labeling Reagent into the sample tube (for 600 µl supernatant).
    b. For labeling serum or plasma: Add 10 µl of Labeling Reagent into the sample tube (for 10 µl serum/plasma *in 600 µl labeling buffer*).
    c. For labeling cell or tissue lysates: Add 5 µl of 1X Labeling Reagent into the sample tube (for 100 µg lysate *in 500 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 more or less amount sample is labelled, adjust this volume proportionally.
7. Add 5 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. Blocking and Incubations

8. Place each membrane printed side up into a Plastic Incubation Tray (provided). 1 membrane per tray.

   Note: The printed membrane will have a "-" mark in the upper left corner of the membrane.

   Note: Up to 4 membranes can be incubated together within one tray with proportional amount of reaction buffer. Rotate the membrane sequence at least once during sample incubation if more than one membrane is incubated in one tray.

9. Dilute 4X Blocking Buffer (Item F) with deionized or distilled water to prepare the 1X Blocking Buffer. Add 6 ml of 1X Blocking Buffer to each membrane and cover with the lid. Incubate at room temperature with gentle shaking for 1 hour.

10. Aspirate the Blocking Buffer from each tray. Add 6 ml of diluted sample onto each membrane and cover with the lid. Incubate at room temperature with gentle shaking for 2 hours.

    Note: It is recommended to use 10-20 folds diluted biotin-labeled culture supernatant, 10-20 folds diluted biotin-labeled serum/plasma, 100 folds diluted biotin-labeled cell/tissue lysate, or 10-20 folds for other body fluid. Dilute sample using 1X Blocking Buffer. The optimal concentration of sample used will depend on the abundance of target proteins. The samples can be concentrated if the overall signals are too weak. If the overall signals are too strong, the sample can be diluted further.

    Note: Incubation may be done at room temperature with gentle shaking for 2 hours or overnight at 4°C.

11. Dilute 20X Wash Buffer 1 (Item G) with deionized or distilled water to prepare the 1X Wash Buffer 1. Aspirate the samples from each tray and then wash by adding 20 ml of 1X Wash Buffer I at room temperature with gentle shaking (5 min per wash). Repeat the wash 2 more times for a total of 3 washes.

12. Aspirate the 1X Wash Buffer 1 from each tray. Dilute 20X Wash Buffer 2 (Item H) with deionized or distilled water to prepare the 1X Wash Buffer 2. Wash 3 times with 20 ml of 1X Wash Buffer 2 at room temperature with gentle shaking.
13. Aspirate the 1X Wash Buffer 2 from each tray.
14. Prepare the HRP-Conjugated Streptavidin. Briefly spin down the tube containing the 500X HRP-Conjugated Streptavidin (Item I) immediately before use. Dilute the 500X HRP-Conjugated Streptavidin with 1X Blocking Buffer to prepare the 1X HRP-Conjugated Streptavidin. Pipette up and down to mix gently. Add 6 ml of 1X HRP-Conjugated Streptavidin to each membrane.

    Note: Ensure that the vial containing the 500X HRP-Conjugated Streptavidin is mixed well before use, as precipitation can form during storage.
15. Incubate at room temperature with gentle shaking for 2 hours.

    Note: incubation may be done overnight at 4°C.
16. Wash as directed in steps 11 through 13.

D. Detection

Note: Do not let the membrane dry out during detection. The detection process must be completed within 40 minutes without stopping.

17. For detection of 2 membranes, add 4.2 ml of Detection Buffer C and 4.2 ml of Detection buffer D into a tube and mix both solutions. Drain off excess wash buffer. Place membrane antibody side up (There is a "-" symbol on the top left corner of each membrane) on a clean plastic plate or its cover (provided in the kit). Pipette 4 ml of the mixed Detection Buffers onto each membrane and incubate at room temperature for 2 minutes with gentle shaking. Ensure that the detection mixture is evenly covering the membrane without any air bubbles.
18. Gently place the membrane with forceps (antibody side up) on a plastic sheet (provided) and cover the membrane with another plastic sheet. Gently smooth out any air bubbles. Avoid using pressure on the membrane. Work as quickly as possible.
19. The signal can be detected directly from the membrane using a chemiluminescence imaging system or by exposing the array to x-ray film (we recommend using Kodak X-Omat™ AR film) with subsequent development.

Expose the membranes for 40 seconds. Then re-expose the film according to the intensity of signals. If the signals are too strong (background too high), reduce the exposure time (e.g., 5-30 seconds). If the signals are too weak, increase the exposure time (e.g., 5-20 min or overnight) or re-incubate membranes overnight with 1X HRP-Conjugated Streptavidin, and repeat detection on the second day.

20. Save membranes at -20°C to -80°C for future reference.

V. Antibody 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
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VI. Antibody Array Target List

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

VII. Interpretation of Results:

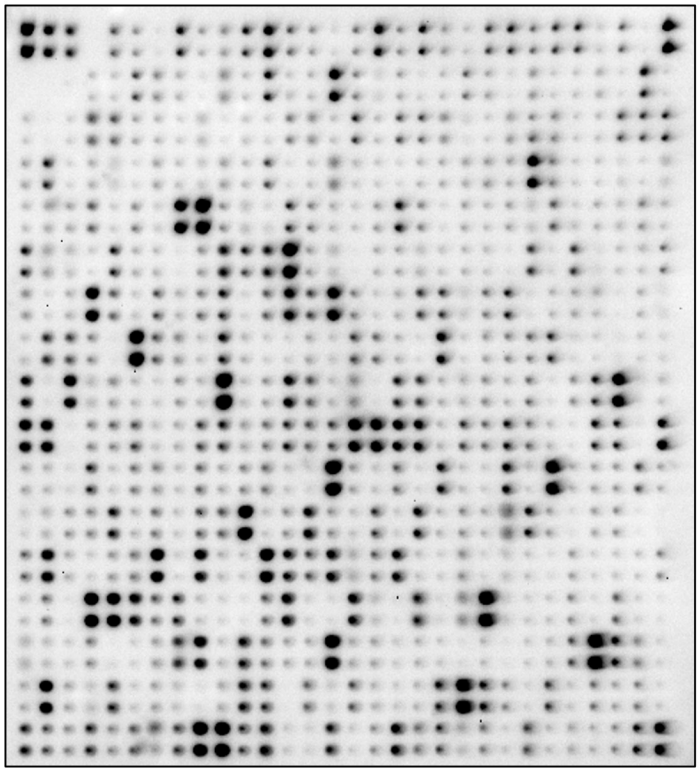
A. Explanation of Controls Spots

To obtain optimal results using a chemiluminescence imaging system (UVP BioImaging Systems), it is suggested to try several different exposure times until the best one is determined. Then, by comparing the signal intensities, relative expression levels of the target proteins can be made. The intensities of signals can be quantified by densitometry. There are three Positive Controls (POS1, POS2, POS3) in each array. These are three levels of standardized anti-HRP antibodies, which will produce positive control signals after incubation with HRP-conjugated Streptavidin. With all other variables being equal, the Positive Control intensities will be the same for each sub-array, which allows for inter-array normalization. Antibody affinity to its target varies significantly between antibodies. The intensity detected on the array with each antibody depends on this affinity; therefore, signal intensity comparison can be performed only within the same antibody/antigen system and not between different antibodies. Some arrays may have beta-actin and GAPDH as internal controls, much as "housekeeping" genes or proteins are used to normalize results in PCR or Western blots, respectively.

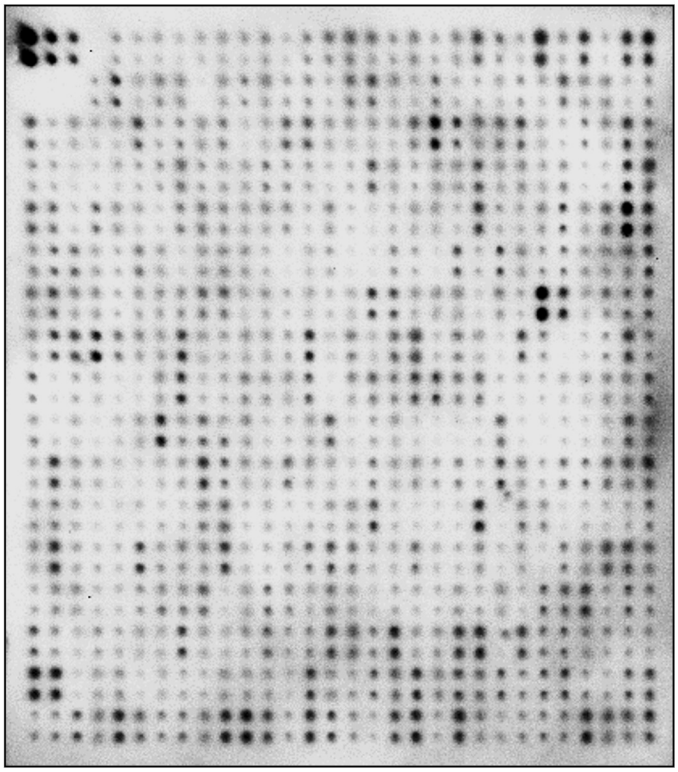
B. Typical Results

The following figure shows the typical result of this array probed with sample(s).

Human Serum



Sample A



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 densitometry data, it is recommended to subtract the local background and normalize to the Positive Control signals before proceeding to analysis.

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 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.

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	Taking too much time for detection	The whole detection process must be completed within 30 min
	Film developer does not work properly	Fix film developer
	Did not mix HRP-Streptavidin well before use	Mix tube containing HRP-Conjugated Streptavidin well before use since precipitates may form during storage
	Sample is too diluted	Increase sample concentration
	Labeling reagent does not function well	Labeling reagent needs to be saved in -20°C and avoid freeze thaw cycle. Always use fresh labeling reagent for sample labelling.
	Other	Check if there were any contamination with any solution containing amines in biotin-labeling step
		Slightly increase HRP concentrations
		Work as quickly as possible after mix Detection Buffer C and D
Uneven signal	Bubble formed during incubation	Remove bubbles during incubation
	Membranes were not completely covered with solution	Completely cover membranes with solution
	Insufficient wash	Use more stringent wash
High background	Exposure time is too long	Decrease exposure time
	Membranes dry out during experiment	Completely cover membranes with solution during experiment. Cover tray with lid.
	Sample is too concentrated	Dilute sample

IX. Selected References

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