RayBio® Biotin Label-based Mouse Antibody Array I

For the Simultaneous Detection of the Expression Levels of 308 Mouse Proteins in Cell Culture Supernates.

User Manual
(Revised Apr 1, 2009)

(Cat#: AAM-BLM-1-2;
AAM-BLM-1-4)

RayBiotech, Inc.

As the Protein Array Pioneer Company,
Excellence and Innovation Is Our Goal

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RayBiotech, Inc

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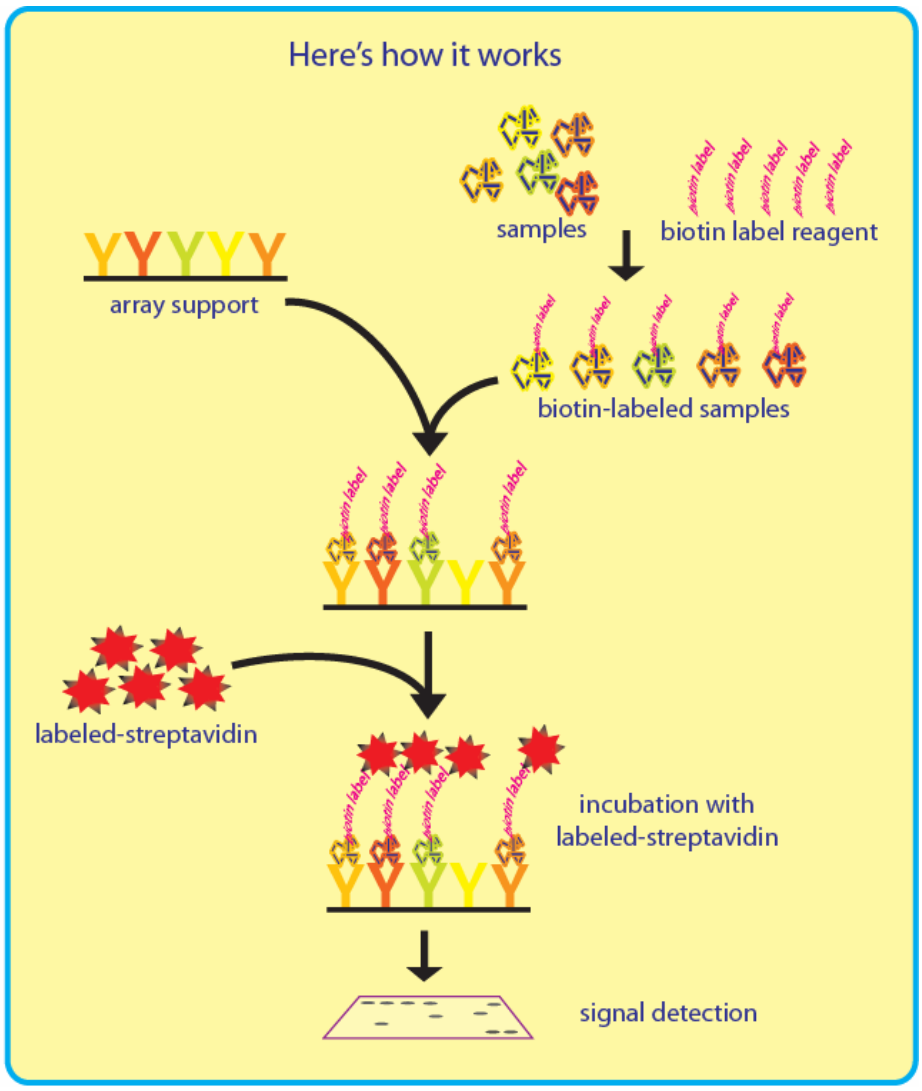
I. Introduction

Recent technological advances by Raybiotech have enabled the largest commercially available antibody array to date. With the L Series 308, researchers can now obtain a broad, panoramic view of cytokine expression. The expression levels of 308 mouse target proteins can be simultaneously detected, including cytokines, chemokines, adipokine, growth factors, angiogenic factors, proteases, soluble receptors, sobuble adhesion molecules and other proteins in cell culture supernate and serum. Furthermore, an internal control is used to monitor the whole process including biotin-labeling, so this massive array will accurately reflect the available cytokines in your sample.

The first step in using the RayBio® Biotin label-based mouse antibody array 1 is to biotinylate the primary amine of the proteins in cell culture supernates. The biotin-labeled sample is then added onto array membrane and incubated at room temperature. After incubation with HRP-streptavidin, the signals can be visualized by chemiluminescence.



II. Materials Provided

Upon receipt, the Box 1 should be stored at -20 °C and Box 2 should be stored at 4 °C. Please use within 6 months from the date of shipment. After initial use, the Blocking Buffer, Stop solution, HRP-Conjugated Streptavidin, Detection Buffer C and D should be stored at 4 °C to avoid repeated freeze-thaw cycles. The Array Membrane and Internal Control should be kept at -20 °C.

Box-1 (store at -20 °C):

- Labeling Reagent (Item B, 1 tube for 2 array membranes , and 2 for 4 array membranes)
- Internal control (Item C, 1 tube for 2 array membranes , and 2 for 4 array membranes)
- Stop Solution (Item D, 50 µl)
- RayBio® Biotin label-based mouse antibody array I (Item E, 2 for Cat#: AAM-BLM-1-2**,** and 4 for Cat#: AAM-BLM-1-4)
- Blocking Buffer (Item F, 30 ml for each bottle, 2 bottles for 2 array membranes , and 4 for 4 array membranes)
- 500X HRP-Conjugated Streptavidin Concentrate (Item I, 100 µl)
- Detection Buffer C (Item K, 5 ml for 2 membranes, and 10 ml for 4 membranes)
- Detection Buffer D (Item L, 5 ml for 2 membranes, and 10 ml for 4 membranes)
- Plastic sheet

Box 2 (store at 4 °C):

- Dialysis tube and Floating Rack (Item A, 2 tubes for 2 array membranes, and 4 for 4 array membranes, dialysis tube is from EMD product)
- 20X Wash Buffer I (Item G, 30ml)
- 20X Wash Buffer II (Item H, 30ml)
- Spin Column (Item J, 2 columns for 2 array membranes, and 4 for 4 array membranes)
- Plate (2 plates for 2 array membranes, and 4 for 4 array membranes)

III. Additional Materials Required

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

IV. Overview and General Considerations

A. Handling Array Membranes

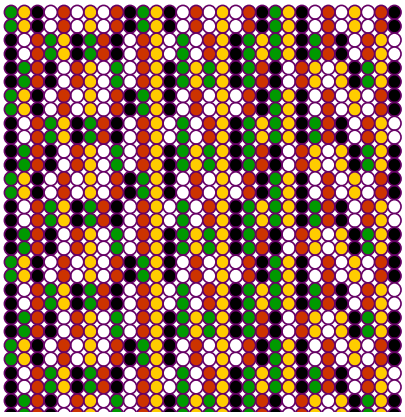
- Always use forceps to handle membranes, and grip the membranes by the edges only.
- Never allow array membranes to dry during experiments.
- Avoid touch Array membrane by hand, tips or any sharp tools.

B. Incubation

- Completely cover membranes with sample or buffer during incubation, and cover eight-well 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-streptavidin incubation) may be done at 4 °C for overnight.

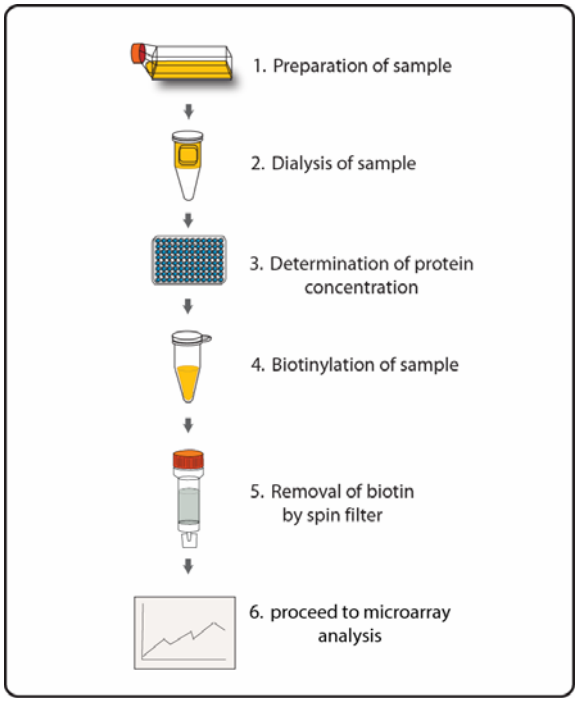
V. Protocol

Layout of Array Membrane



30 columns x 30 rows

Assay Diagram



A. Preparation of Samples

The cell culture supernates can be prepared in the following conventional manner:

To prepare cell culture supernates (cell conditioned media), cells are plated in 100 mm tissue culture dishes at a density of $1x10^6$ cells* per dish. The cells are then cultured with complete culture medium for 24~48 hours**. The complete culture medium is replaced with lower serum medium such as 0.2% FCS serum, and then the cells are cultured for 48 hour** again once more. The supernates are collected, centrifuged at 1,000 g, aliquoted and stored at –80 ^{0}C until use. Meanwhile, the cells are also collected and the total protein concentration is determined. For each sample it is recommended that the concentration of the supernates and cell lysate (help nomalize different cell culture supernates) be determined using a total protein assay (BCA Protein Assay Kit, Pierce, Prod# 23227).

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

*** Culture times may vary depending on your cell lines and research.*

B. Dialysis of Sample

The cell culture supernates should be dialyzed with a Dialysis tube (Item A) before the biotin-labeling procedure. We recommend loading 2.5~3.0 ml cell culture supernates into a dialyzer and dialyzing with at least 2,000 ml 1X PBS buffer (pH = 8) at 4 ^{0}C. Change the 1X PBS buffer and dialyze again. Allow at

least 3 h for each dialysis step, stir gently. The sample total volume may be changed after dialysis.

Note: Preparation of 1X PBS, pH=8.0, 1.0 g KCl, 40 g NaCl, 1.0 g KH_2PO_4, 5.75 g Na2HPO4 dissolve in 4,500 ml deionized or distilled water. Adjust pH=8.0 with 1M NaOH and adjust final volume to 5,000 ml with deionized or distilled water.

C. Biotin-labeling Sample

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

1. Briefly spin down Internal Control tube (Item C*)* before use. Add 100 µl 1X PBS, pH=8.0 into the Internal Control tube, pipette up and down to dissolve the powder. Transfer 2 ml dialyzed sample into a new tube. Add 40 µl prepared Internal Control into the tube. Mix well.

2. Immediately before use, briefly spin down the Labeling Reagent tube (Item B). Add 100 µl 1X PBS into the tube, pipette up and down or vortex to dissolve the powder to prepare 1X Labeling Reagent solution.

3. Add an appropriate amount* of prepared Labeling Reagent into above tube with sample in step 2, mix well immediately. Incubate the reaction solution at room temperature for 30 min with gentle shaking. Gently tap the tube to mix the reaction solution every 5 min.

* *7.2 µl of 1X Labeling Reagent for labeling 1 mg total protein*

in supernates .

Note: You need to re-calculate the total protein concentration if cell culture supernatet volume is changed after dialysis and you measure the total protein concentration before dialysis step.

4. Add 5 µl Stop Solution into the above reaction solution and then use the spin column to remove free biotin.

a). Twist off the spin column's bottom closure and loosen the cap. Place the column into a 50 ml collection tube.

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

Note: The resin will appear compacted after centrifugation.

c). Add 5 ml 1X PBS into column, centrifuge at 1,000 g for 3 minutes to 1X PBS. Repeat additional 2 times to wash the column.

d). Place the column in a new collection tube, slowly load the sample to the center of the compact resin bed.

e). Centrifuge the column at 1,000 g for 3 minutes to collect sample. Stored at –80 ^{0}C until testing. Discard column after use.

D. Blocking and Incubation

1. Place each membrane into the provided tray ("-" mark is on the antibody printed side).

   Note: *The printed side should be facing upward.*

2. Add 8 ml Blocking Buffer and incubate at room temperature for 1 hour to block membranes.

3. Decant Blocking Buffer from each container. Add 8 ml of sample into each array membrane, and cover with the lid. Incubate at room temperature with gentle shaking for 2 hours. Dilute sample using Blocking Buffer.

   Note: 1). We recommended using 8 ml of 5-fold diluted cell culture supernates which have been biotin-labeled. Dilute sample using Blocking Buffer.

   Note: 2). The amount of sample used depends on the abundance of protein. More of the sample can be used if signals are too weak. If signals are too strong, the sample can be diluted further.

   Note: 3). Incubation may be done at room temperature for 2 hours. Over night at 4°C

4. Decant the samples from each container, and wash 3 times with 20 ml of 1X Wash Buffer I at room temperature with shaking. 5

min per wash. Dilute 20X Wash Buffer I with deionized or distilled water.

5. Decant the 1X Wash Buffer I from each container. Wash 3 times with 20 ml of 1X Wash Buffer II at room temperature.

6. Decant the 1X Wash Buffer II. Add 8 ml of 500 fold diluted HRP-conjugated streptavidin (e.g. add 36 µl of HRP-conjugated streptavidin to 18 ml of Blocking Buffer) to each membrane.

   Note: *Mix tube containing 500X HRP-Conjugated Streptavidin well before use since precipitation may form during storage.*

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

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

8. Wash as directed in steps 4 and 5.

E. Detection

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

1. Add 4.2 ml of Detection Buffer **C** and 4.2 ml of Detection Buffer D into a tube (for detecting 2 membranes); Mix both solutions; Drain off excess wash buffer. Place membrane protein side up ("-" mark is on the protein side top left corner) on a clean plastic plate or its cover (provided in the kit). Pipette 4ml of the mixed

Detection Buffer on to each membrane and incubate at room temperature with shaking for 2 minutes. Ensure that the detection mixture is evenly covering the membrane without any air bubbles.

2. Gently place the membrane with forceps, protein side up, on a piece of plastic sheet ("-" mark is on the protein side top left corner). Cover the array with another piece of plastic sheet. Gently smooth out any air bubbles. Avoid using pressure on the membrane. Work as quickly as possible.

3. 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.

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

VI. Interpretation of Results:

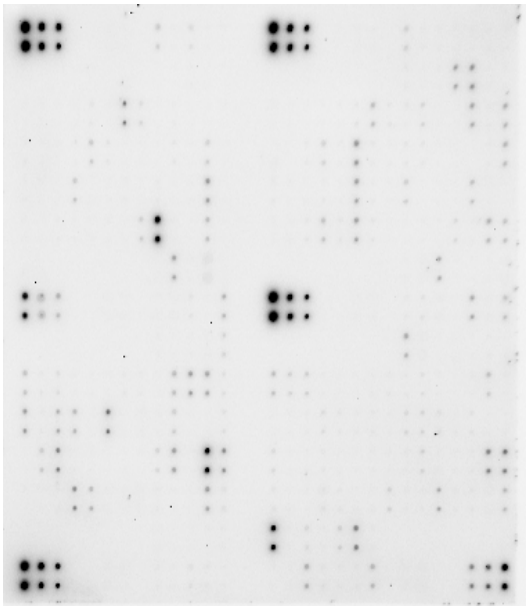
The following figure shows the RayBio® **Biotin-label-based Mouse Antibody Array 1** probed with cell culture supernates. The image was captured using a chemiluminescene imaging system. One important parameter is the background signal. To obtain the

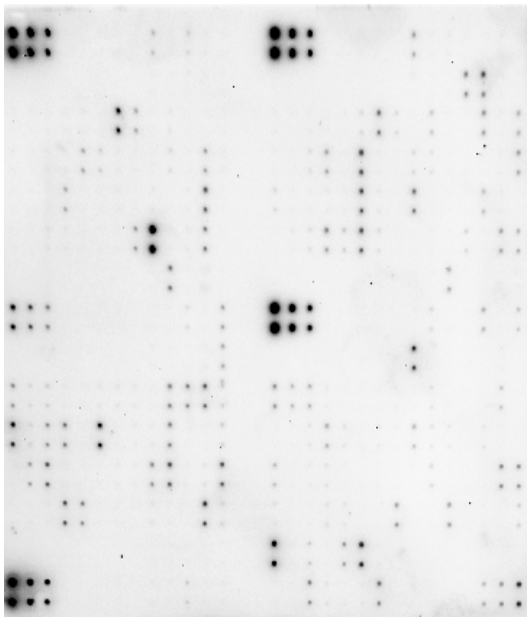
best results, we suggest that several exposures be attempted. By comparing the signal intensities, relative expression levels of target proteins can be made. The intensities of signals can be quantified by densitometry. A biotinylated protein and internal control will produce positive control signals, 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® Biotin Label-based 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.

Sample-1 **Sample-2**





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

P-1a	P-1b	P-1c	Blank	Blank	NEG	NEG	Blank	9	10	11	12	13	Blank	Blank	P-2a	P-2b	P-2c	Blank	Blank	NEG	NEG	Blank	24	25	26	27	28	29	30
P-1a	P-1b	P-1c	Blank	Blank	NEG	NEG	Blank	9	10	11	12	13	Blank	Blank	P-2a	P-2b	P-2c	Blank	Blank	NEG	NEG	Blank	24	25	26	27	28	29	30
Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	39	40	41	42	43	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	54	55	56	57	58	59	60
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IC-1a	IC-1b	IC-1c	Blank	Blank	NEG	NEG	Blank	219	220	221	222	223	Blank	Blank	P-3a	P-3b	P-3c	Blank	Blank	NEG	NEG	Blank	234	235	236	237	238	239	240
IC-1a	IC-1b	IC-1c	Blank	Blank	NEG	NEG	Blank	219	220	221	222	223	Blank	Blank	P-3a	P-3b	P-3c	Blank	Blank	NEG	NEG	Blank	234	235	236	237	238	239	240
Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	249	250	251	252	253	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	264	265	266	267	268	269	270
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P-4a	P-4b	P-4c	Blank	Blank	NEG	NEG	Blank	429	430	431	432	433	Blank	Blank	436	437	438	439	440	441	442	Blank	NEG	NEG	Blank	Blank	IC-2c	IC-2b	IC-2a

RayBio® Biotin Label-based Mouse Antibody List

1	P-1a	61	CCL8 / MCP-2	121	Fas Ligand	181	Blank
2	P-1b	62	CCR10	122	FCrRIIB / CD32b	182	Blank
3	P-1c	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	CRP	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	P-2a	76	CD30	136	Galectin-3	196	Blank
17	P-2b	77	CD40	137	G-CSF	197	Blank
18	P-2c	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	IC-1a
32	Blank	92	CXCR2 / IL-8 RB	152	Granzyme G	212	IC-1b
33	Blank	93	CXCR3	153	Gremlin	213	IC-1c
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	P-3a
47	Blank	107	EGF R	167	IFN-gamma	227	P-3b
48	Blank	108	EG-VEGF / PK1	168	IFN-gamma R1	228	P-3c
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® Biotin Label-based Mouse Antibody List...continued

241	Blank	301	LIX	361	Spinesin Ectodomain	421	P-4a
242	Blank	302	LRP-6	362	TACI / TNFRSF13B	422	P-4b
243	Blank	303	L-Selectin	363	TARC	423	P-4c
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	IC-2c
269	IL-17E	329	Neurturin	389	TNF-alpha	449	IC-2b
270	IL-17F	330	NGF R / TNFRSF16	390	TNF-beta / TNFSF1B	450	IC-2a
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		

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.Reduce blocking concentration by diluting in 1X Wash Buffer II.
		2. Slightly increase HRP concentrations.
		3. Slightly increase biotinylate-antibody concentrations.
		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 to x-ray file is too long.	1. Decrease exposure time.
	2. Membranes were allowed to dry out during experiment.	2. Completely cover membranes with solution during experiment.
	3. Sample is too concentrated.	3. Use more diluted sample.

VIII. Reference List

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