RayBio® Label-based (L-Series) Mouse Antibody Array L-308 Membrane Kit

Patent Pending Technology
User Manual (Revised Apr 11, 2013)

For the simultaneous detection of the relative expression of 308 (L-308) mouse proteins in cell culture supernatants.

Cat# AAM-BLM-1-2 (2 Sample Kit)
Cat# AAM-BLM-1-4 (4 Sample Kit)

Please read manual carefully
before starting experiment

RayBiotech, Inc.
the protein array pioneer company

Your Provider for Excellent Protein Array Systems and Services

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RayBiotech, Inc

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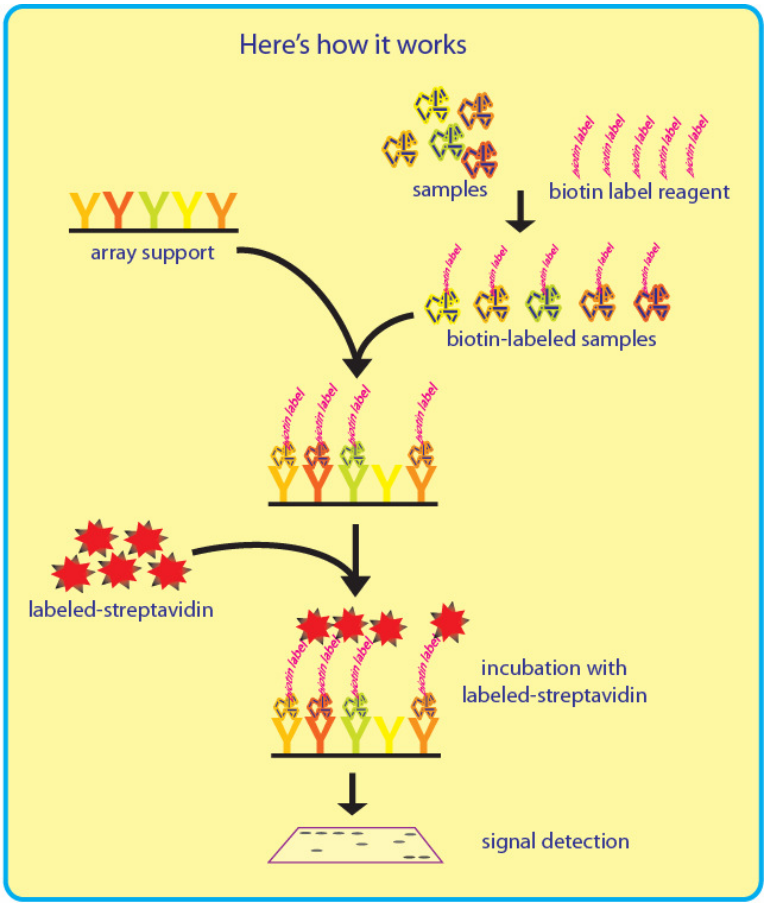
I. Introduction

Recent technological advances by RayBiotech have enabled the largest commercially available antibody array to date. With the RayBio® L-Series Mouse Antibody Array 308, researchers can now obtain a broad, panoramic view of cytokine expression. The expression levels of 308 mouse proteins can be simultaneously detected, including cytokines, chemokines, adipokine, growth factors, angiogenic factors, proteases, soluble receptors, soluble adhesion molecules and other proteins in cell culture supernatants.

The first step in using the Mouse L-308 is to biotinylate the primary amine of the proteins in the sample. The membrane arrays are then blocked, similar to 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	cat#: AAM-BLM-1-2	Cat#: AAM-BLM-1-4
B	Labeling Reagent	1 vials	2 vials
D	Stop Solution	1 vial (50 ul)	
E	RayBio® L-Series Mouse 308 Antibody Array Membranes	2 membranes L-308	4 membranes L-308
F	Blocking Buffer	2 vials (30 ml/ea)	4 vials (30 ml/ea)
I	500X HRP-Conjugated Streptavidin Concentrate	1 vial (100 ul)	2 vials (100 ul/ea)
K	Detection Buffer C	1 vial (10 ml)	2 vials (10 ml/ea)
L	Detection Buffer D	1 vial (10 ml)	2 vials (10 ml/ea)
Other Kit Components:		Plastic Sheets	

Box 2 (store at 4 °C):

ITEM	DESCRIPTION	Cat#: AAM-BLM-1-2	Cat#: AAM-BLM-1-4
A	Dialysis Vials	2 vials	4 vials
G	20X Wash Buffer 1 Concentrate	1 vial (30 ml)	1 vials (30 ml/ea)
H	20X Wash Buffer 2 Concentrate	1 vial (30 ml)	1 vials (30 ml/ea)
J	Spin Columns	2 columns	4 columns
N/A	Plastic Incubation Trays (w/lid)	2 trays	4 trays
M	Floating Dialysis Rack	1 rack	

B. Additional Materials Required

- 1X PBS, pH=8.0
- Shaker
- 2~5 ml tube
- 50 ml conical collection tubes
- Distilled water
- Kodak X-Omat™ AR film (REF 165 1454) and film processor or Chemiluminescence imaging system
- large beaker
- stir plate
- Eppendorf tube

III. Overview and General Considerations

A. Handling 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.

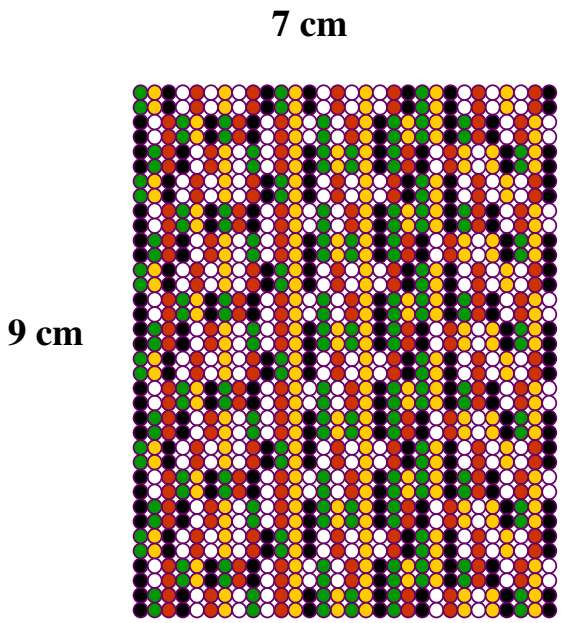
B. Incubation

- Completely cover membranes with sample or buffer during incubation and cover Plastic Incubation Tray with 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 in page 10 (sample incubation) or step 7 in page 11 (HRP-Conjugated Streptavidin incubation) may be done at 4 °C for overnight.

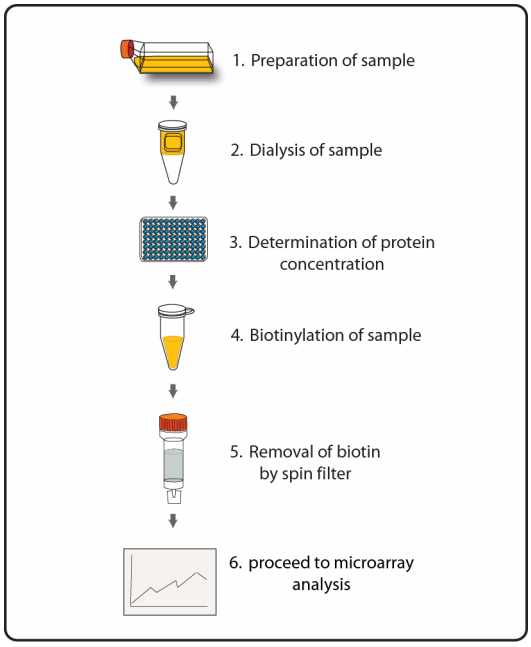
IV. Protocol

Layout of L-308 Array Membrane



30 columns x 30 rows

Assay Diagram



A. Preparation of Samples

1). Seed cells at a density of $1x10^6$ cells in 100 mm tissue culture dishes.*

2) Culture in complete culture medium for ~24–48 hours.**

3) Replenish with serum-free or low-serum medium, such as 0.2% FCS/FBS, and then re-incubate cells for ~48 hours***

4) Collect the cell culture supernatant and centrifuge at 1,000 g for 10 minutes and store in ≤1 ml aliquots at -80 °C until needed.

5) Measure the total wet weight of the cultured cells in the pellet and/or culture dish. 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). Normalization can also be done between arrays by determining the total protein concentration using a total protein assay (RayBiotech recommends the Pierce BCA Protein Assay Kit, cat# 23227).

Note: * The density of cells per dish used is dependent on the cell type. More or less cells may be required but should be determined empirically.

** Optimal culture time may be different and depends on cell lines, treatment conditions, and other factors.

***Bovine serum proteins produce detectable signals on the RayBio® L-Series membrane arrays at concentrations as low as 0.2%. When testing serum-containing media, it is recommended test an uncultured media blank sample for comparison with sample results.

B. Dialysis of Sample

Note: Samples must be dialyzed prior to biotin-labeling (Steps 5–7).

1. Prepare dialysis buffer (1X PBS) by dissolving 0.6 g KCl, 24 g NaCl, 0.6 g KH_2PO_4 and 3.45 g Na_2HPO_4 in 2500 ml de-ionized or distilled water. Adjust to a pH of 8.0 with 1M NaOH and adjust final volume to 3000 ml with de-ionized or distilled water.

2. Load each sample into a separate Dialysis Vials (Item A), 2.5-3.0 ml of sample per vial for dialyzing. Carefully place all Dialysis Vials into the Floating Rack.

3. Place the Floating Rack into ≥500 ml dialysis buffer in a large beaker. Place beaker on a stir plate and dialyze for at least 3 hours at 4 °C, occasionally gently stirring the dialysis buffer. Then exchange the dialysis buffer with fresh buffer and repeat dialysis for at least 3 hours at 4 °C. Transfer dialyzed samples into a clean eppendorf tube. Centrifuge dialyzed samples for 5 minutes at 10,000 rpm to remove any particulates or precipitates and then transfer and combine each sample into one clean eppendorf tube. Mix well by gently pipetting.

Note: The sample volume may change during dialysis.

Note: Dialysis procedure may proceed overnight.

C. Biotin-labeling of Sample

Avoid contamination with any solution containing amines (i.e., Tris, glycine) as well as azides during the biotinylation process.

4. Immediately before use, prepare 1X Labeling Reagent by briefly centrifuging down the Labeling Reagent vial (Item B) and add 100 µl 1X PBS (pH=8.0) into the vial. Pipette up and down or vortex briefly to dissolve the powder.

5. Add an appropriate amount* of 1X Labeling Reagent into the tube containing the sample and immediately mix the reaction solution. Incubate the reaction solution at room temperature for 30 minutes with gentle shaking. Gently tap the tube to mix the reaction solution every 5 minutes.

* Use 7.2 µl of 1X Labeling Reagent for labeling 1 mg of total protein in samples. For example, if sample's total protein concentration is 0.5 mg/ml you need to add 10.8 µl 1X Labeling Reagent to 3 ml dialyzed sample.

Note: The total protein concentration needs to be determined if the sample volume changes after dialysis or if the total protein concentration was determined before the dialysis step.

6. Add 5 µl Stop Solution (Item D) into the reaction solution and then use the Spin Column (Item J) to remove any unbound biotin.

a). Twist off the bottom closure of the Spin Column and loosen the cap (but keep the cap on). Place the Spin Column into a 50 ml conical collection tube.

b). Centrifuge the Spin Column at 1,000 g for 3 minutes to remove storage solution.

Note: The resin should appear compacted after centrifugation.

c). Add 5 ml 1X PBS (pH=8.0) into the Spin Column and centrifuge at 1,000 x g for 3 minutes to remove the 1X PBS. Repeat an

additional 2 times to wash the Spin Column.

d). Place the Spin Column in a new 50 ml conical collection tube and slowly load 3.5 ml of sample to the center of the compact resin bed.

Note: The maximal sample volume is 4 ml for each Spin Column. Do not load over 4 ml of sample into a Spin Column.

e). Centrifuge the Spin Column at 1,000 x g for 3 minutes. The sample should filter through the resin and deposit into the 50 ml conical collection tube. Store at -80 ^{0}C until needed. Discard the Spin Column after use.

D. Blocking and Incubation

7. 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.

8. Add 8 ml of Blocking Buffer (Item F) to each membrane and cover with the lid. Incubate at room temperature with gentle shaking for 1 hour.

9. Aspirate Blocking Buffer from each tray. Add 8 ml of diluted* or undiluted sample onto each membrane and cover with the lid. Incubate at room temperature with gentle shaking for 2 hours.

Note: 1). It is recommended to use 8 ml of 5-fold diluted biotin-labeled cell culture supernatant. Dilute sample using Blocking Buffer.

Note: 2). The concentration of sample used depends on the abundance of 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: 3). Incubation may be done at room temperature with gentle shaking for 2 hours or overnight at 4°C.

10. Dilute 20X Wash Buffer 1 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.

11. Aspirate the 1X Wash Buffer 1 from each tray. Dilute 20X Wash Buffer 2 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.

12. Aspirate the 1X Wash Buffer 2 from each tray. Dilute the 500X HRP-Conjugated Streptavidin with Blocking Buffer to prepare

the 1X HRP-Conjugated Streptavidin. Add 8 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.

13. Incubate at room temperature with gentle shaking for 2 hours.

Note: incubation may be done at 4 ^{0}C for overnight.

14. Wash as directed in steps 10 and 11.

E. Detection

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

15. 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 ("-" symbol is marked in 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 on to 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.

16. 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.

17. 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 exposure time (eg, 5–30 seconds). If the signals are too weak, increase exposure time (eg, 5–20 min or overnight). Or re-incubate membranes overnight with 1X HRP-Conjugated Streptavidin, and repeat detection on the second day.

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

V. Antibody Array Map

RayBio® L-Series Human Antibody Array 308 Maps – if needed, larger versions of these maps can obtained by contacting technical support at 770-729-2992 or techsupport@raybiotech.com.

RayBio® Biotin Label-based Mouse Antibody Array I Map – Larger versions of this can obtained by contacting technical support at 770-729-2992.

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
P-1a	P-2a	P-3a	Blank	Blank	NEG	NEG	Blank	9	10	11	12	13	Blank	Blank	Blank	Blank	Blank	Blank	Blank	NEG	NEG	Blank	24	25	26	27	28	29	30
P-1a	P-2a	P-3a	Blank	Blank	NEG	NEG	Blank	9	10	11	12	13	Blank	Blank	Blank	Blank	Blank	Blank	Blank	NEG	NEG	Blank	24	25	26	27	28	29	30
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RayBio® L-Series Mouse Antibody Array 308 (L-308) List

Number	Name	Number	Name	Number	Name	Number	Name
1	P-1a	61	CCL8 / MCP-2	121	Fas Ligand	181	Blank
2	P-2a	62	CCR10	122	FCrRIIB / CD32b	182	Blank
3	P-3a	63	CCR3	123	FGF R3	183	Blank
4	Blank	64	CCR4	124	FGF R4	184	Blank
5	Blank	65	CCR6	125	FGF R5 beta	185	Blank
6	NEG	66	CCR7	126	FGF-21	186	Blank
7	NEG	67	CCR9	127	Fit-3 Ligand	187	Blank
8	Blank	68	CD11b	128	FLRG (Follistatin)	188	Blank
9	6Ckine	69	CD14	129	Follistatin-like 1	189	IL-1 R9
10	Activin A	70	CD195	130	Fractalkine	190	IL-1 RI
11	Activin C	71	CD27 / TNFRSF7	131	Frizzled-1	191	IL-1 RII
12	Activin RIB / ALK-4	72	CD27 Ligand / TNFSF7	132	Frizzled-6	192	IL-2
13	Adiponectin / Acrp30	73	CD30 L	133	Frizzled-7	193	IL-2 R alpha
14	Blank	74	Blank	134	Blank	194	Blank
15	Blank	75	Blank	135	Blank	195	Blank
16	Blank	76	CD30	136	Galectin-3	196	Blank
17	Blank	77	CD40	137	G-CSF	197	Blank
18	Blank	78	CD40 Ligand / TNFSF5	138	GDF-1	198	Blank
19	Blank	79	Cerberus 1	139	GDF-3	199	Blank
20	Blank	80	Chordin-Like 2	140	GDF-5	200	Blank
21	NEG	81	Coagulation Factor III / Tissue Factor	141	GDF-8	201	Blank
22	NEG	82	Common gamma Chain / IL-2 R gamma	142	GDF-9	202	Blank
23	Blank	83	CRG-2	143	GFR alpha-2 / GDNF R alpha-2	203	Blank
24	AgRP	84	Cripto	144	GFR alpha-3 / GDNF R alpha-3	204	IL-2 R beta
25	ALCAM	85	Crossveinless-2	145	GFR alpha-4 / GDNF R alpha-4	205	IL-3
26	Angiopoietin-like 2	86	Cryptic	146	GITR	206	IL-3 R alpha
27	Angiopoietin-like 3	87	Csk	147	GITR Ligand / TNFSF18	207	IL-3 R beta
28	AR (Amphiregulin)	88	CTACK	148	Glut2	208	IL-4
29	Artemin	89	CTLA-4 / CD152	149	GM-CSF	209	IL-4 R
30	Axl	90	CXCL14 / BRAK	150	Granzyme B	210	IL-5
31	Blank	91	CXCL16	151	Granzyme D	211	Blank
32	Blank	92	CXCR2 / IL-8 RB	152	Granzyme G	212	Blank
33	Blank	93	CXCR3	153	Gremlin	213	Blank
34	Blank	94	CXCR4	154	Growth Hormone R	214	Blank
35	Blank	95	CXCR6	155	HGF R	215	Blank
36	Blank	96	DAN	156	HGF	216	NEG
37	Blank	97	Decorin	157	HVEM / TNFRSF14	217	NEG
38	Blank	98	DKK-1	158	ICAM-1	218	Blank
39	b FGF	99	Dkk-3	159	ICAM-2 / CD102	219	IL-5 R alpha
40	B7-1/CD80	100	Dkk-4	160	ICAM-5	220	IL-6
41	BAFF R / TNFRSF13C	101	DPPIV / CD26	161	ICK	221	IL-6 R
42	BCMA / TNFRSF17	102	DR3 / TNFRSF25	162	IFN-alpha / beta R1	222	IL-7
43	beta-Catenin	103	Dtk	163	IFN-alpha / beta R2	223	IL-7 R alpha
44	Blank	104	Blank	164	Blank	224	Blank
45	Blank	105	Blank	165	Blank	225	Blank
46	Blank	106	EDAR	166	IFN-beta	226	Blank
47	Blank	107	EGF R	167	IFN-gamma	227	Blank
48	Blank	108	EG-VEGF / PK1	168	IFN-gamma R1	228	Blank
49	Blank	109	Endocan	169	IGFBP-1	229	Blank
50	Blank	110	Endoglin / CD105	170	IGFBP-2	230	Blank
51	Blank	111	Endostatin	171	IGFBP-3	231	NEG
52	Blank	112	Eotaxin	172	IGFBP-5	232	NEG
53	Blank	113	Eotaxin-2	173	IGFBP-6	233	Blank
54	BLC	114	Epigen	174	IGFBP-rp1 / IGFBP-7	234	IL-9
55	BTC (Betacellulin)	115	Epiregulin	175	IGF-I	235	IL-9 R
56	Cardiotrophin-1	116	Erythropoietin (EPO)	176	IGF-II	236	IL-10
57	CCL1 / I-309 / TCA-3	117	E-Selectin	177	IL-1 alpha	237	IL-10 R alpha
58	CCL28	118	FADD	178	IL-1 beta	238	IL-11
59	CCL4 / MIP-1 beta	119	FAM3B	179	IL-1 R4 / ST2	239	IL-12 p40/p70
60	CCL7 / MCP-3 / MARC	120	Fas / TNFRSF6	180	IL-1 R6 / IL-1 R rp2	240	IL-12 p70

RayBio® L-Series Mouse Antibody Array 308 (L-308) List continued

Number	Name	Number	Name	Number	Name	Number	Name
241	Blank	301	LIX	361	Spinesin Ectodomain	421	Blank
242	Blank	302	LRP-6	362	TACI / TNFRSF13B	422	Blank
243	Blank	303	L-Selectin	363	TARC	423	Blank
244	Blank	304	Lungkine	364	TCA-3	424	Blank
245	Blank	305	Lymphotactin	365	TCCR / WSX-1	425	Blank
246	Blank	306	Lymphotoxin beta R / TNFRSF3	366	TECK	426	NEG
247	Blank	307	MAdCAM-1	367	TFPI	427	NEG
248	Blank	308	MCP-1	368	TGF-beta 1	428	Blank
249	IL-12 R beta 1	309	MCP-5	369	TGF-beta 2	429	Urokinase
250	IL-13	310	M-CSF	370	TGF-beta 3	430	VCAM-1
251	IL-13 R alpha 2	311	MDC	371	TGF-beta RI / ALK-5	431	VE-Cadherin
252	IL-15	312	MFG-E8	372	TGF-beta RII	432	VEGF
253	IL-15 R alpha	313	MFRP	373	Thrombospondin	433	VEGF R1
254	Blank	314	Blank	374	Blank	434	Blank
255	Blank	315	Blank	375	Blank	435	Blank
256	Blank	316	MIG	376	Thymus Chemokine-1	436	VEGF R2
257	Blank	317	MIP-1 alpha	377	Tie-2	437	VEGF R3
258	Blank	318	MIP-1 gamma	378	TIMP-1	438	VEGF-B
259	Blank	319	MIP-2	379	TIMP-2	439	VEGFC
260	Blank	320	MIP-3 alpha	380	TIMP-4	440	VEGF-D
261	Blank	321	MIP-3 beta	381	TL1A / TNFSF15	441	WIF-1
262	Blank	322	MMP-2	382	TLR1	442	WISP-1 / CCN4
263	Blank	323	MMP-3	383	TLR2	443	Blank
264	IL-16	324	MMP-9	384	TLR3	444	NEG
265	IL-17	325	MMP-12	385	TLR4	445	NEG
266	IL-17BR	326	MMP-14 / LEM-2	386	TMEFF1 / Tomoregulin-1	446	Blank
267	IL-17C	327	MMP-24 / MT5-MMP	387	TNF RI / TNFRSF1A	447	Blank
268	IL-17D	328	Neuregulin-3 / NRG3	388	TNF RII	448	P-3b
269	IL-17E	329	Neurturin	389	TNF-alpha	449	P-2b
270	IL-17F	330	NGF R / TNFRSF16	390	TNF-beta / TNFSF1B	450	P-1b
271	IL-17R	331	NOV / CCN3	391	Blank		
272	IL-17RC	332	Osteoactivin / GPNMB	392	Blank		
273	IL-17RD	333	Osteopontin	393	Blank		
274	IL-18 R alpha/IL-1 R5	334	Osteoporotegerin	394	Blank		
275	IL-20	335	OX40 Ligand / TNFSF4	395	Blank		
276	IL-20 R alpha	336	PDGF C	396	Blank		
277	IL-21	337	PDGF R alpha	397	Blank		
278	IL-21 R	338	PDGF R beta	398	Blank		
279	IL-22	339	Pentraxin3 / TSG-14	399	TPO		
280	IL-22BP	340	PF-4	400	TRAIL / TNFSF10		
281	IL-23	341	PlGF-2	401	TRAIL R2 / TNFRSF10B		
282	IL-23 R	342	Progranulin	402	TRANCE / TNFSF11		
283	IL-24	343	Prolactin	403	TREM-1		
284	Blank	344	Blank	404	Blank		
285	Blank	345	Blank	405	Blank		
286	IL-27	346	P-Selectin	406	TROY		
287	IL-28 / IFN-lambda	347	RAGE	407	TSLP		
288	IL-31	348	RANTES	408	TSLP R		
289	IL-31 RA	349	RELM beta	409	TWEAK / TNFSF12		
290	Insulin	350	Resistin	410	TWEAK R / TNFRSF12		
291	Integrin beta 2 / CD18	351	S100A10	411	Ubiqultin		
292	I-TAC	352	SCF	412	uPAR		
293	KC	353	SCF R / c-kit	413	Blank		
294	Kremen-1	354	SDF-1	414	Blank		
295	Kremen-2	355	Serum Amyloid A1	415	Blank		
296	Lefty-1	356	Shh-N	416	Blank		
297	Leptin R	357	SIGIRR	417	Blank		
298	LEPTIN(OB)	358	SLPI	418	Blank		
299	LIF	359	Soggy-1	419	Blank		
300	LIGHT / TNFSF14	360	SPARC	420	Blank		

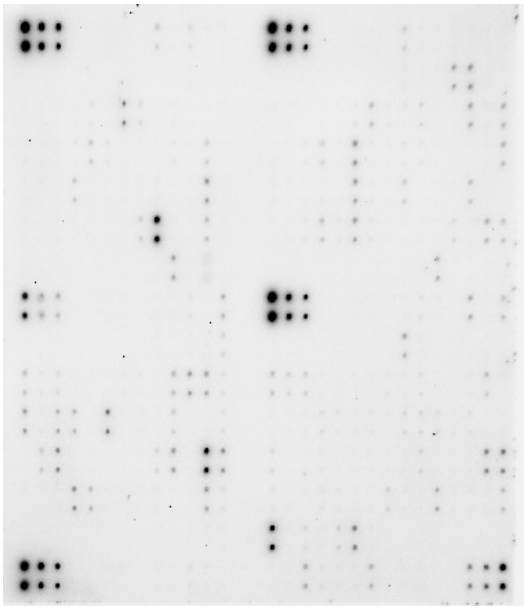
VI. Interpretation of Results

The following images show the RayBio® L-Series Mouse Antibody Array 308 captured using a chemiluminescence imaging system (UVP BioImaging Systems). To obtain optimal results, 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. Anti-HRP (P-1a, P-2a, P-3a) and anti-streptavidin (P-1b, P-2b, P-3b) will produce positive control signals, which can be used to identify the orientation and help normalize the results from different arrays being compared.

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.

The RayBio® Analysis Tool is a program specifically designed for analysis of RayBio® L-Series Mouse Antibody Antibody Arrays. This tool will not only assist in compiling and organizing your data, but also reduces your calculations to a "copy and paste." Call RayBiotech, Inc. at 770-729-2992 for ordering information.

L-308 image



VII. Troubleshooting Guide

Problem	Cause	Recommendation
Weak signal or no signal	1. Taking too much time for detection.	1. The whole detection process must be completed in 30 min.
	2. Film developer does not work properly.	2. Fix film developer.
	3. Did not mix HRP-streptavidin well before use.	3. Mix tube containing HRP-Conjugate Streptavidin well before use since precipitates may form during storage.
	4. Sample is too dilute.	4. Increase sample concentration
	5. Other.	1.Check if there were any contamination with any solution containing amines in biotin-labeling step
		2. Slightly increase HRP concentrations.
		3. Work as quickly as possible after mix Detection Buffer C and D
		4. Expose film for overnight to detect weak signal.
Uneven signal	1. Bubbles formed during incubation.	1. Remove bubbles during incubation.
	2. Membranes were not completely covered by solution.	2. Completely cover membranes with solution.
High background	1. Exposure time is too long.	1. Decrease exposure time.
	2. Membranes dry out during experiment.	2. Completely cover membranes with solution during experiment. Cover tray w/ lid
	3. Sample is too concentrated.	3. Dilute sample.

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