RayBio® Biotin Label-based Human Obesity Antibody Array 1

For the Simultaneous Detection of the Expression Levels of 182 Human Proteins in Cell Culture Supernates.

User Manual
(Revised May 28, 2010)

**(Cat#: AAH-BLM-ADI-1-2;
AAH-BLM-ADI-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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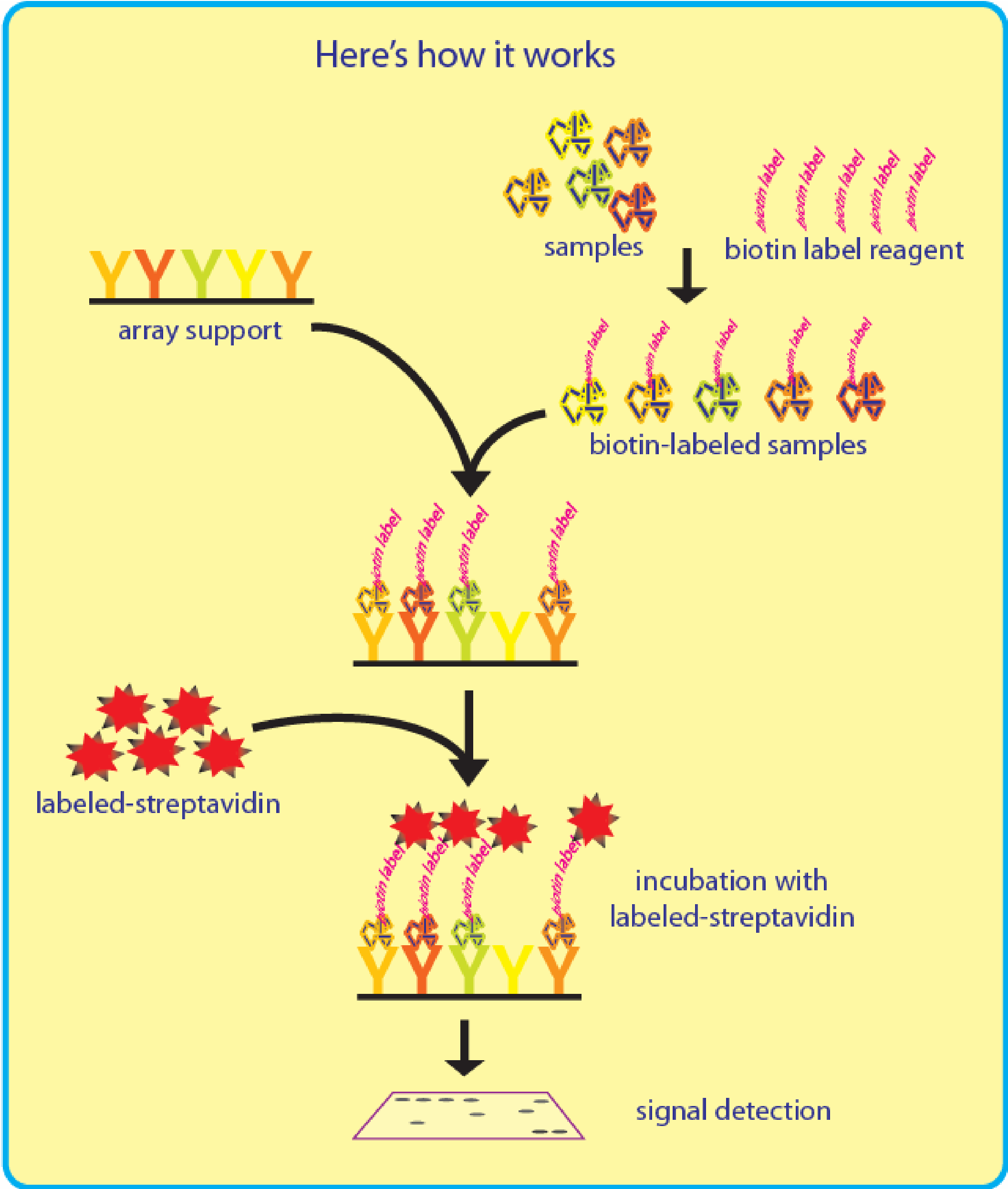
I. Introduction

The area of obesity research is getting hotter ever over the past years. One of the key driving force is that adipose tissue is found no longer to be an inert energy storage organ, but is emerging as an active participant in regulating physiological and pathologic processes. Many soluble factors have been identified from the adipose tissue and are so called as adipocytokines or adipokines. Some of the adipokines are mainly produced by the adipose tissue like leptin and resistin, while others are also synthesized in other tissues like TNF-alpha, IL-6, MCP-1, and IL-1. Because all of these factors can act in an autocrine, paracrine or endocrine manner in the organisms, adipokines are thought to serve as mediators linking obesity, inflammation, immunity and other obesity related diseases.

Recent technological advances by Raybiotech have enabled the largest commercially available antibody array to date. With the L Series Human Obesity Antibody Array 1, researchers can now obtain a broad, panoramic view of adipokine expression. The expression levels of 182 human target proteins can be simultaneously detected in cell culture supernates 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 adipokines in your sample.

The first step in using the RayBio® Biotin Label-based Human Obesity 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.


Here's how it works
samples
biotin label reagent
array support
biotin-labeled samples
labeled-streptavidin
incubation with
labeled-streptavidin
signal detection

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 Human Obesity Antibody Array 1 (Item E, 2 array membranes for Cat#: AAH-BLM-ADI-1-2**,** and 4 for Cat#: AAH-BLM-ADI-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)
- 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

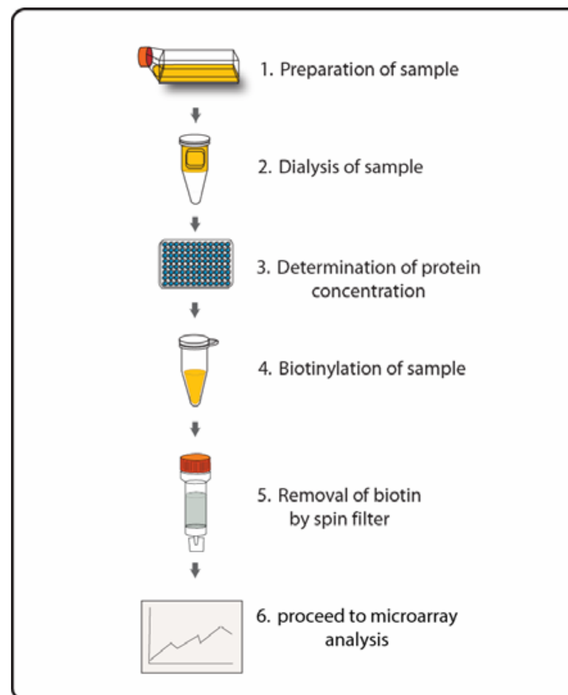
- Always use forceps to handle membranes, and grip the membranes by the edges only.
- Never allow array membranes to dry during experiments.
- Avoid touch Array membrane by hand, tips or any sharp tools.

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

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 supernates 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:

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.

RayBio® Biotin Label-based Human Obesity Antibody Array 1 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
1	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
2	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
3	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
4	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
5	61	62	63	64	65	66	67	68	69	70	71	72	73	Blank	Blank	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90
6	61	62	63	64	65	66	67	68	69	70	71	72	73	Blank	Blank	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90
7	91	92	93	94	95	96	97	98	99	100	101	102	103	Blank	Blank	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120
8	91	92	93	94	95	96	97	98	99	100	101	102	103	Blank	Blank	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120
9	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	129	130	131	132	133	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	144	145	146	147	148	149	150
10	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	129	130	131	132	133	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	144	145	146	147	148	149	150
11	IC-1a	IC-1b	IC-1c	Blank	Blank	NEG	NEG	Blank	159	160	161	162	163	Blank	Blank	P-3a	P-3b	P-3c	Blank	Blank	NEG	NEG	Blank	174	175	176	177	178	179	180
12	IC-1a	IC-1b	IC-1c	Blank	Blank	NEG	NEG	Blank	159	160	161	162	163	Blank	Blank	P-3a	P-3b	P-3c	Blank	Blank	NEG	NEG	Blank	174	175	176	177	178	179	180
13	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	189	190	191	192	193	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	204	205	206	207	208	209	210
14	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	189	190	191	192	193	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	204	205	206	207	208	209	210
15	241	242	243	244	245	246	247	248	249	250	251	252	253	Blank	Blank	256	257	258	259	260	261	262	263	264	265	266	267	268	269	270
16	241	242	243	244	245	246	247	248	249	250	251	252	253	Blank	Blank	256	257	258	259	260	261	262	263	264	265	266	267	268	269	270
17	271	272	273	274	275	276	277	278	279	280	281	282	283	Blank	Blank	286	287	288	289	290	291	292	293	294	295	296	297	298	299	300
18	271	272	273	274	275	276	277	278	279	280	281	282	283	Blank	Blank	286	287	288	289	290	291	292	293	294	295	296	297	298	299	300
19	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	309	310	311	312	313	Blank	Blank	316	317	318	319	320	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank
20	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	309	310	311	312	313	Blank	Blank	316	317	318	319	320	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank
21	P-4a	P-4b	P-4c	Blank	Blank	NEG	NEG	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	NEG	NEG	Blank	Blank	IC-2c	IC-2b	IC-2a
22	P-4a	P-4b	P-4c	Blank	Blank	NEG	NEG	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	NEG	NEG	Blank	Blank	IC-2c	IC-2b	IC-2a

RayBio® Biotin-label-based Human Obesity Antibody Array 1 List

No.	Name	No.	Name	No.	Name
1	P-1a	61	BMP-3	121	Blank
2	P-1b	62	BMP-3b / GDF-10	122	Blank
3	P-1c	63	BMP-4	123	Blank
4	Blank	64	BMP-5	124	Blank
5	Blank	65	BMP-6	125	Blank
6	NEG	66	BMP-7	126	Blank
7	NEG	67	BMP-8	127	Blank
8	Blank	68	BMP-15	128	Blank
9	ACE	69	BMPR-IA / ALK-3	129	IGFBP-1
10	ACE-2	70	BMPR-IB / ALK-6	130	IGFBP-2
11	ACTH	71	BMPR-II	131	IGFBP-3
12	ADFP	72	b-NGF	132	IGF-II
13	Acrp 30	73	C3a des Arg	133	IL-1 R1
14	Blank	74	Blank	134	Blank
15	Blank	75	Blank	135	Blank
16	P-2a	76	CART	136	Blank
17	P-2b	77	CD137 (4-1BB)	137	Blank
18	P-2c	78	CD36	138	Blank
19	Blank	79	Clusterin	139	Blank
20	Blank	80	CNTF	140	Blank
21	NEG	81	C-peptide	141	Blank
22	NEG	82	CRP	142	Blank
23	Blank	83	Cystatin C	143	Blank
24	Adipsin (Factor D)	84	Dtk	144	IL-1 R4
25	AgRP	85	EGF	145	IL-1a
26	AMPKa1	86	EGF-R	146	IL-1b
27	Amylin	87	ENA-78	147	IL-1ra
28	Angiopoietin-1	88	Endophin Beta	148	IL-6
29	Angiopoietin-2	89	Epiregulin	149	IL-6 sR
30	Angiotensinogen / Angiotensin II	90	E-selectin	150	IL-8
31	Blank	91	ET-1 (Endothelin)	151	IC-1a
32	Blank	92	FABP4	152	IC-1b
33	Blank	93	FAM3B	153	IC-1c
34	Blank	94	FAS / Apo-1	154	Blank
35	Blank	95	FGF-10	155	Blank
36	Blank	96	FGF-6	156	NEG
37	Blank	97	FSH	157	NEG
38	Blank	98	Galectin -1	158	Blank
39	Ang-like Factor	99	GH (Growth Hormone)	159	IL-10
40	ANGPTL1	100	Ghrelin	160	IL-11
41	ANGPTL2	101	GITR	161	IL-12
42	ANGPTL3	102	GITRL	162	IL-25 / IL-17E
43	ANGPTL4	103	GLP-1	163	INSL3
44	Blank	104	Blank	164	Blank
45	Blank	105	Blank	165	Blank
46	Blank	106	Glucagon	166	P-3a
47	Blank	107	Glut1	167	P-3b
48	Blank	108	Glut2	168	P-3c
49	Blank	109	Glut3	169	Blank
50	Blank	110	Glut5	170	Blank
51	Blank	111	Glutathione peroxidase 1	171	NEG
52	Blank	112	Glutathione peroxidase 3	172	NEG
53	Blank	113	GROa	173	Blank
54	Apelin Receptor	114	HCC4	174	INSRR
55	ApoB	115	HGF	175	Insulin
56	ApoE	116	HSD-1	176	Insulin R (CD220)
57	Axl	117	ICAM1	177	Leptin
58	BDNF	118	IFNg	178	Leptin R
59	bFGF	119	IGF-1	179	LH (Luteinizing Hormone)
60	BMP-2	120	IGF-1 sR	180	LIF

RayBio® Biotin Label-based Human Obesity Antibody Array List…contin

181 Blank
182 Blank
183 Blank
184 Blank
185 Blank
186 Blank
187 Blank
188 Blank
189 LOX
190 Lymphotactin
191 MCP-1
192 MCP-3
193 M-CSF
194 Blank
195 Blank
196 Blank
197 Blank
198 Blank
199 Blank
200 Blank
201 Blank
202 Blank
203 Blank
204 MIF
205 MIP-1a
206 MIP-1b
207 MIP-3b
208 MMP-2
209 MMP-9
210 MMP-11
211 MMP-19
212 MSHa
213 MSPa
214 Myostatin
215 NAIP
216 NeuroD1
217 Neurophilin-2
218 NGF R
219 NPY (Neuropeptide Y)
220 Obestatin R (GPR-39)
221 Orexin A
222 Orexin B
223 OSM
224 Blank
225 Blank
226 Osteocalcin
227 Osteonectin
228 Osteoprotegerin
229 PARC
230 PDGF
231 PDGF-AA
232 PDGF-AB
233 PDGF-C
234 PDGF-D
235 PEDF
236 Pentraxin-3
237 PPARg2 / NRIC3
238 Pref-1
239 Prohibitin
240 Prolactin
241 PYY
242 RANTES
243 RBP4
244 RELMb
245 Resistin
246 S100
247 S100 A8+A9
248 S100 A10
249 SAA
250 SDF-1
251 SEMA3A
252 Serotonin
253 Syndecan-3
254 Blank
255 Blank
256 TACE
257 TDAG51
258 TECK
259 TGF-a
260 TGF-b
261 Thrombospondin 1
262 Thrombospondin 2
263 Thrombospondin 4
264 TIMP-1
265 TIMP-2
266 TIMP-3
267 TIMP-4
268 Tissue factor (CD142)
269 TLR2
270 TLR4
271 Blank
272 Blank
273 Blank
274 Blank
275 Blank
276 Blank
277 Blank
278 Blank
279 TNF alpha
280 TNF sRI
281 TNF sRII
282 TSG-6
283 TSH
284 Blank
285 Blank
286 Vaspin
287 VCAM1
288 VEGF
289 Visfatin/PBEF1
290 XEDAR
291 Blank
292 Blank
293 Blank
294 Blank
295 Blank
296 Blank
297 Blank
298 Blank
299 Blank
300 Blank
301 P-4a
302 P-4b
303 P-4c
304 Blank
305 Blank
306 NEG
307 NEG
308 Blank
309 Blank
310 Blank
311 Blank
312 Blank
313 Blank
314 Blank
315 Blank
316 Blank
317 Blank
318 Blank
319 Blank
320 Blank
321 Blank
322 Blank
323 Blank
324 NEG
325 NEG
326 Blank
327 Blank
328 IC-2c
329 IC-2b
330 IC-2a

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

1. Profiling of cytokine expression by biotin-labeled-based protein arrays. Ying Lin, Ruochun Huang, Lipai Chen *et al.* ***Proteomics.*** 2003, 3: 1750-1757.

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