RayBio® Label-Based (L-Series) Human L12 Array, Membrane

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.

AAH-BLM-12-2 (2 Sample Kit)
AAH-BLM-12-4 (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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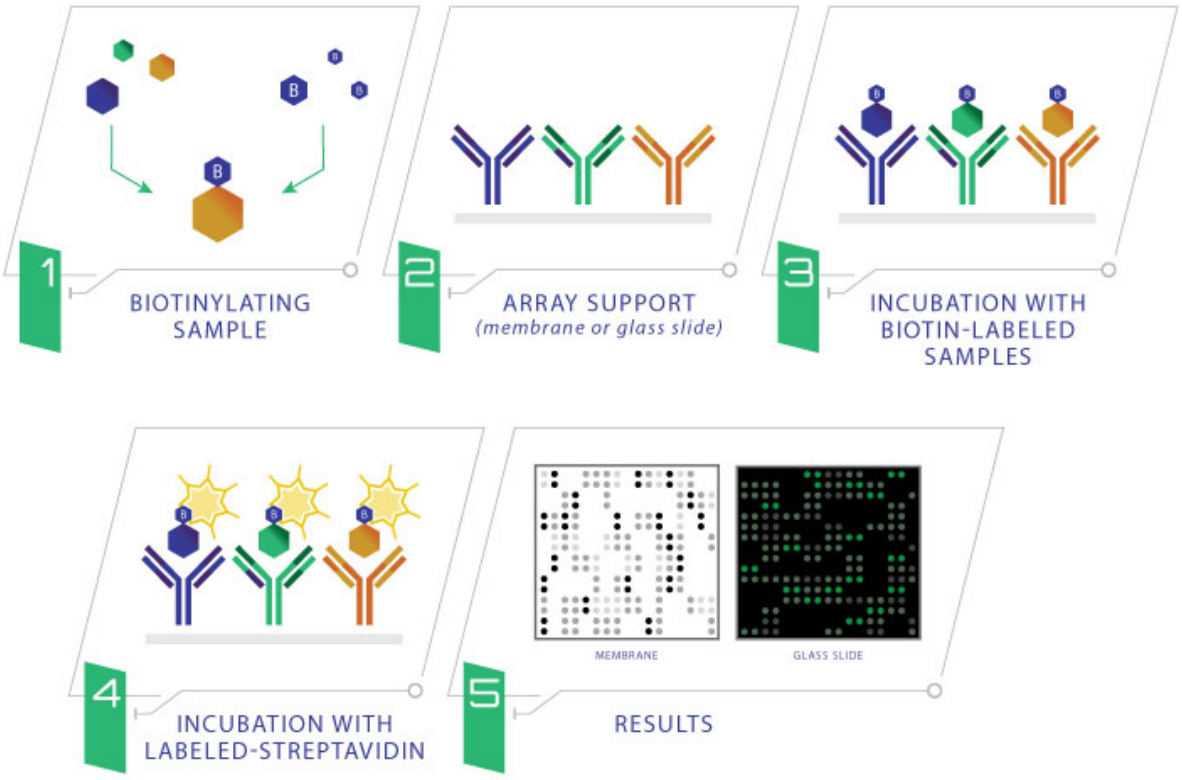
I. Introduction

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).

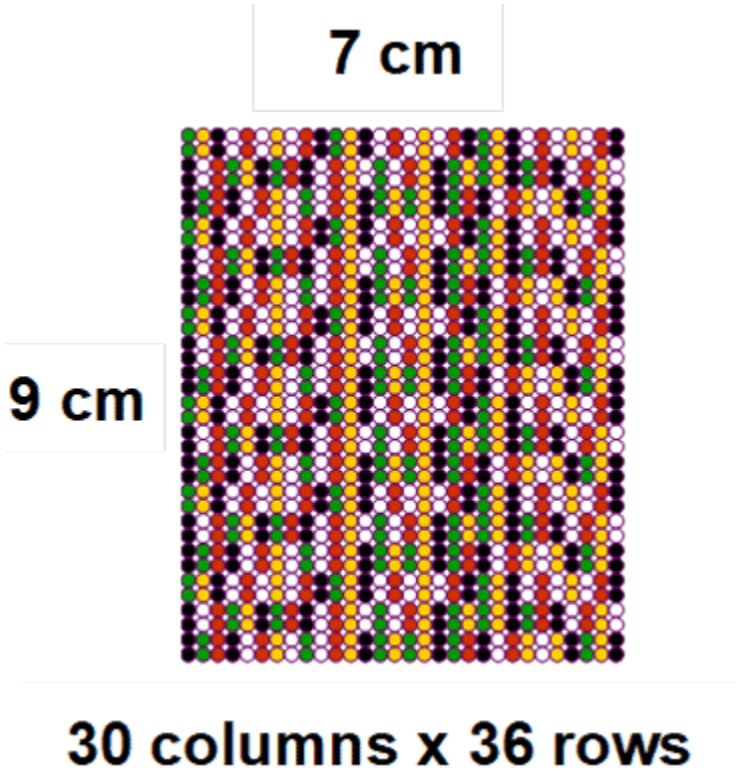
B. Handling the Array Membranes

- 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.

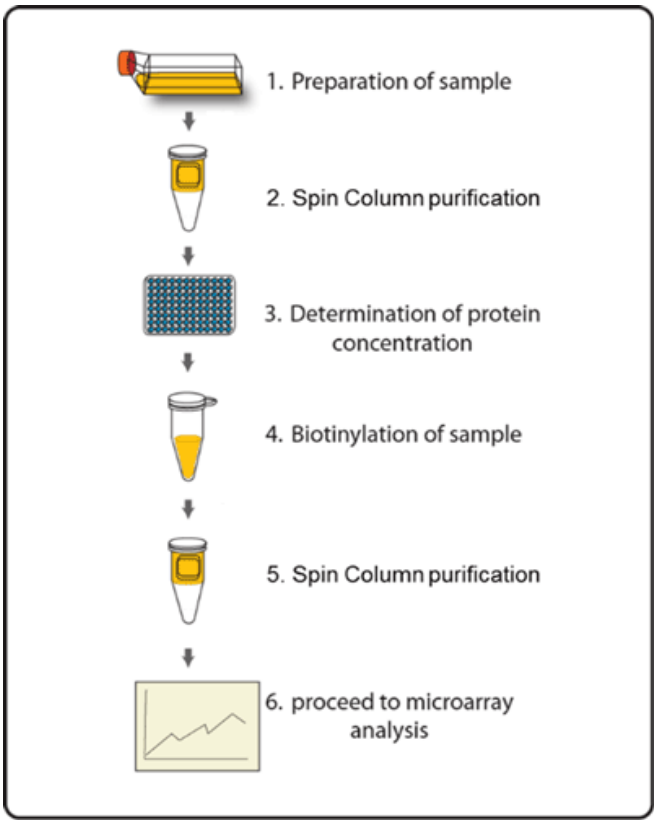
D. Layout of Array Membrane



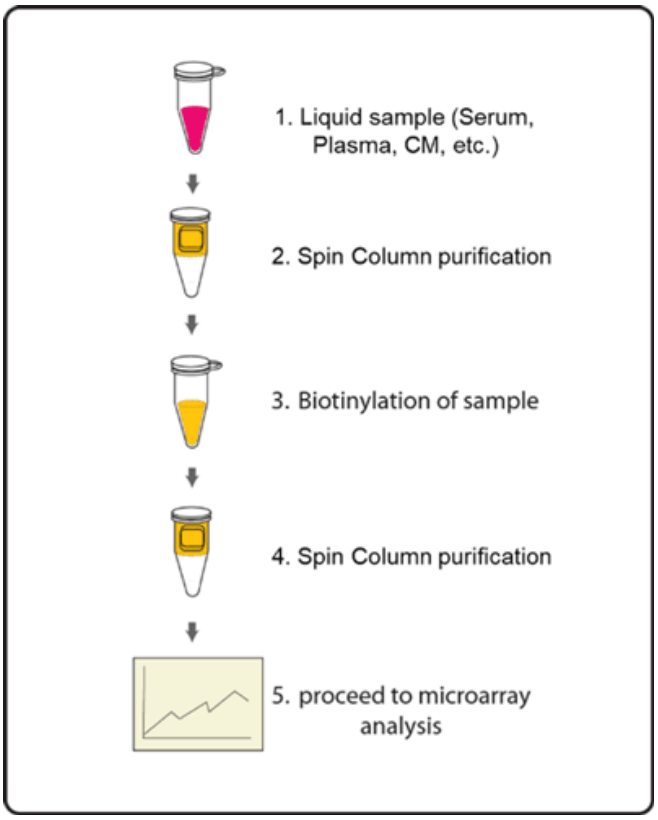
IV. Protocol

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	5T4	73	CD79B	145	DNASE1	217	GRID2	289	LRRTM1	361	PODXL2	433	SORCS2
2	ABP1	74	CD86	146	DNMT3A	218	GRK1	290	LXN	362	POGLUT1	434	SORCS3
3	ACP5	75	CD99	147	DPP7	219	GRK5	291	LY86	363	PPA1	435	SORT1
4	ACP6	76	CD99L2	148	DRAK1	220	GRM3	292	MATN2	364	PRKCA	436	SOX10
5	ACYP1	77	CDC14B	149	DSCAML1	221	GRM4	293	MATN3	365	PRKN	437	SOX15
6	ADA	78	CDC25B	150	DSG3	222	GRM7	294	MD-2	366	PRTG	438	SOX21
7	ADAM12	79	CDCP1	151	DSG4	223	GUSB	295	MDGA1	367	PSMA	439	SOX9
8	ADAM23	80	CDH11	152	DUSP3	224	GYPA	296	MDGA2	368	PSMB8	440	SPHK1
9	ADAM28	81	CDH15	153	DYRK3	225	GZMB	297	MDM2	369	PTGES2	441	SPI1
10	ADGRE2	82	CDH17	154	EDEM2	226	HAND1	298	MEF2C	370	PTGIS	442	SPOCK1
11	AGR3	83	CDH4	155	EDIL3	227	HAPLN1	299	MEP1B	371	PTH1R	443	SPON1
12	AIF1L	84	CDO1	156	EDNRB	228	HAPLN4	300	MESD	372	PTP1B	444	SPRY1
13	AK1	85	CDX4	157	EFNA4	229	HCN4	301	METAP1D	373	PTPN11	445	SRGN
14	AKR1C1	86	CEACAM3	158	EFNA5	230	HIF1A	302	METTL11A	374	PXN	446	ST3GAL1
15	AKR1C4	87	CEACAM6	159	EGR1	231	HIN1	303	MIA	375	RAB5A	447	ST8SIA1
16	AMIGO2	88	CEACAM7	160	ENPP5	232	HK1	304	MISRII	376	RAET1E	448	ST8SIA2
17	ANTXR2	89	CES2	161	ENPP7	233	HNF4A	305	MKK7	377	RALA	449	STAB2
18	ARG1	90	CES3	162	ENTK	234	HPGD	306	MLANA	378	RALB	450	STAMBP
19	ARNT	91	CFC1	163	ENTPD1	235	HPN	307	MOG	379	RCOR1	451	STC1
20	ARSA	92	CFL2	164	ENTPD2	236	HS3ST1	308	MSPR	380	REN	452	STUB1
21	ASAH2	93	CHAT	165	ESR2	237	HS3ST3B1	309	MYD88	381	RGMA	453	STX2
22	ASB17	94	CHI3L2	166	ETS1	238	HS3ST4	310	NAAA	382	RGMB	454	STX4
23	ASF1B	95	CHMP2B	167	EXTL3	239	HSPH1	311	NAGA	383	RGMC	455	STX6
24	ATCAY	96	CHST1	168	FABP6	240	ICK	312	NAPSA	384	RLN1	456	SULT1B1
25	BAI3	97	CHST15	169	FABP7	241	ICOS	313	NCK1	385	RNF113A	457	SULT1C2
26	BAMBI	98	CHST2	170	FABP8	242	IDS	314	NCR2	386	RNF31	458	SULT2A1
27	BCCIP	99	CHST3	171	FAM3A	243	IFNE	315	NCR3	387	RNF43	459	SULT2B1
28	BCL2	100	CHST4	172	FAM3D	244	IGSF11	316	NCSTN	388	RNLS	460	SULT4A1
29	BCL2L10	101	CIB1	173	FCAR	245	IGSF3	317	NDP	389	ROBO2	461	SUMF1
30	BCS1L	102	CITED2	174	FCER1A	246	IKBKG	318	Nectin-2	390	ROBO3	462	SUMF2
31	BIRC2	103	CLEC4C	175	FCGR1A	247	IL28RA	319	Nectin-4	391	RSPO2	463	SUMO1
32	BMP8A	104	CLEC4M	176	FCN1	248	INA	320	NEDD9	392	RSPO3	464	SUMO2
33	BOC	105	CLEC7A	177	FES	249	ING1	321	NENF	393	RTN4R	465	TBX18
34	BSG	106	CLIP1NTF	178	FGF3	250	INHA	322	NEUROG3	394	S100A13	466	TCF7L1
35	BTN3A1	107	CLSTN2	179	FGR	251	IPP2	323	NFASC	395	S100A2	467	TCN2
36	C1QTNF4	108	CNP	180	FHL1	252	IRF1	324	NFATC3	396	SARS2	468	TCPTP
37	C1QTNF5	109	CNTN5	181	FKBP4	253	IRF2	325	NFKB1	397	SBDS	469	TDP43
38	C1R	110	COL2A1	182	FLOT2	254	IRF4	326	NgR3	398	SCARF1	470	TFPI2
39	CA12	111	COMT	183	FLRT1	255	ISL1	327	NKp46	399	SCGN	471	TGM2
40	CA14	112	CPB1	184	FLRT2	256	ITGA1	328	NLGN1	400	SEMA3C	472	TGM4
41	CA4	113	CRELD2	185	FLRT3	257	ITGA11	329	NLGN2	401	SEMA3E	473	TGM7
42	CA5B	114	CRISP2	186	FMOD	258	ITGA2	330	NLGN4X	402	SEMA4A	474	THSD1
43	CA6	115	CRISP3	187	FOSB	259	ITGA3	331	NLRP2	403	SEMA4C	475	TINAGL1
44	CA8	116	CRK	188	FOXC2	260	ITGA4	332	NMNAT1	404	SEMA4D	476	TNR
45	CADM4	117	CSNK2A1	189	FOXJ1	261	ITGA5	333	NOS3	405	SEMA4G	477	TP63
46	CALR3	118	CSPG4	190	FOXM1	262	ITGB4	334	NOTCH3	406	SEMA5A	478	TPH1
47	CANT1	119	CTRC	191	FSTL4	263	ITGB7	335	NPDC1	407	SEMA6D	479	TPST2
48	CANX	120	CTSE	192	FUT11	264	JAG1	336	NPTN	408	SERINC3	480	TRAF3
49	CASP1	121	CTSV	193	FUT3/5	265	JAG2	337	NQO1	409	SERPINB2	481	TREM2
50	CASP2	122	CXADR	194	FUT7	266	JAM2	338	NR1H4	410	SH2B1	482	TTK
51	CASP9	123	CXCL17	195	FUT8	267	JAM3	339	NRXN3	411	SH2D1A	483	TXNL5
52	CASPR2	124	CXXC1	196	GAD1	268	JUN	340	NTHL1	412	SIGLEC10	484	UCHL3
53	CAV2	125	CYFIP2	197	Galectin-8	269	JUNB	341	NTRK1	413	SIGLEC11	485	UGT1A1
54	CBLN2	126	CYP2W1	198	GALNT10	270	KIRREL3	342	NUDT5	414	SIGLEC6	486	ULBP3
55	CCNE1	127	CYP4B1	199	GALNT3	271	KLK1	343	NUMB	415	SIRPG	487	USE1
56	CD164	128	CYP4F11	200	GATA1	272	KLK12	344	NUP85	416	SIRT1	488	VAMP1
57	CD2	129	CYP4F12	201	GATA2	273	KLK15	345	OLIG1	417	SIRT2	489	VAMP2
58	CD200R1	130	CYTH2	202	GATA5	274	KPNA2	346	OLIG2	418	SIRT3	490	VAPB
59	CD205	131	CYTH3	203	GBA3	275	KRT12	347	ONPEP	419	SIRT5	491	VDAC2
60	CD226	132	DAB2	204	GCK	276	KRT2	348	PAR1	420	SLAMF1	492	VIAAT
61	CD28	133	DAPK3	205	GIF	277	L1CAM	349	PAX3	421	SLC15A1	493	VISTA
62	CD300A	134	DAPP1	206	GLA	278	LGALS4	350	PAX4	422	SLITRK2	494	VTCN1
63	CD34	135	DAZL	207	GLB1	279	LGALS9	351	PCDH1	423	SLITRK4	495	WNT4
64	CD39L2	136	DBN1	208	GLI3	280	LILRA2	352	PCK1	424	SLITRK5	496	WNT9A
65	CD39L3	137	DCTN1	209	GLRX3	281	LILRB1	353	PGDS	425	SMAD2	497	WTAP
66	CD4	138	DCTN2	210	GNMT	282	LILRB2	354	PGLYRP4	426	SMAD3	498	WWP2
67	CD43	139	DCTN4	211	GPA33	283	LILRB4	355	PILRA	427	SMOC2	499	WWTR1
68	CD5	140	DCTN5	212	GPC1	284	LOXL2	356	PIR	428	SMPD1	500	XPNPEP2
69	CD58	141	DDR1	213	GPC2	285	LRRC32	357	PLA2G2A	429	SMPD3		
70	CD6	142	DDX21	214	GPC4	286	LRRC4	358	PLA2G4A	430	SNAP25		
71	CD7	143	DFF45	215	GRAP2	287	LRRC4C	359	PLXNA2	431	SNCB		
72	CD70	144	DMD	216	GRIA2	288	LRRC8D	360	PNPLA2	432	SORCS1		

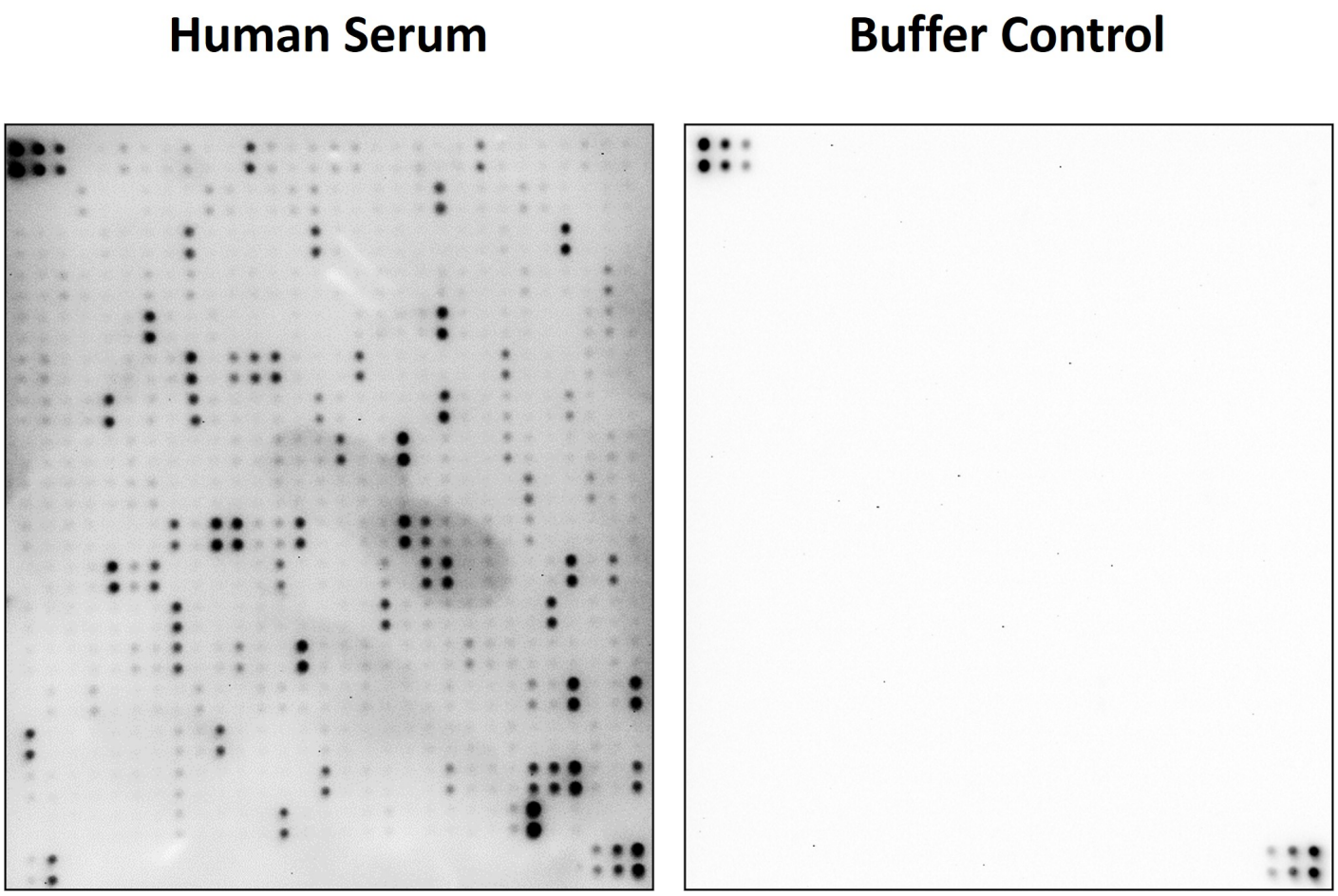
VII. Interpretation of Results:

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).



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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