RayBio® Label-Based (L-Series) Human Obesity Antibody Array 182 (L-182)

Patent Pending Technology
User Manual (Revised September 9, 2014)

For the simultaneous detection of the relative expression of 182 (L-182) human obesity proteins in serum, plasma, cell culture supernatants, cell/tissue lysates or other body fluids.

L-Series Human Antibody Array L-182
Cat# AAH-BLG-ADI-2 (2 Sample Kit)
Cat# AAH-BLG-ADI-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

Tel: (Toll Free) 1-888-494-8555 or +1-770-729-2992; Fax: +1-770-206-2393;
Website: www.raybiotech.com Email: info@raybiotech.com

TABLE OF CONTENTS

I. Introduction

Obesity research has seen a surge in interest over the past 10 years. One of the key driving forces 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 known as adipocytokines or *adipokines*. Some adipokines, such as leptin and resistin, are produced mainly by the adipose tissues while others, such as TNF-alpha, IL-6, MCP-1, and IL-1, are also synthesized in other tissues. Because all of these factors can act in an autocrine, paracrine or endocrine manner, 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 Antibody Array 182, researchers can now obtain a broad, panoramic view of adipokine expression. The expression levels of 182 human target proteins can be simultaneously detected, including cytokines, chemokines, adipokines, growth factors, angiogenic factors, proteases, soluble receptors, soluble adhesion molecules and other proteins in cell culture supernatants, serum and plasma.

The first step in using the RayBio® L-Series Human Obesity Antibody Array 182 is to biotinylate the primary amine of the proteins in serum or plasma samples, cell culture supernatants, cell lysates or tissue lysates. The glass slide arrays are then blocked, just like a Western blot, and the biotin-labeled sample is added onto the glass slide, which is pre-printed with capture antibodies, and incubated to allow for interaction of target proteins. Streptavidin-conjugated fluorescent dye (Cy3 equivalent) is then applied to the array. Finally, the glass slide is dried, and laser fluorescence scanning is used to visualize the signals.

II. Materials Provided

A. Storage Recommendations

Upon receipt, the kit should be stored at -20°C until needed. Please use within 6 months from the date of shipment. After initial use, remaining reagents should be stored at 4°C and may be stored for up to 3 months (Labeling Reagent, Item B, should be prepared fresh each time before use). Unused glass slides should be kept at -20 °C and repeated freeze-thaw cycles should be avoided (slides may be stored for up to 6 months).

ITEM	DESCRIPTION	AAH-BLG-ADI-1-2	AAH-BLG-ADI-1-4
A	Dialysis Vials & Floating Dialysis Rack	4 vials	8 vials
B	Labeling Reagent	1 vial	2 vials
D	Stop Solution	1 vial (50 µl)	
E	RayBio® L-Series Human Antibody Array L-182 Glass Slide*	1 slide (L-182)	1 slides (L-182))
F	Blocking Buffer	1 bottle (8 ml)	1 bottle (8 ml)
G	20X Wash Buffer I	1 bottle (30 ml)	1 bottle (30 ml)
H	20X Wash Buffer II	1 bottle (30 ml)	1 bottle (30 ml)
I	Cy3-Conjugated Streptavidin**	1 vial	2 vials
J	Adhesive Plastic Strips		
K	Labeling Buffer	1 bottle (8 ml)	
n/a	2X Cell Lysis Buffer***	1 bottle (10 ml)	
M	30 ml Centrifuge Tube	1 tube	

*Each slide contains 2 or 4 identical subarrays

**HiLyte Plus™ 555

***Only needed if testing cell or tissue lysates

B. Additional Materials Required

- KCl, NaCl, KH_2PO_4, Na_2HPO_4 and ddH_2O
- 1 ml tube, small plastic or glass containers
- Orbital shaker or oscillating rocker
- Beaker, stir plate and stir bar
- Pipettors, pipette tips and other common lab consumables
- Laser scanner for fluorescence detection (list available online)
- Aluminum foil

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.**,† The membrane-based array is recommended if high serum medium such as 10% FCS/FBS is used, as high background can occur on glass slide arrays with high serum containing media samples.
4. To collect supernatants, centrifuge at 1,000 g for 10 min and store as ≤1 ml aliquots at -80°C until needed.
5. Measure the total wet weight of cultured cells in the pellet and/or culture dish. You may then 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). 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. Centrifuge 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. Proceed to b. Cells in Suspension.
    b. Cells in Suspension: Pellet the cells by centrifuging using a microcentrifuge at 1500 rpm for 10 min.
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 min 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 min 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.

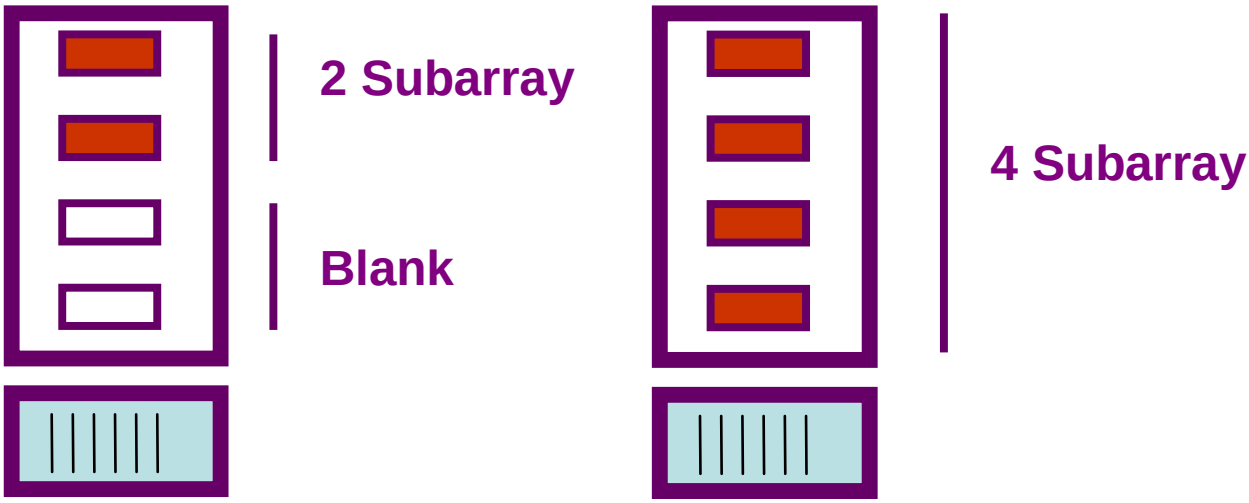
4. Transfer supernatant to a clean tube and store at -80°C.

B. Handling the Glass Slides

- The microarray slides are delicate. Please do not touch the array surface with pipette tips, forceps or your fingers. Hold the slides by the edges only.
- Handle the slides with powder-free gloves and in a clean environment.
- Do not remove the glass slide from the chamber assembly until step 20 on page 14, and take great care not to break the glass slide when doing so.
- Remove reagents/sample by gently applying suction with a pipette to corners of each chamber. Do not touch the printed area of the array, only the sides as seen in image below.

C. Layout of Human L-182 Glass Slide

Two or four identical sub-arrays on one slide



D. Incubations and Washes

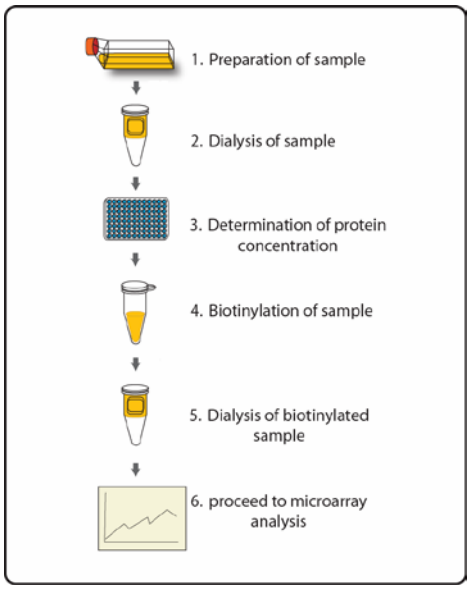
- Cover incubation chamber with a Plastic Adhesive Strip (Item J) to prevent evaporation during incubation or wash steps, particularly those steps lasting 2 hours or longer.
- During incubation and wash steps avoid foaming and remove all bubbles from the sub-array surface.
- Perform all incubation and wash steps under gentle rotation or rocking motion (~0.5 to 1 cycle/sec).
- Wash steps in Wash Buffer II and all incubation steps may be performed overnight at 4°C.
- Avoid cross-contamination of samples to neighboring wells. To remove Wash Buffers and other reagents from chamber wells, you may invert the Glass Slide Assembly to decant, and aspirate the remaining liquid.

- Unlike most Cy3 fluors, the HiLyte Plus™ 555 used in this kit is very stable at room temperature (RT) and resistant to photobleaching on the hybridized glass slides. However, please protect glass slides from directly strong light and temperatures above RT.

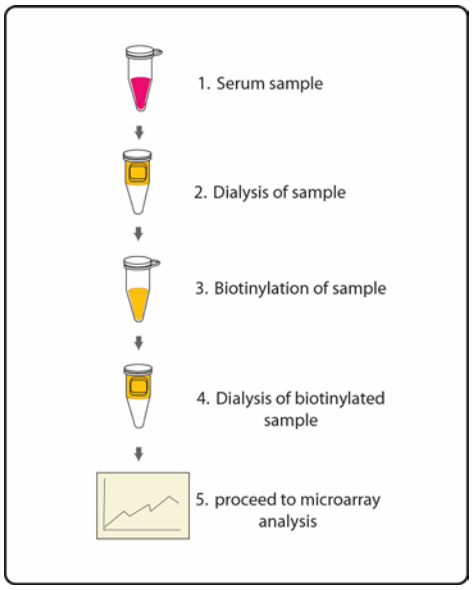
IV. Protocol

Assay Diagram

1. Cell culture supernatants or cell/tissue lysates



2. Serum or plasma



Note: If using cell or tissue lysates, start at "Dialysis of sample"

A. Dialysis of Sample

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

1. To prepare dialysis buffer (1X PBS, pH=8.0), dissolve 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 pH=8.0 with 1M NaOH and adjust final volume to 3000 ml with ddH_2O.

2. Add each sample into a separate Dialysis Tube (Item A). Loading volumes are as follows: 200 µl cell culture supernatant; 100 µl cell or tissue lysate (1~2 mg/ml total protein) or 20 µl serum or plasma + 80 µl 1X PBS, pH=8 (5-fold dilution). Carefully place Dialysis Tubes into Floating Dialysis Rack.

   Note for cell culture supernatants: if using a 2-fold dilution of biotin-labeled sample in the array incubation step (page 12, step 11), you will need to load a total of 400 µl of original cell culture supernatant into 2 separate Dialysis Tubes (200 µl /tube).

   Note: If the samples appear to be cloudy, transfer the samples to a clean tube, centrifuge at 13,000 rpm for 20 minutes at 2-8°C. If the samples are still not clear, store them at -20°C for 20 minutes. Remove from the freezer, immediately centrifuge at 13,000 rpm for 20 minutes at 2-8°C.

3. Place Floating Dialysis 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, stirring buffer gently. Then exchange the 1X PBS buffer and repeat dialysis for at least 3 h at 4°C. Transfer dialyzed sample to a clean microfuge tube. Spin dialyzed samples for 5 min at 10,000 rpm to remove any particulates or precipitates, and then transfer the supernatants to a clean tube.

Note: The sample volume may change during dialysis.

Note: Dialysis procedure may proceed overnight.

Note: Determine the total protein concentration for cell culture supernatants or cell/tissue lysate after dialysis procedure (Step 3). We recommended using a BCA total protein assay (eg, Pierce, Catalog # 23227).

B. Biotin-labeling Sample

Note: Amines (e.g., Tris, glycine) and azides quench the biotinylation reaction. Avoid contaminating samples with these chemicals prior to biotinylation.

4. Immediately before use, prepare 1X Labeling Reagent. Briefly spin down the Labeling Reagent tube (Item B). Add 100 µl 1X PBS into the tube, then pipette up and down or vortex slightly to dissolve the lyophilized reagent.

5. Add 1X Labeling Reagent to dialyzed samples.

    a. For labeling cell culture supernatants: transfer 180 µl dialyzed sample into a new tube. Add 36 µl of 1X Labeling Reagent Solution per 1 mg total protein in dialyzed cell culture supernatant. Mix well. For example, if sample's total protein concentration is 0.5 mg/ml you need to add 3.24 µl 1X Labeling Reagent to the tube of 180 µl dialyzed sample.

Note: You need to biotin-label 360 µl of dialyzed sample if dilution of the biotin-labeled samples is 2 fold in step 11 on page 11.

    b. For labeling serum or plasma: Add 22 µl of 1X Labeling Reagent Solution into a new tube containing 35 µl dialyzed serum or plasma sample and 155 µl Labeling Buffer (Item K).

    c. For labeling cell or tissue lysates: transfer 30 µg (15 µl of 2 mg/ml) cell or tissue lysates into a tube and add labeling buffer (Item K) for a total volume of 300 µl. Then add 3.3 µl of 1X Labeling Reagent Solution.

Note: To normalize serum/plasma or cell/tissue lysate concentrations during biotinylation, measure sample volume before and after dialysis. Then adjust the volumes of dialyzed serum/plasma or cell/tissue lysates and Labeling Buffer to compensate. For example, if the sample volume doubles after dialysis, then use twice as much serum/plasma in the labeling reaction (70 µl) and reduce the Labeling Buffer to 120 µl.

6. 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.

7. Add 3 µl Stop Solution (Item D) into each reaction tube and immediately dialyze as directed in Steps 1–3 on pages 8-9.

Note: Biotinylated samples can be stored at -20°C or -80°C until you are ready to proceed with the assay.

C. Drying the Glass Slide

8. Remove the package containing the Assembled Glass Slide (Item E) from the freezer. Place unopened package on the bench top for approx. 15 min, and allow the Assembled Glass Slide to equilibrate to RT.

9. Open package, and take the Assembled Glass Slide out of the sleeve (Do not disassemble the Glass Slide from the chamber assembly). Place glass slide assembly in laminar flow hood or similar clean environment for 1-2 hours at RT.

Note: Protect the slide from dust or other contaminants.

D. Blocking and Incubations

Note: Glass slide should be completely dry before adding Blocking Buffer to wells.

10. Block sub-arrays by adding 400 µl of Blocking Buffer (Item F) into each well of Assembled Glass Slide and incubating at RT for 30 min. Ensure there are no bubbles on the array surfaces.

11. Immediately prior to sample incubation, spin biotin-labeled samples for 5 min at 10,000 rpm to remove any particulates or precipitates. Dilute samples with Blocking Buffer. Recommended dilution of the biotin-labeled samples with Blocking Buffer is 2-10 fold for cell culture supernatants, 20-fold for serum/plasma and 30 fold cell/tissue lysate.

Note: Optimal sample dilution factor will depend on the abundance of target proteins. If the background or antigen-specific antibody signals are too strong, the sample can be diluted further in subsequent experiments. If the signal is too weak, more concentrated samples can be used.

12. Completely remove Blocking Buffer from each well. Add 400 µl of diluted samples into appropriate wells. Remove any bubbles on array surfaces. Incubate arrays with gentle rocking or shaking for 2 hours at RT or overnight at 4°C.

Note: Avoid the flow of sample into neighboring wells.

13. Based on number of samples and remaining protocol, calculate the amount of 1X Wash Buffer I and 1X Wash Buffer II needed to complete the experiment. Separately dilute the required amounts of 20X Wash Buffer I Concentrate (Item G) 20-fold and 20X Wash Buffer II Concentrate (Item H) with ddH_2O.

14. Decant the samples from each well, and wash 3 times with 800 µl of 1X Wash Buffer I at RT with gentle rocking or shaking for 5 min per wash.

15. Obtain a clean container (e.g., pipette tip box or slide-staining jar), place the Assembled Glass Slide into the container with enough volume of 1X Wash Buffer I to completely cover the entire assembly, and remove any bubbles in wells. Wash 2 times at RT with gentle rocking or shaking for 10 min per wash.

16. Decant the Wash Buffer I from each well, place the Assembled Glass Slide into the container with enough volume of 1X Wash Buffer II to completely cover the entire assembly, and remove any bubbles in wells. Wash 2 times at RT with gentle rocking or shaking for 5 min per wash.

17. Prepare 1X Cy3-Conjugated Streptavidin:

    a) Briefly spin down tube containing the Cy3-Conjugated Streptavidin (Item I) immediately before use.

    b) Add 1000 µl of Blocking Buffer into the tube to prepare a concentrated Cy3-Conjugated Streptavidin stock solution. Pipette up and down to mix gently (do not store the stock solution for later use).

    c) To prepare 1X Cy3-Conjugated Streptavidin add 200 µl of the concentrated Cy3-Conjugated Streptavidin stock solution into a tube with 800 µl of Blocking Buffer. Mix gently.

18. Carefully remove Assembled Glass Slide from container. Remove all of Wash Buffer II from the wells. Add 400 µl of 1X Cy3-Conjugated Streptavidin to each sub-array. Cover the incubation chamber with the plastic adhesive strips.

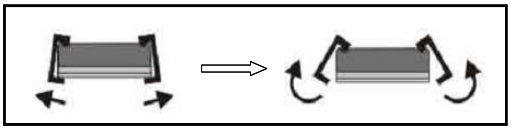
Note: Avoid exposure to light in Steps 19–25 by covering the Glass Slide Assembly with aluminum foil or incubate in a dark room.

19. Incubate with 1X Cy3-Conjugated Streptavidin at RT for 2 hours with gentle rocking or shaking.

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

20. Decant the solution and disassemble the glass slide from the incubation frame and chamber. Disassemble the device by pushing clips outward from the side, as shown below. Carefully remove the glass slide from the gasket.

Note: Be careful not to touch the printed surface of the glass slide, which is on the same side as the barcode.



21. Gently place the glass slide into 30 ml Centrifuge Tube (Item M). Add enough 1X Wash Buffer I to cover the entire glass slide (about 30 ml). Wash with gentle rocking or shaking for 10 min. Remove the wash buffer. Repeat 2 times for a total of 3 washes.

22. Repeat step 20, this time with 1X Wash Buffer II. Repeat one time for a total of two washes for 5 min per wash.

23. Finally, wash the glass slide with 30 ml of ddH_2O for 5 min. Remove glass slide and decant water from Centrifuge Tube.

24. Remove buffer droplets from the slide completely by one of the following ways:

    - Put the glass slide into the Slide Washer/Dryer, and dry the glass slide by centrifuge at 1,000 rpm for 3 minutes without cap.
    - Or, dry the glass slide by a compressed N2 stream.
    - Or gently apply suction with a pipette to remove buffer droplets. Do not touch the array, only the sides.

Note: Make sure the finished glass slide is completely dry before scanning or storage.

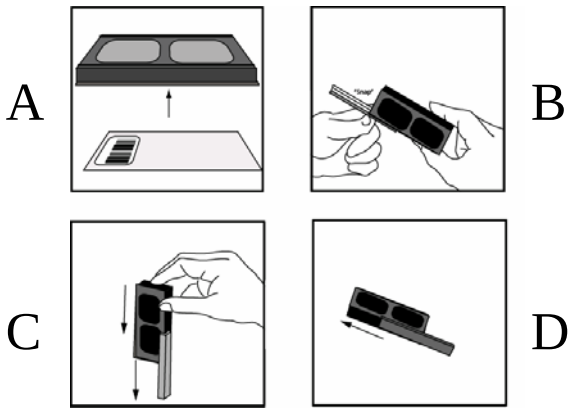
E. Fluorescence Detection

25. You may proceed immediately to scanning or you may store the slide at -20 °C in the Centrifuge Tube provided or at RT to scan at a later time.

Note: <u>Please protect the finished glass slides from temperatures above RT and store them in the dark</u>. Do not expose glass slide to strong light, such as sunlight or a UV lamp.

Note: If you need to repeat any of the incubation steps after finishing the experiment, you must first re-assemble the glass slide into the incubation chamber by following the steps as described below. To avoid breaking the printed glass slide, you may first want to practice assembling the device with a blank glass slide.

1. *Apply slide to incubation chamber barcode facing upward (image A).*
2. *Gently snap one edge of a snap-on side (image B).*
3. *Gently press other of side against lab bench and push in lengthwise direction (image C).*
4. *Repeat with the other side (image D)*



RayBio® Biotin Label-based Human Adipokine Antibody Array 1 Map

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	POS-1	POS-1	POS-2	POS-2	POS-3	POS-3	NEG	NEG	ACE / CD43	ACE / CD43	ACE-2	ACE-2	ACTH	ACTH	ADFP
2	Angiotensinogen / Angiotensin II	Angiotensinogen / Angiotensin II	Ang-like Factor	Ang-like Factor	ANGPTL1	ANGPTL1	ANGPTL2	ANGPTL2	ANGPTL3	ANGPTL3	ANGPTL4	ANGPTL4	APJ	APJ	ApoB
3	BMP-4	BMP-4	BMP-5	BMP-5	BMP-6	BMP-6	BMP-7	BMP-7	BMP-8	BMP-8	BMP-15	BMP-15	BMPR-IA / ALK-3	BMPR-IA / ALK-3	BMPR-IB / ALK-6
4	CNTF	CNTF	C-peptide	C-peptide	CRP	CRP	Cystatin C	Cystatin C	Dtk	Dtk	EGF	EGF	EGF-R	EGF-R	ENA-78
5	FGF-10	FGF-10	FGF-6	FGF-6	FSH	FSH	Galectin -1	Galectin -1	GH (Growth Hormone)	GH (Growth Hormone)	Ghrelin	Ghrelin	GITR	GITR	GITRL
6	Glutathione peroxidase 3	Glutathione peroxidase 3	GROa	GROa	HCC4	HCC4	HGF	HGF	HSD-1	HSD-1	ICAM1	ICAM1	IFNg	IFNg	IGF-1
7	IL-1a	IL-1a	IL-1b	IL-1b	IL-1ra	IL-1ra	IL-6	IL-6	IL-6 sR	IL-6 sR	IL-8	IL-8	IL-10	IL-10	IL-11
8	POS-1	POS-1	POS-2	POS-2	POS-3	POS-3	NEG	NEG	Leptin R	Leptin R	LH (Luteinizing Hormone)	LH (Luteinizing Hormone)	LIF	LIF	LOX
9	MIP-3b	MIP-3b	MMP-2	MMP-2	MMP-9	MMP-9	MMP-11	MMP-11	MMP-19	MMP-19	MSHa	MSHa	MSPa	MSPa	Myostatin
10	Orexin B	Orexin B	OSM	OSM	Osteocalcin	Osteocalcin	Osteonectin	Osteonectin	Osteoprotegerin	Osteoprotegerin	PARC	PARC	PDGF-BB	PDGF-BB	PDGF-AA
11	Prohibitin	Prohibitin	Prolactin	Prolactin	PYY	PYY	RANTES	RANTES	RBP4	RBP4	RELMb	RELMb	Resistin	Resistin	S100b
12	TACE	TACE	TDAG51	TDAG51	TECK	TECK	TGF-a	TGF-a	TGF-b	TGF-b	Thrombospondin 1	Thrombospondin 1	Thrombospondin 2	Thrombospondin 2	Thrombospondin 4
13	TNF alpha	TNF alpha	TNF sRI	TNF sRI	TNF sRII	TNF sRII	TSG-6	TSG-6	TSH	TSH	Vaspin	Vaspin	VCAM1	VCAM1	VEGF

	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1	ADFP	Adiponectin / Acrp30	Adiponectin / Acrp30	Adipsin (Factor D)	Adipsin (Factor D)	AgRP	AgRP	AMPKa1	AMPKa1	Amylin	Amylin	Angiopoietin-1	Angiopoietin-1	Angiopoietin-2	Angiopoietin-2
2	ApoB	ApoE	ApoE	Axl	Axl	BDNF	BDNF	bFGF	bFGF	BMP-2	BMP-2	BMP-3	BMP-3	BMP-3b / GDF-10	BMP-3b / GDF-10
3	BMPR-IB / ALK-6	BMPR-II	BMPR-II	b-NGF	b-NGF	C3a des Arg	C3a des Arg	CART	CART	CD137 (4-1BB)	CD137 (4-1BB)	CD36	CD36	Clusterin	Clusterin
4	ENA-78	Endophin Beta	Endophin Beta	Epiregulin	Epiregulin	E-selectin	E-selectin	ET-1 (Endothelin)	ET-1 (Endothelin)	FABP4	FABP4	FAM3B	FAM3B	FAS / Apo-1	FAS / Apo-1
5	GITRL	GLP-1	GLP-1	Glucagon	Glucagon	Glut1	Glut1	Glut2	Glut2	Glut3	Glut3	Glut5	Glut5	Glutathione peroxidase 1	Glutathione peroxidase 1
6	IGF-1	IGF-1 sR	IGF-1 sR	IGFBP-1	IGFBP-1	IGFBP-2	IGFBP-2	IGFBP-3	IGFBP-3	IGF-II	IGF-II	IL-1 R1	IL-1 R1	IL-1 R4	IL-1 R4
7	IL-11	IL-12	IL-12	IL-25 / IL-17E	IL-25 / IL-17E	INSL3	INSL3	INSRR	INSRR	Insulin	Insulin	Insulin R (CD220)	Insulin R (CD220)	Leptin	Leptin
8	LOX	Lymphotactin	Lymphotactin	MCP-1	MCP-1	MCP-3	MCP-3	M-CSF	M-CSF	MIF	MIF	MIP-1a	MIP-1a	MIP-1b	MIP-1b
9	Myostatin	NAIP	NAIP	NeuroD1	NeuroD1	Neurophilin-2	Neurophilin-2	NGF R	NGF R	NPY (Neuropeptide Y)	NPY (Neuropeptide Y)	Obestatin R (GPR-39)	Obestatin R (GPR-39)	Orexin A	Orexin A
10	PDGF-AA	PDGF-AB	PDGF-AB	PDGF-C	PDGF-C	PDGF-D	PDGF-D	PEDF	PEDF	Pentraxin-3	Pentraxin-3	PPARg2 / NRIC3	PPARg2 / NRIC3	Pref-1	Pref-1
11	S100b	S100 A8+A9	S100 A8+A9	S100 A10	S100 A10	SAA	SAA	SDF-1	SDF-1	SEMA3A	SEMA3A	Serotonin	Serotonin	Syndecan-3	Syndecan-3
12	Thrombospondin 4	TIMP-1	TIMP-1	TIMP-2	TIMP-2	TIMP-3	TIMP-3	TIMP-4	TIMP-4	Tissue factor (CD142)	Tissue factor (CD142)	TLR2	TLR2	TLR4	TLR4
13	VEGF	Visfatin/PBEF1	Visfatin/PBEF1	XEDAR	XEDAR	NEG	NEG	NEG	NEG	Pos 3	Pos 3	Pos 2	Pos 2	Pos 1	Pos 1

VI. Interpretation of Results:

A. Explanation of Controls Spots

1) **Positive Control spots (POS1, POS2, POS3)** are standardized amounts of biotinylated IgGs printed directly onto the array. All other variables being equal, the Positive Control intensities will be the same for each sub-array. This allows for normalization based upon the relative fluorescence signal responses to a known control, much as "housekeeping" genes or proteins are used to normalize results in PCR or Western blots, respectively.

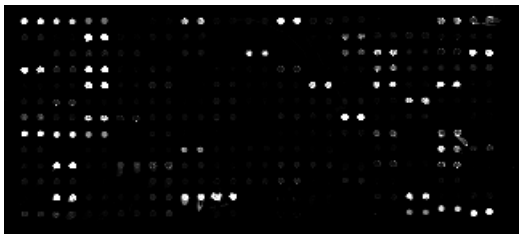
2) **Negative Control (NEG) spots** contain a protein-containing buffer (used to dilute antibodies printed on the array). Their signal intensities represent non-specific binding of Biotin-conjugated anti-Cytokines and/or the Cy3-Conjugated Streptavidin. Negative control signal intensities are usually very close to background signals in each sub-array.

B. Typical Results

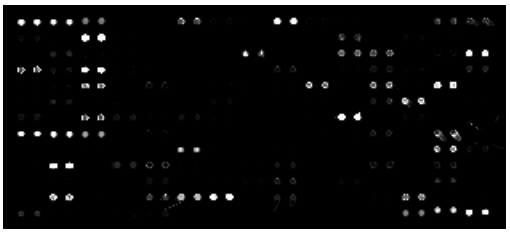
The following figure shows the RayBio® L-Series Human Obesity Antibody Array 182 probed with serum sample. The images were captured using a Axon GenePix laser scanner. The strong signals in row 20 and the upper left and lower right corners of each array are Positive Controls, which can be used to identify the orientation and help normalize the results between arrays.

RayBio® L-Series Human Obesity Antibody Array 182

Sample-1



Sample-2



If scanned using optimal settings, 3 distinct signal intensities will be seen: POS1>POS2>POS3. If all of these signals are of similar intensity, try increasing or decreasing laser power and/or signal gain settings.

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 (ie, concentrations of the various analytes in your samples), try using our Quantibody® Arrays instead.

C. Background Subtraction

Once you have obtained fluorescence intensity data, you should subtract the background and normalize to the Positive Control signals before proceeding to analysis.

Most laser fluorescence scanner software have an option to automatically measure the local background around each spot. For best results, we recommend comparing signal intensities representing the MEDIAN background signals minus local background. If your resulting fluorescence signal intensity reports do not include these values (e.g., a column labeled as “MED532-B532”), you may need to subtract the background manually or change the default settings on your scanner’s data report menu.

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.

To order the Analysis Tool, please contact us at +1-770-729-2992 or info@raybiotech.com for more information.

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 ≥1.5-fold increase or ≤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 ≈ 95%).

VII. Troubleshooting Guide

Problem	Cause	Recommendation
Weak Signal	Inadequate detection	Increase laser power and PMT parameters
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
	Short incubation time	Ensure sufficient incubation time and change sample incubation step to overnight
	Too low protein concentration in sample	Dilute starting sample less or concentrate sample
	Improper storage of kit	Store kit as suggested temperature. Don't freeze/thaw the slide.
Uneven signal	Bubble formed during incubation	Handle and pipette solutions more gently; De-gas solutions prior to use
	Arrays are not completed covered by reagent	Prepare more reagent and completely cover arrays with solution
	Reagent evaporation	Cover the incubation chamber with adhesive film during incubation
General	Cross-contamination from neighboring wells	Avoid overflowing wash buffer between wells
	Comet tail formation	Air dry the slide for at least 1 hour before usage
	Inadequate detection	Increase laser power so the highest standard concentration for each cytokine receives the highest possible reading yet remains unsaturated
High background	Overexposure	Lower the laser power
	Dark spots	Completely remove wash buffer in each wash step
	Insufficient wash	Increase wash time and use more wash buffer
	Dust	Minimize dust in work environment before starting experiment
	Slide is allowed to dry out	Take additional precautions to prevent slides from dying out during experiment

VIII. Selected References

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