

RayBio[®] Biotin Label-based Human Adipokine Antibody Array 1

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

**User Manual
(Revised Apr 1, 2009)**

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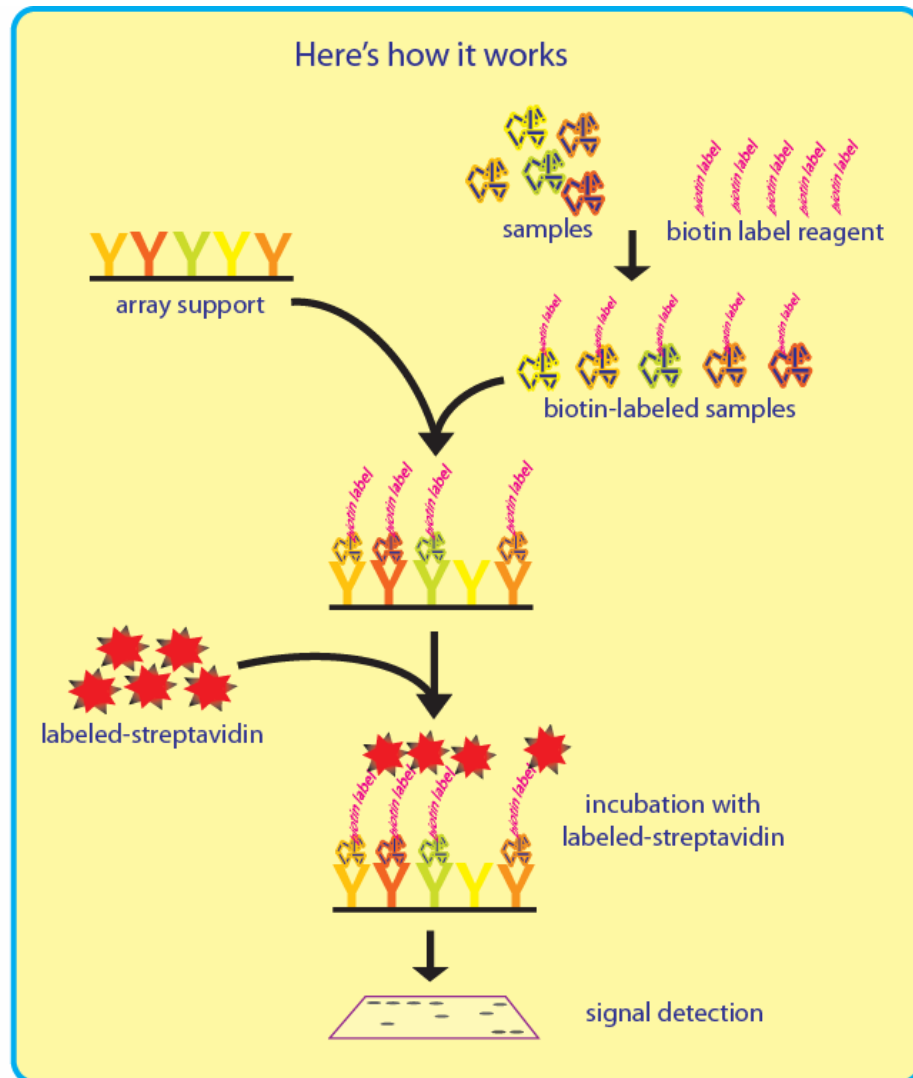
I. Introduction

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 Adipokine 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 antibody array I is to biotinylate the primary amine of the proteins in cell culture supernates and serum. The biotin-labeled sample is then added onto glass chip and incubated at room temperature.

Fluorescent dye-Conjugated Streptavidin (cy3 equivalent) is used to visualize the signals.



II. Materials Provided

Upon receipt, the kit should be stored at -20 °C. Please use within 6 months from the date of shipment. After initial use, the Blocking Buffer, Stop Solution, 20X Wash Buffer I and II, Serum Buffer and Fluorescent dye-Conjugated Streptavidin should be stored at 4 °C to avoid repeated freeze-thaw cycles. The Array I Glass Chip and internal control should be kept at -20 °C.

- Dialysis tube (Item A, 4 tubes for 2-subarray chips, and 8 for 4-subarray chips, dialysis tube is from EMD product)
- Labeling Reagent (Item B, 1 tube for 2-subarray chips, and 2 for 4-subarray chips)
- Internal control (Item C, 1 tube for 2-subarray chips, and 2 for 4-subarray chips)
- Stop Solution (Item D, 50 µl)
- RayBio® Biotin label-based human antibody array 1 Glass Chip with Frame (Item E, 1 slide with 2 subarrays for AAH-BLG-ADI-1-2 or 4 subarrays for AAH-BLG-ADI-1-4)
- Blocking Buffer (Item F, 8 ml)
- 20X Wash Buffer I (Item G, 30ml)
- 20X Wash Buffer II (Item H, 30ml)
- Fluorescent dye-Conjugated Streptavidin (Item I, cy3 equivalent, 1 tube for 2-subarray chips, and 2 for 4-subarray chips)
- Adhesive film (Item J)
- Serum Buffer (Item K, 8 ml)
- D-Tube Floating Rack
- 30 ml centrifuge tube

III. Additional Materials Required

- 1X PBS, pH=8.0
- Shaker and plastic or glass box
- 1 ml tube
- Laser scanner for fluorescence detection
- Aluminum foil
- Distilled water

IV. Overview and General Considerations

A. Handling glass chips

- The microarray slides are sensitive, so do not touch the array surface by tips, forceps or hand. Hold the slides by the edges only.
- Handle the slides with latex free gloves.
- Avoid breaking the glass slide.
- A clean environment is essential
- Remove the final buffer by gently applying suction with a pipette to corners of each chamber. Do not touch the array, only the sides.

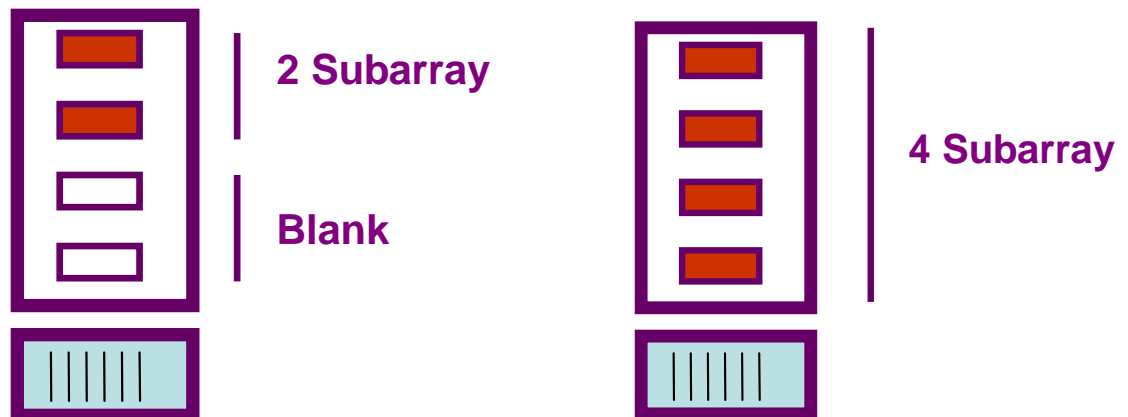


B. Incubation of Antibody Array

- Completely cover the array area with sample or buffer during incubation, and cover the incubation chamber with adhesive film or a plastic sheet protector to avoid drying, particularly when incubation is more than 2 hours.
- Avoid foaming during incubation steps.
- Perform all incubation and wash steps under gentle rotation.
- Avoid cross-contamination by preventing overflow to neighboring wells.
- Several incubation steps such as step 2 (sample incubation) in page 10, or step 7 (Fluorescent dye-Conjugated Streptavidin incubation) in page 11 may be done at 4 °C for overnight. Please make sure to cover the incubation chamber tightly to prevent evaporation.
- Avoid array slide exposure to light since step 6 in page 11.

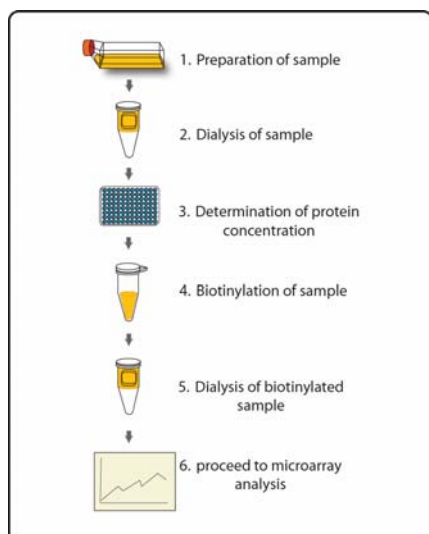
V. Protocol

Layout of Array Glass Chip

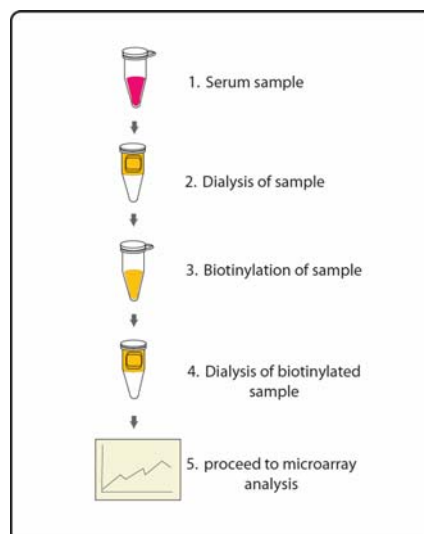


Assay Diagram

1. Cell Culture Supernates



2. Serum



A. Preparation of Cell Culture Supernates

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 1×10^6 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 for 10 min, aliquoted and stored at $-80\text{ }^{\circ}\text{C}$ until use. Meanwhile, the cells are also collected and the total protein concentration is determined. For each sample it is recommended that the concentrations of the supernates and cell lysates (help normalize different cell culture supernates) are

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.*

*** The culture time may be different and depends on your cell lines and research.*

B. Dialysis of Sample

The cell culture supernates, serum or plasma should be dialyzed with a Dialysis tube (Item A) before the biotin-labeling procedure. We recommend loading 200 μ l cell culture supernates or 100 μ l 5-fold diluted serum/plasma with 1X PBS (pH=8) (20 μ l serum or plasma + 80 μ l 1X PBS) into a dialyzer and dialyzing with at least 500 ml 1X PBS buffer (pH= 8) at 4 $^{\circ}$ 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, 0.6 g KCl, 24 g NaCl, 0.6 g KH_2PO_4 , 3.45 g Na_2HPO_4 dissolve in 2500 ml deionized or distilled water. Adjust pH=8.0 with 1M NaOH and adjust final volume to 3000 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 μ l 1X PBS, pH=8.0 into the Internal Control tube, pipette up and down to dissolve the powder.
 - a) For labeling cell culture supernates: transfer 180 μ l dialyzed cell culture supernates into a new tube. Add 40 μ l prepared Internal Control into the tube. Mix well.
 - b) For labeling serum or plasma: add 40 μ l prepared Internal Control into a new tube containing 35 μ l dialyzed serum or plasma sample and 155 μ l Serum Buffer (Item K).

Note: Check serum total volume has been changed or not after dialysis step, and then adjust adding dialyzed serum and Serum buffer volume to keep same serum concentration in Biotin-labeling step. For example, you need to use 70 μ l dialyzed serum and 120 μ l serum buffer if serum volume become 200 μ l from 100 μ l after dialysis.

2. Immediately before use, briefly spin down the Labeling Reagent tube (Item B). Add 100 μ l 1X PBS into the tube, pipette up and down or vortex slightly 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 with gentle shaking for 30 min. Gently tap the tube to mix the reaction solution every 5 min.

* *For labeling cell culture supernates: 36 μ l of 1X Labeling Reagent Solution for labeling 1 mg total protein in the cell culture 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.

For labeling serum or plasma: Add 22 μ l of Labeling Reagent Solution into each prepared serum or plasma sample in page 8 step 1.

4. Add 5 μ l Stop Solution (Item D) into above reaction solution and immediately dialyze as directed in Step B.
5. Samples should be centrifuged at 10,000 g for 5 min (4°C) after Dialysis.

Note: Samples (supernates) can be stored at -20 or -80 °C until microarray assay.

D. Dry the Glass Chip

Open the box containing glass chip, and take it out, and then let it air dry for 1 hour in a clean environment before use.

Note: Protect the chip from dust or other contaminants.

E. Blocking and Incubation of Antibody Array

1. Add 400 μ l of Blocking Buffer (Item F) into each well (Glass Chip with Frame, Item E) and incubate at room temperature for 30 min to block slides. Make sure there are no bubbles are in the well.
2. Decant Blocking Buffer from each well (make sure to remove all of buffer). Add 400 μ l of each sample into appropriate wells. Incubate arrays with sample at room temperature for 2 hours with gentle shaking or 4 °C for overnight.

Note: a. We recommended dilution of the biotin-labeled cell culture supernates 2-10 fold or the biotin-labeled serum/plasma 40-fold with blocking buffer. Make sure there are no bubbles in the wells.

b. The dilution used depends on the abundance of target proteins. The sample can be diluted further if the background or signal is too strong. If the signal is too weak, more sample can be used.

c. Incubation may be done at 4 °C overnight.

d. Avoid the flow of sample into neighboring wells.

3. Decant the samples from each well, and wash 3 times with 800 μ l of 1X Wash Buffer I (Item G) at room temperature with gentle shaking for 5 min per wash.
4. Put the glass chip with frame into a box with 1X Wash Buffer I (cover the whole glass slide and frame with Wash Buffer I), and wash 2 times at room temperature with gentle shaking for 10 min per wash.

5. Decant the Wash Buffer I from each well, Put the glass chip with frame into the box with Wash Buffer II (cover the whole glass slide and frame with Wash Buffer II), and wash 2 times at room temperature with gentle shaking for 5 min.
6. Remove all of Wash Buffer II in the well. Add 400 μ l of 1X Fluorescent dye-Conjugated Streptavidin (cy3 equivalent) to each subarray. Cover the incubation chamber with Adhesive film, and then cover the plate with aluminum foil to avoid exposure to light or incubate in dark room (avoid array slide exposure to light in the following steps 7, 8, 9, 10 and 11).

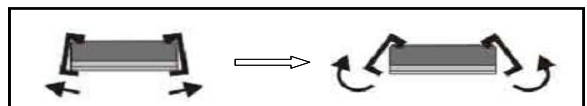
Note: briefly spin down the Fluorescent dye-Conjugated Streptavidin (cy3 equivalent) (Item I) before use. Add 1000 μ l of Blocking Buffer into the tube to prepare a Streptavidin Concentrate. Pipette up and down to mix gently (don't store the Concentrate for next day use). Add 200 μ l of Streptavidin Concentrate into a tube with 800 μ l ml of Blocking Buffer. Mix gently to prepare 1 X Streptavidin solution.

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

Note: Incubation may be done at 4 °C for overnight.

8. Decant the solution and disassemble the slide out of the incubation frame and chamber. Disassemble the device by pushing clips outward from the slide side. Carefully remove the slide from the gasket.

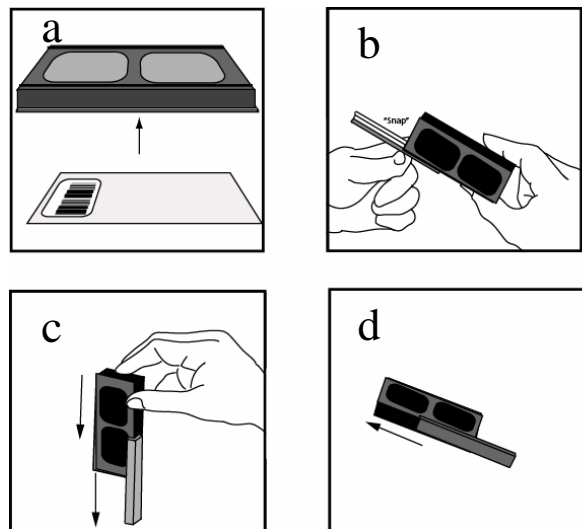
Note: Be careful not to touch the surface of the array side



9. Gently put the glass chip into a 30 ml centrifuge tube provided with 30 ml of 1X Wash Buffer I (add enough Wash Buffer I to cover the whole slide). Gently shake for 10 min. Remove the wash buffer. Repeat 2 times for a total of 3 washes.
10. Wash the glass chip with 30 ml of 1X Wash Buffer II. Repeat one time for a total of two washes for 5 min per wash.
11. Finally wash the glass chip with 30 ml of deionized or distilled water for 5 min.

Note: You may assemble the glass chip into an incubation chamber by following step if necessary as shown in the figure B. You may want to practice assembling the device with a blank glass slide:

- a. Apply slide to incubation chamber barcode facing upward as in step (a).
- b. Gently snap one edge of a snap-on side as shown in step (b).
- c. Gently press other of side against lab bench and push in direction shown in step (c)
- d. Repeat with the other side.



F. Fluorescence Detection

Put the glass chip into a 30 ml centrifuge tube provided, and dry the glass chip by centrifuge at 1,000 rpm for 3 minutes, or, dry the glass chip by a compressed N₂ stream. You can also let the glass chip dry completely in a clean environment (protect from light). Make sure the slides are absolutely dry before the scanning procedure. The signals can be visualized with a laser scanner, such as the Axon GenePix, using the cy3 channel.

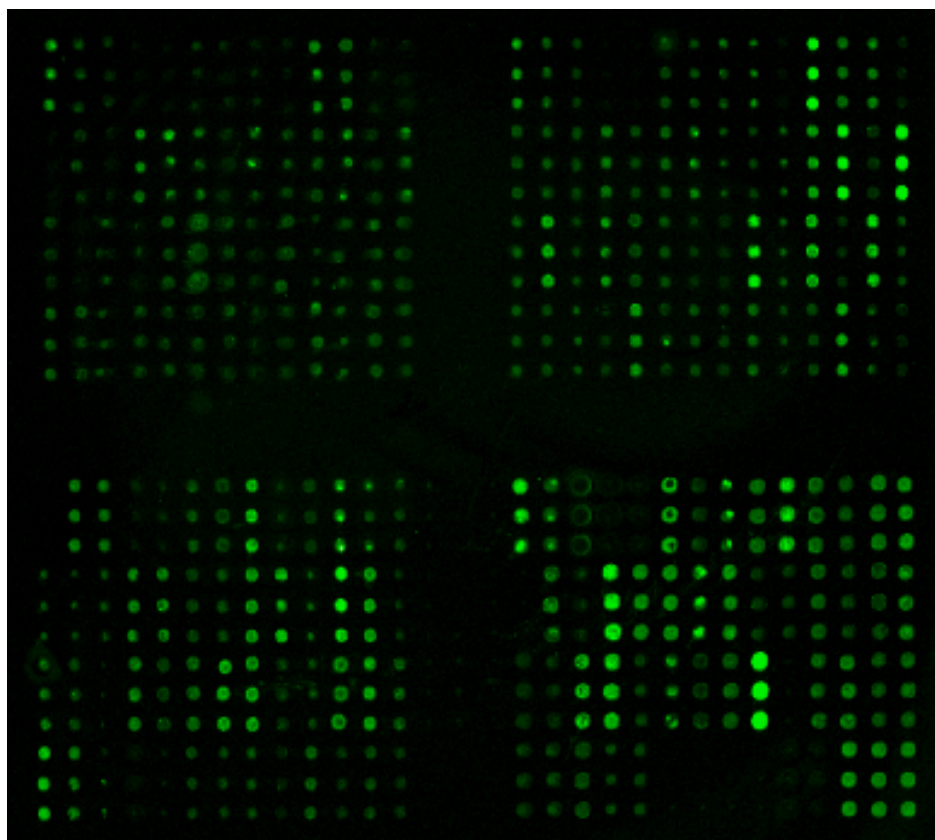
Note: We recommend scanning the slides right after the experiment. You also can store the slides at -20 °C in the dark for several days. If you do not have a laser scanner, we can provide this service for you. Just simply send your slide to us and we will take care of it.

VI. Interpretation of Results:

The following figure shows the RayBio[®] **Biotin-label-based Human Adipokine Antibody Array 1** probed with serum sample. The images was captured using a Axon GenePix laser scanner. 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 varies significantly. The fluorescence intensity detected on the array with each antibody depends on its 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 Adipokine Antibody Array 1 List

	Target Protein		Target Protein		Target Protein		Target Protein		Target Protein
1	POS-1a	61	Cystatin C	121	Blank	181	MMP-11	241	POS-2a
2	POS-1b	62	Dtk	122	Blank	182	MMP-19	242	POS-2b
3	POS-1c	63	EGF	123	Blank	183	MSH-alpha	243	POS-2c
4	NEG	64	EGF-R	124	Blank	184	MSP-alpha	244	NEG
5	NEG	65	ENA-78	125	Blank	185	Myostatin	245	NEG
6	ACE / CD43	66	Endorphin, Beta	126	Blank	186	NAIP	246	Tissue factor (CD142)
7	ACE-2	67	Epiregulin	127	Blank	187	NeuroD1	247	TLR2
8	ACTH	68	E-selectin	128	Blank	188	Neurophilin-2	248	TLR4
9	ADFP	69	ET-1 (Endothelin)	129	Blank	189	NGF R	249	TNF alpha
10	Adiponectin / Acrp30	70	FABP4	130	Blank	190	NPY (Neuropeptide Y)	250	TNF sRI
11	Adipsin (Factor D)	71	FAM3B	131	Blank	191	Orestatin R (GPR-39)	251	TNF sRII
12	AgRP	72	FAS / Apo-1	132	Blank	192	Orexin A	252	TSG-6
13	AMPK-alpha-1	73	FGF-10	133	Blank	193	Orexin B	253	TSH
14	Blank	74	Blank	134	Blank	194	Blank	254	Blank
15	Blank	75	Blank	135	Blank	195	Blank	255	Blank
16	Blank	76	Blank	136	Blank	196	Blank	256	Blank
17	POS-1a	77	FGF-6	137	Blank	197	OSM	257	Vaspin
18	POS-1b	78	FSH	138	Blank	198	Osteocalcin	258	VCAM1
19	POS-1c	79	Galectin -1	139	Blank	199	Osteonectin	259	VEGF
20	NEG	80	GH (Growth Hormone)	140	Blank	200	Osteoprotegerin	260	Visfatin/PBEF1
21	NEG	81	Ghrelin	141	Blank	201	PARC	261	XEDAR
22	Amylin	82	GITR	142	Blank	202	PDGF	262	Blank
23	Angiopoietin-1	83	GITRL	143	Blank	203	PDGF-AA	263	Blank
24	Angiopoietin-2	84	GLP-1	144	Blank	204	PDGF-AB	264	Blank
25	Angiotensinogen / Angiotensin II	85	Glucagon	145	Blank	205	PDGF-C	265	Blank
26	Ang-like Factor	86	Glut1	146	Blank	206	PDGF-D	266	NEG
27	ANGPTL1	87	Glut2	147	Blank	207	PEDF	267	NEG
28	ANGPTL2	88	Glut3	148	Blank	208	Pentraxin-3	268	IC-3
29	ANGPTL3	89	Glut5	149	Blank	209	PPARg2 / NRIC3	269	IC-2
30	ANGPTL4	90	Glutathione peroxidase 1	150	Blank	210	Pref-1	270	IC-1
31	Apelin Receptor	91	Glutathione peroxidase 3	151	IC-1	211	Prohibitin		
32	ApoB	92	GRO-alpha	152	IC-2	212	Prolactin		
33	ApoE	93	HCC4	153	IC-3	213	PYY		
34	Axl	94	HGF	154	NEG	214	RANTES		
35	BDNF	95	HSD-1	155	NEG	215	RBP4		
36	bFGF	96	ICAM1	156	Insulin	216	RELMb		
37	BMP-2	97	IFN-gamma	157	Insulin R (CD220)	217	Resistin		
38	BMP-3	98	IGF-1	158	Leptin	218	S100		
39	BMP-3 beta / GDF-10	99	IGF-1 sR	159	Leptin R	219	S100 A8+A9		
40	BMP-4	100	IGFBP-1	160	LH (Luteinizing Hormone)	220	S100 A10		
41	BMP-5	101	IGFBP-2	161	LIF	221	SAA		
42	BMP-6	102	IGFBP-3	162	LOX	222	SDF-1		
43	BMP-7	103	IGF-II	163	Lymphotactin	223	SEMA3A		
44	Blank	104	Blank	164	Blank	224	Blank		
45	Blank	105	Blank	165	Blank	225	Blank		
46	Blank	106	Blank	166	Blank	226	Blank		
47	BMP-8	107	IL-1 R1	167	POS-2a	227	Serotonin		
48	BMP-15	108	IL-1 R4	168	POS-2b	228	Syndecan-3		
49	BMPR-1A / ALK-3	109	IL-1 alpha	169	POS-2c	229	TACE		
50	BMPR-1B / ALK-6	110	IL-1 beta	170	NEG	230	TDAG51		
51	BMPR-II	111	IL-1ra	171	NEG	231	TECK		
52	b-NGF	112	IL-6	172	MCP-1	232	TGF-alpha		
53	C3a des Arg	113	IL-6 sR	173	MCP-3	233	TGF-beta		
54	CART	114	IL-8	174	M-CSF	234	Thrombospondin 1		
55	CD137 (4-1BB)	115	IL-10	175	MIF	235	Thrombospondin 2		
56	CD36	116	IL-11	176	MIP-1 alpha	236	Thrombospondin 4		
57	Clusterin	117	IL-12	177	MIP-1 beta	237	TIMP-1		
58	CNTF	118	IL-25 / IL-17E	178	MIP-3 beta	238	TIMP-2		
59	C-peptide	119	INSL3	179	MMP-2	239	TIMP-3		
60	CRP	120	INSRR	180	MMP-9	240	TIMP-4		

VII. Troubleshooting Guide

Problem	Cause	Recommendation
Weak signal	Inadequate detection	Check laser power and PMT parameters
	Inadequate reagent volumes or improper dilution	Check pipettors and ensure correct preparation
	Short incubation times	Ensure sufficient incubation time and change sample incubation step to overnight
	Too low protein concentration in sample	Don't make too low dilution Or concentrate sample
	Improper storage of kit	Store kit at suggested temperature
High background	Sample is too concentrated	Use more diluted sample
	Excess of streptavidin	Make sure to use the correct amount of streptavidin
	Inadequate detection	Check laser power and PMT parameters
	Inadequate wash	Increase the volume of wash buffer and incubation time
Uneven signal	Bubbles formed during incubation	Avoid bubble formation during incubation
	Arrays are not completely covered by reagent	Completely cover arrays with solution

VIII. Reference List

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