

Quantibody® Human Angiogenesis Array 1

-Quantitative measurement of 10 Angiogenesis cytokines

Patent Pending Technology

User Manual (Version Sept06)

Cat # QAH-ANG-1



RayBiotech, Inc.

**We Provide You With Excellent
Protein Array Systems And Service**

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Quantibody[®] Human Angiogenesis Array I

Cytokines (10)	Angiogenin, ANG-2, EGF, bFGF, HB-EGF, HGF, Leptin, PDGF-BB, PIGF, VEGF
Format	One standard glass slide is spotted with 16 wells of identical cytokine antibody arrays. Each antibody is arrayed in quadruplicate.
Detection Method	Fluorescence with laser scanner: Cy3 wavelength equivalent dye
Sample Volume	50 – 100 μ l
Reproducibility	CV <20%
Assay duration	4 hrs

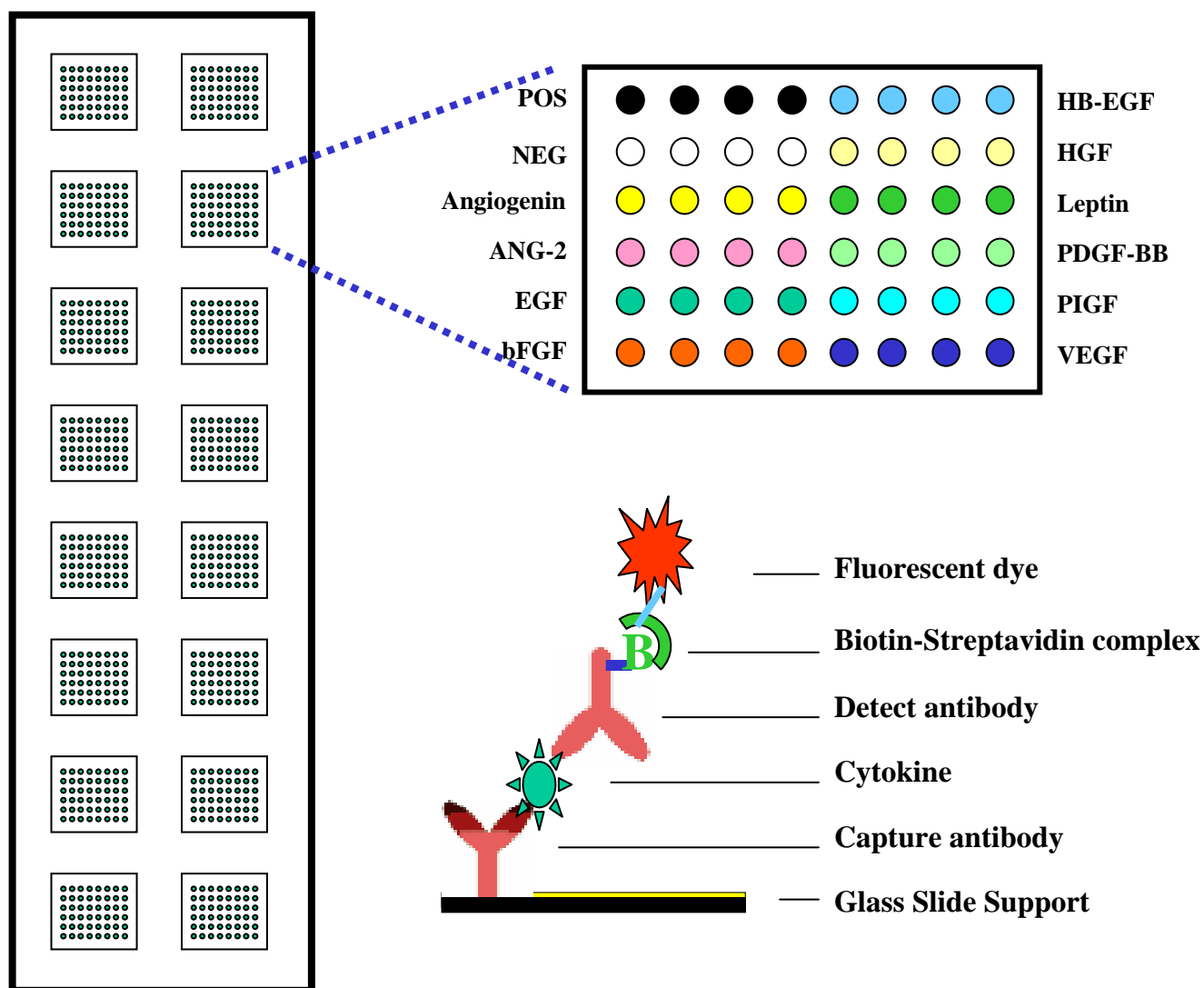




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I. Introduction

Cytokines play an important role in innate immunity, apoptosis, angiogenesis, cell growth and differentiation. They are involved in interactions between different cell types, cellular responses to environmental conditions, and maintenance of homeostasis. In addition, cytokines are also involved in most disease processes, including cancer and cardiac diseases.

The traditional method for cytokine detection and quantification is through the use of an enzyme-linked immunosorbent array (ELISA). In this method, target protein is first immobilized to a solid support. The immobilized protein is then complexed with an antibody that is linked to an enzyme. Detection of the enzyme-complex can then be visualized through the use of a substrate that produces a detectable signal. While the traditional method works well for a single protein, the overall procedure is time consuming and requires a lot of sample. With little sample to work with, conservation of precious small quantities becomes a risky task. Take the advantage of advancement of microarray technology over the last decade; more and more choices are available to the scientist today. A long-standing leader in the field, Raybiotech, has pioneered the development of semi-quantitative cytokine antibody array, in which multiple cytokine antibodies are arrayed on solid support (membrane or glass slide). Detection of multiple cytokines is achieved through a sandwich-like ELISA procedure. Our current RayBio[®] Human Cytokine Antibody Array C or G series 2000 enables scientists to detect 174 human cytokines in a single experiment rapidly and inexpensively. The array data can be further validated and quantified by using RayBiotech ELISA kits.

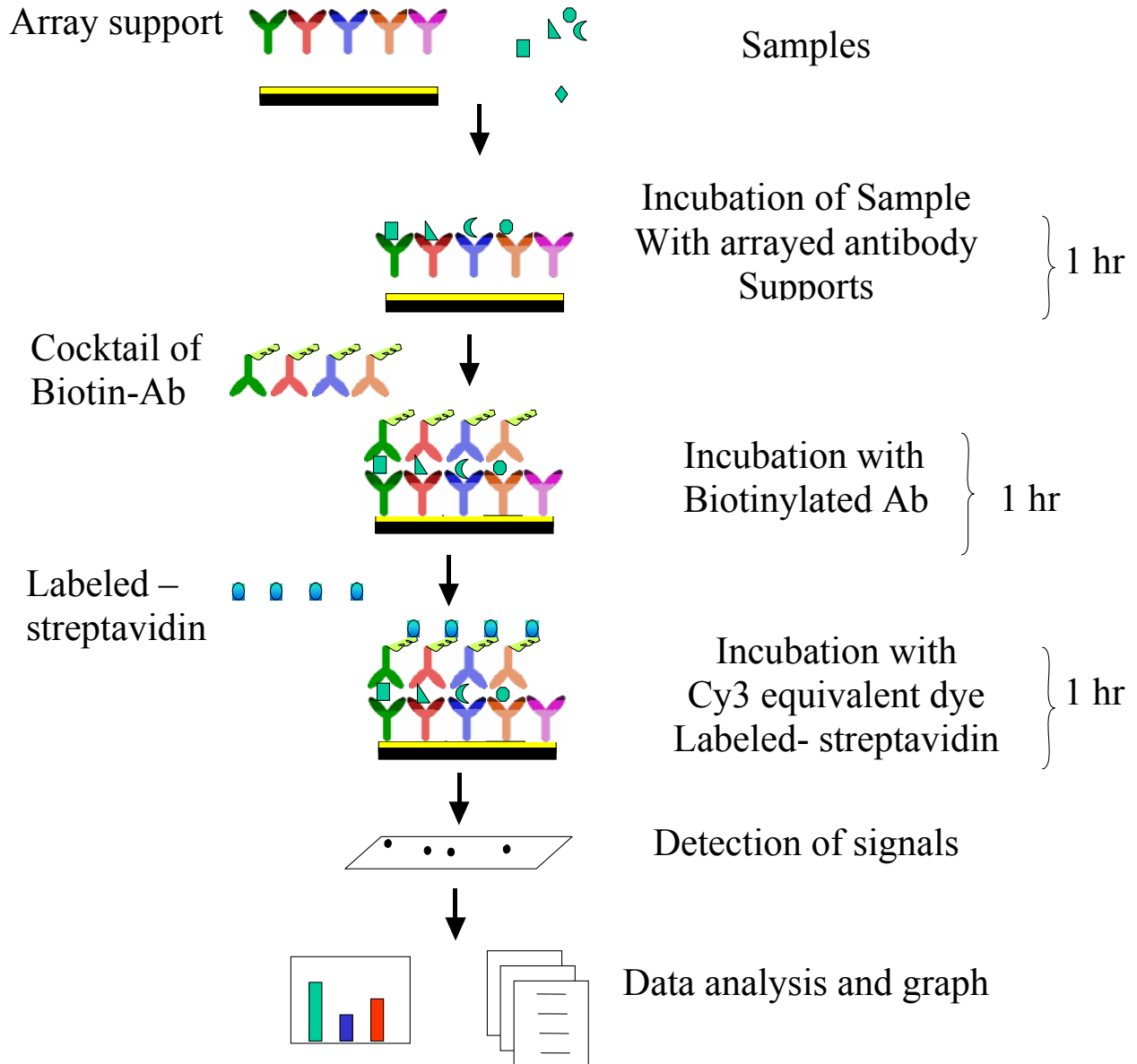
Our new multiplex Quantibody[®] Array is another quantum leap forward in protein microarray technology. This glass-chip-based multiplexed sandwich ELISA system enables researchers to accurately determine the concentration of 10 cytokines simultaneously. The system is relatively rapid and simple compared to the traditional ELISAs, which requires large sample volumes and significant processing time. Furthermore, with this system, 48 times more data can be obtained in four hours and with as little as 50 μ l of samples.

Included in the kit is a glass slide on which 16 arrays of antibody have been spotted through the use of a non-contact arrayer. A specially designed 16 well gasket has been attached to slide to delineate the wells and aid in the application of samples. Each of the 10 cytokine specific capture antibody is arrayed in quadruplicate, together with positive and negative controls. The kit also provides a purified cytokine standard mixture of these 10 cytokines, whose concentration has been predetermined. The serial dilution of the cytokine standard will be used to generate a five-point standard curve.

During the procedure, standard cytokines and samples are assayed in each well simultaneously through a method similar to a sandwich-ELISA. The signals will then be detected using a fluorescence-based detection method for consistency and reliability. By comparing signals from unknown samples to the standard curve generated for each of the 10 cytokines, the unknown cytokine concentration in the samples will be determined. The standard curve requires six wells (including a blank), leaving 10 wells for experimental samples.

The kit provides a highly sensitive approach to simultaneously detect 10 cytokine expression levels from cell culture supernatant, patient's serum, tissue lysate and other sources. The sensitivity of each of the 10 cytokines is within Pico gram range. The experimental procedure is simple and can be performed in any laboratory.

How it works



II. Materials Provided

Upon receipt, all components of the Quantibody® Human Angiogenesis Array 1 kit should be stored at -20°C. At -20°C the kit will retain complete activity for up to 6 months. Once thawed, the glass chip, cytokine standard mix, detection antibody cocktail and Cy3 equivalent dye-conjugated Streptavidin should be kept at -20°C and all other components should be stored at 4°C. The entire kit should be used within 6 months of purchase.

Components:

Item	Description	1 Slide	2 slides
1	Quantibody® Human Angiogenesis I Glass Chip	1	2
2	Sample Diluent	1	1
3	20X Wash Buffer I	2	3
4	20X Wash Buffer II	1	1
5	Lyophilized human cytokine standard mix *	1	1
6	Detection antibody cocktail	1	2
7	Cy3 equivalent dye-conjugated Streptavidin	1	2
8	Slide Washer/Dryer	1	1
9	Adhesive device sealer	5	10
10	Manual	1	1

**Contains the following amount of antigens per vial (pg)*

Antigen	Angiogenin	ANG-2	EGF	bFGF	HB-EGF	HGF	Leptin	PDGF-BB	PIGF	VEGF
Amount (pg)	2000	4000	100	4000	4000	4000	4000	1000	4000	4000

Additional Materials Required

- Orbital shaker
- Laser scanner for fluorescence detection
- Aluminum foil
- Distilled water
- 1.5ml Polypropylene microcentrifuge tubes

III. General Considerations

A. Preparation of Samples

- Use serum-free conditioned media if possible.
- If serum-containing conditioned media is required, it is highly recommended that complete medium be used as a control since many types of sera contains cytokines.
- We recommend the following parameters for your samples:
 - 50 to 100 µl of cell culture supernatant
 - or 50 to 100 µl of original or 5-fold diluted serum or plasma
 - or 20-200 µg of protein for cell lysates and tissue lysates.

If you experience high background or the readings exceed the detection range, further dilution your sample is recommended.

B. Handling glass chips

- Do not touch the surface of the slides, as the microarray slides are very sensitive. Hold the slides by the edges only.
- Handle all buffers and slides with latex free gloves.
- Avoid breaking glass slide.
- Handle glass chip in clean environment.

C. Incubation

- Completely cover array area with sample or buffer during incubation.
- Avoid foaming during incubation steps.
- Perform all incubation and wash steps under gentle rotation.
- Cover the incubation chamber with adhesive film during incubation, particularly when incubation is more than 2 hours or <70 µl of sample or reagent is used.
- Avoid cross-contamination from overflowing solution to neighboring wells.
- Several incubation steps such as step 6 (blocking), step 7 (sample incubation), step 10 (Detection antibody incubation) or step 13 (Cy3 equivalent dye-streptavidin incubation) may be done at 4⁰C for overnight. Please make sure to cover the incubation chamber tightly to prevent evaporation.

IV. Protocol

A. Complete air dry the glass chip

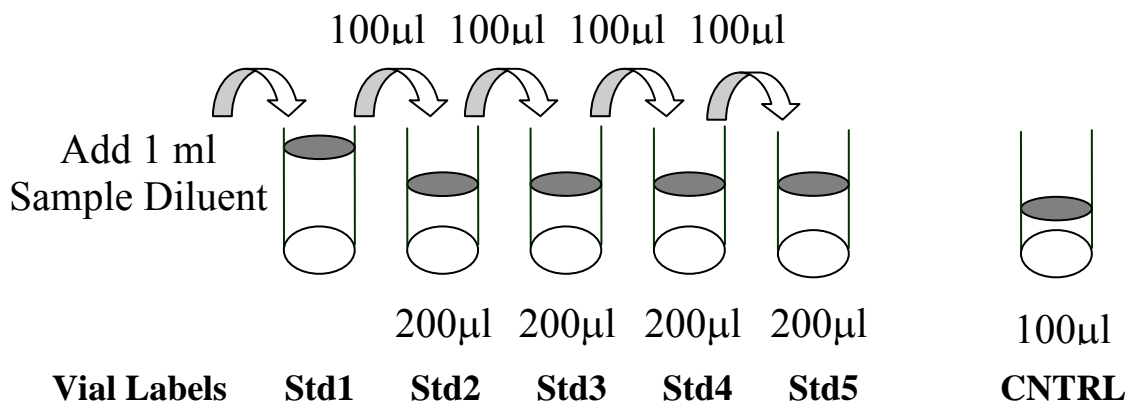
1. Take out the glass chip from the box; remove it from the plastic bag; peel off the covering film, and let it air dry at room temperature for at least 1-2 hours.

Note: Incomplete drying of slides before use may cause the formation of “comet tails”.

B. Prepare Cytokine Standard Dilutions

Note: There is only one vial of standard provided in the two-slide kit, which is enough for making two standard curves. Reconstitute the lyophilized standard within one hour of usage. If you must use the standard for two different days, store only the Std1 dilution at -80°C .

Prepare serial dilution of cytokine standards



2. Reconstitute the Human Cytokine Standard Mix (lyophilized) by adding 1 ml Sample Diluent to the tube. Dissolve the powder thoroughly by a gentle mix. Labeled the tube as Std1.
3. Label 4 clean microcentrifuge tubes as Std 2 to Std 5. Add 200µl Sample Diluent to each of the tubes.

4. Pipette 100µl Std1 into tube Std2 and mix gently. Perform 3 more serial dilutions by adding 100ul Std2 to tube Std3 and so on.
 5. Add 100µl Sample Diluent to another tube labeled as CNTRL. Do not add standard cytokines or sample to the CNTRL tube, which will be used as negative control.
- *Note: Since the starting concentration of each cytokine is different, the serial concentrations from Std1 to Std5 for each cytokine are varied which can be found in section VI.*

C. Blocking and Incubation

6. Add 100µl Sample Diluent into each well and incubate at room temperature for 30 min to block slides.
7. Decant buffer from each well. Add 100µl standard cytokines or samples to each well. Incubate arrays at room temperature for 1 hour.

Note: The sample volume can be 50-100 µl. If sample volume is less than 70 µl, cover the gasket with adhesive sealer to prevent evaporation during incubation.

*Note: We recommend using 50 to 100 µl of conditioned media or 50 to 100 µl of original or 2-5 fold diluted serum or plasma or 10-200 µg of protein for cell lysates and tissue lysates. **Dilute the lysate at least 10 folds with Sample Diluent to make a total volume of 50 to 100 µl.. Make sure there is no bubble in the wells.***

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

Note: Incubation may be done at 4⁰C for overnight.

8. Decant the samples from each well, and wash 5 times with 200 µl of 1x Wash Buffer I and then 2 times with 200 µl of 1x Wash Buffer II at

room temperature with gentle shaking. Completely remove wash buffer in each wash step.

Note: avoid solution flowing into neighboring wells.

9. Reconstitute the Detection Antibody by adding 1.4 ml of Sample Diluent to the tube. Spin briefly.

Note: the diluted Detection antibodies can be stored at 4⁰C for 2-3 days.

10. Add 80 µl of the detection antibody cocktail to each well. Incubate at room temperature for 1 hour.

Note: incubation may be done at 4⁰C for overnight.

11. Wash as directed in step 8.

12. After briefly spinning down, add 1.4 ml of Sample Diluent to Cy3 equivalent dye-conjugated streptavidin tube. Mix gently.

13. Add 80 µl of Cy3 equivalent dye-conjugated streptavidin to each well. Cover the device with aluminum foil to avoid exposure to light or incubate in dark room. Incubate at room temperature for 1 hour.

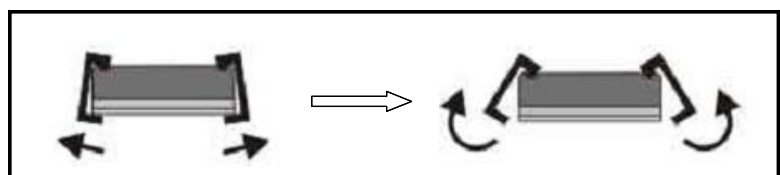
Note: incubation may be done at 4⁰C for overnight.

14. Wash four times with 1x Wash Buffer I.

D. Fluorescence Detection

15. 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



16. Place the slide in the slide washer (50 ml centrifuge tube), add enough 1x Wash Buffer I (about 40 ml) to cover the whole slide, and then gently shake at room temperature for 15 minutes. Decant Wash Buffer I. Wash with 1x Wash Buffer II (about 40 ml) with gentle, and gently shake at room temperature for 5 minutes.

Note: This step can be done using slide chamber.

17. Decant Wash Buffer II and remove water droplets by centrifuging at 1,000 rpm for 3 minutes without cap.

Note: After the rinse step, proceed immediately for the drying step to prevent the deposit of the watermarks on the slide.

18. The signals can be visualized through use of a laser scanner such as Axon GenePix. The settings should be: Excitation: 555 nm; Emission: 565 nm; Resolution: 10 μ m. Make sure that the signal from the standard well containing the highest concentration (Std1) receives the highest possible reading, yet remains unsaturated. Save the image as a high resolution (16-bit) .tif file.

Note: In case the signal intensity for different cytokine varies greatly in the same array, we recommend using multiple scans for the low signal ones.

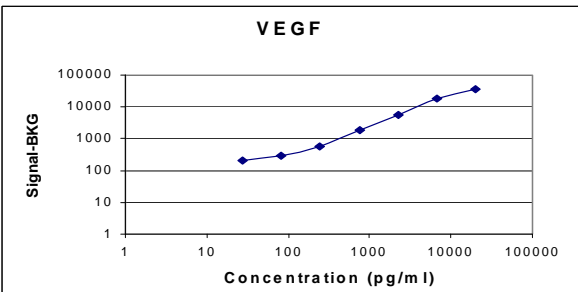
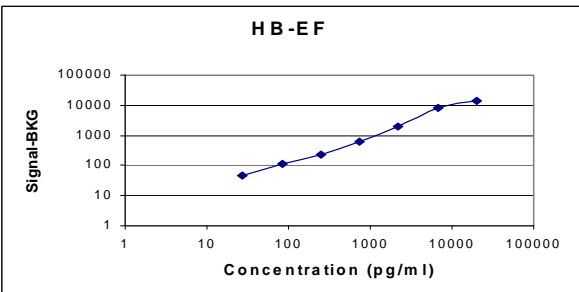
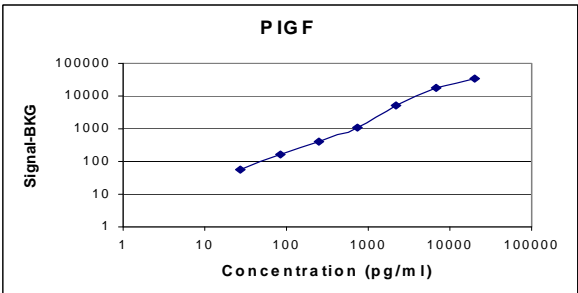
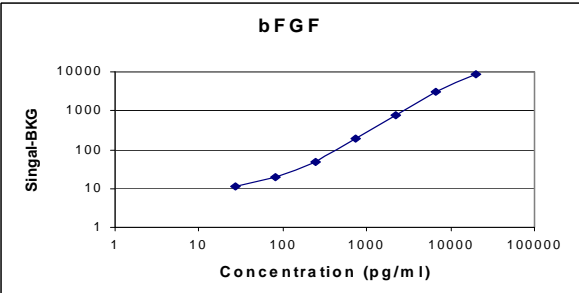
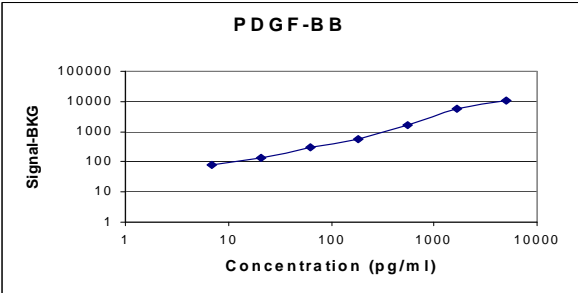
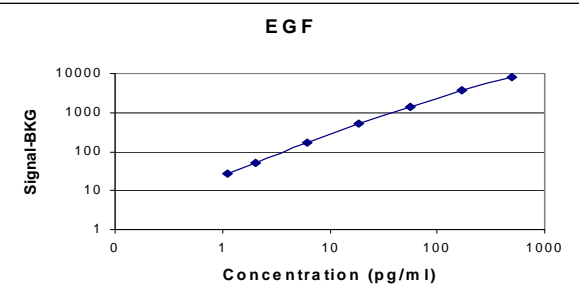
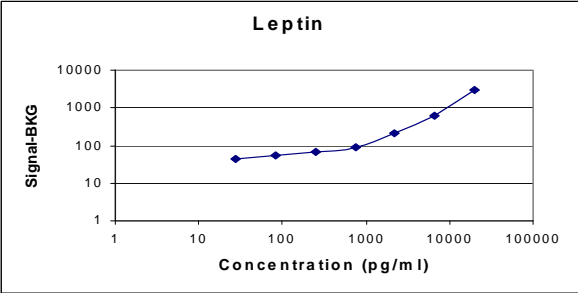
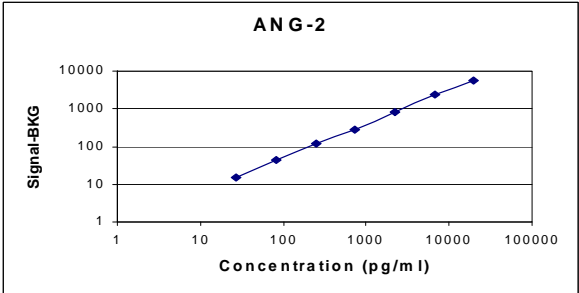
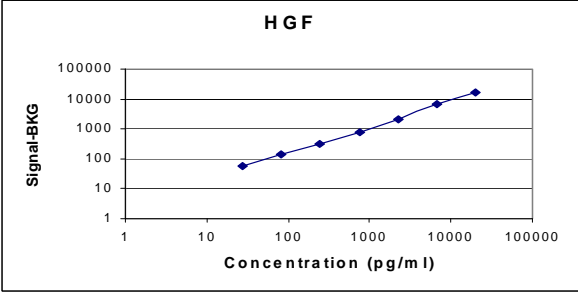
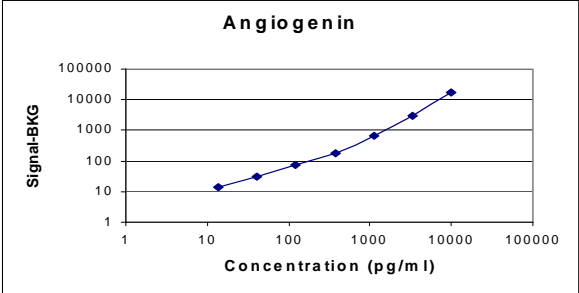
Note: we recommend scanning slide right after experiment. You can also store the slide at 4⁰C in a dry dark container for several days. If you do not have a laser scanner, RayBiotech can provide service for you. Just simply send your slide to us and we will take care of it.

E. Data Analysis

19. Data extraction can be done with most of the microarray analysis software (GenePix, ScanArray Express, ArrayVision, or MicroVigene). For quantitative data analysis, our RayBio[®] Q Analyzer software is available. It gives visual output as well as digital value. More information can be found in section VIII.

V. Standard Curve Range for Individual Cytokines

Log-log plot of Signal Intensity vs. Cytokine Concentration (pg/ml)



VI. System Performance

The Quantibody[®] Human Angiogenesis Array I Kit has been rigorously tested for its performance including: specificity, sensitivity, reproducibility and spiking recovery.

Specificity

The antibody pairs used in the kit have been tested to recognize their specific cytokines. Analysis of samples containing only a single recombinant cytokine found no cross-reactivity with other proteins.

Sensitivity

While the standard curve for a given cytokine ranges from 49 to 4000 pg/ml for most of the cytokines, the quantifiable range for each cytokine can be extended up to five-time wider (see section V). The sensitivity of each cytokine, which is defined as the corresponding concentration at two standard deviations above the median fluorescence of 20 replicates of the negative control (0 pg/ml), is listed at the following table.

Serial standard concentration (pg/ml) and assay sensitivity

(pg/ml)	Std1	Std2	Std3	Std4	Std5	Control	Median Fluorescence	Standard Deviation	Sensitivity (pg/ml)
Angiogenin	2000	667	222	74	25	0	70	11	14.0
ANG-2	4000	1333	444	148	49	0	101	9	31.5
EGF	100	33	11	4	1	0	97	11	0.8
bFGF	4000	1333	444	148	49	0	43	10	46.2
HB-EGF	4000	1333	444	148	49	0	37	12	16.2
HGF	4000	1333	444	148	49	0	35	5	4.8
Leptin	4000	1333	444	148	49	0	310	15	36.7
PDGF-BB	1000	333	111	37	12	0	183	9	1.4
PIGF	4000	1333	444	148	49	0	42	9	9.9
VEGF	4000	1333	444	148	49	0	389	20	6.6

Reproducibility

Eight replicates of human serum samples were spiked with different levels of recombinant human cytokines and assayed along side the standards. The cytokine concentrations in each sample were then determined through the RayBio[®] Q Analyzer. The well-to -well CV was found to be <20%.

	Observed antigen (pg/ml)	Standard Deviation	CV	Observed antigen (pg/ml)	Standard Deviation	CV
Angiogenin	19632.1	404.1	2%	19098.2	855.0	4%
ANG-2	3050.8	209.8	7%	1550.1	34.4	2%
EGF	100.9	3.0	3%	57.5	3.4	6%
bFGF	861.4	58.4	7%	65.8	12.5	19%
HB-EGF	1455.9	126.3	9%	72.1	7.2	10%
HGF	1457.6	107.4	7%	237.4	25.2	11%
Leptin	8600.2	685.7	8%	6425.3	164.8	3%
PDGF-BB	1016.1	156.7	15%	455.4	48.0	11%
PIGF	1755.4	136.9	8%	160.6	26.4	16%
VEGF	2257.9	171.1	8%	327.2	19.8	6%

Recovery

Different levels of recombinant human cytokines were spiked into the 2x diluted human serum samples. All samples together with the non-spiked serum sample as negative control were assayed in sextuplet along side the standards. The recovery rate for each cytokine was then determined by subtracting the endogenous cytokine level from the observed cytokine value and divided by the spiking cytokine concentration.

Angiogenin			
Spiking Ag (pg/ml)	2500	278	0
Observed Ag (pg/ml)	20374	18578	18308
Net Ag (pg/ml)	2066	270	0
Recovery Rate	83%	97%	0

ANG-2			
Spiking Ag (pg/ml)	5000	556	0
Observed Ag (pg/ml)	5935	1882	1200
Net Ag (pg/ml)	4735	682	0
Recovery Rate	95%	123%	0

EGF			
Spiking Ag (pg/ml)	125	14	0
Observed Ag (pg/ml)	188	62	50
Net Ag (pg/ml)	138	12	0
Recovery Rate	110%	86%	0

bFGF			
Spiking Ag (pg/ml)	5000	556	0
Observed Ag (pg/ml)	4245	306	7
Net Ag (pg/ml)	4238	299	0
Recovery Rate	85%	54%	0

HB-EGF			
Spiking Ag (pg/ml)	5000	556	0
Observed Ag (pg/ml)	4437	421	2
Net Ag (pg/ml)	4435	419	0
Recovery Rate	89%	75%	0

HGF			
Spiking Ag (pg/ml)	5000	556	0
Observed Ag (pg/ml)	4115	510	68
Net Ag (pg/ml)	4047	442	0
Recovery Rate	81%	79%	0

Leptin			
Spiking Ag (pg/ml)	5000	556	0
Observed Ag (pg/ml)	9920	5534	5028
Net Ag (pg/ml)	4892	506	0
Recovery Rate	98%	91%	0

PDGF-BB			
Spiking Ag (pg/ml)	1250	139	0
Observed Ag (pg/ml)	1897	541	425
Net Ag (pg/ml)	1472	116	0
Recovery Rate	118%	83%	0

PIGF			
Spiking Ag (pg/ml)	5000	556	0
Observed Ag (pg/ml)	4330	500	0
Net Ag (pg/ml)	4330	500	0
Recovery Rate	87%	90%	0

VEGF			
Spiking Ag (pg/ml)	5000	556	0
Observed Ag (pg/ml)	4149	699	111
Net Ag (pg/ml)	4038	588	0
Recovery Rate	81%	106%	0

Calibration

The RayBio® Cytokine standard concentration was determined by and equalized to the following international reference standards.

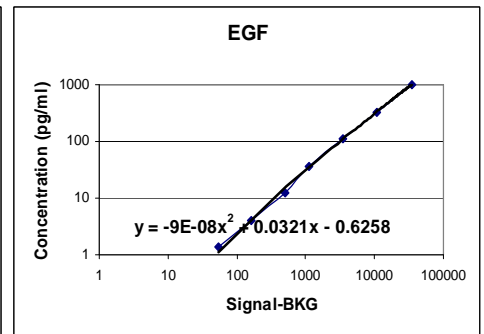
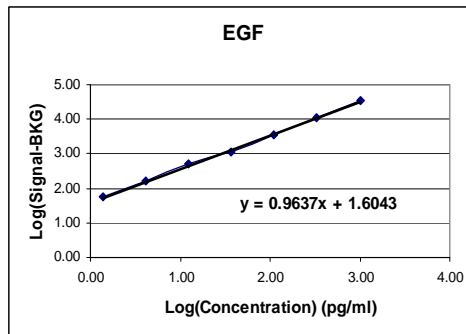
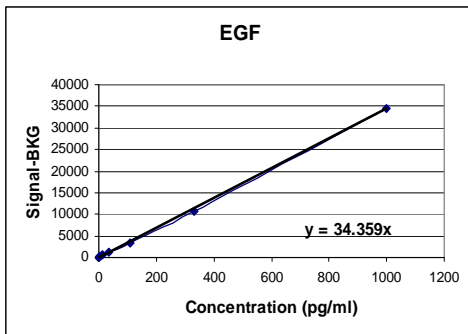
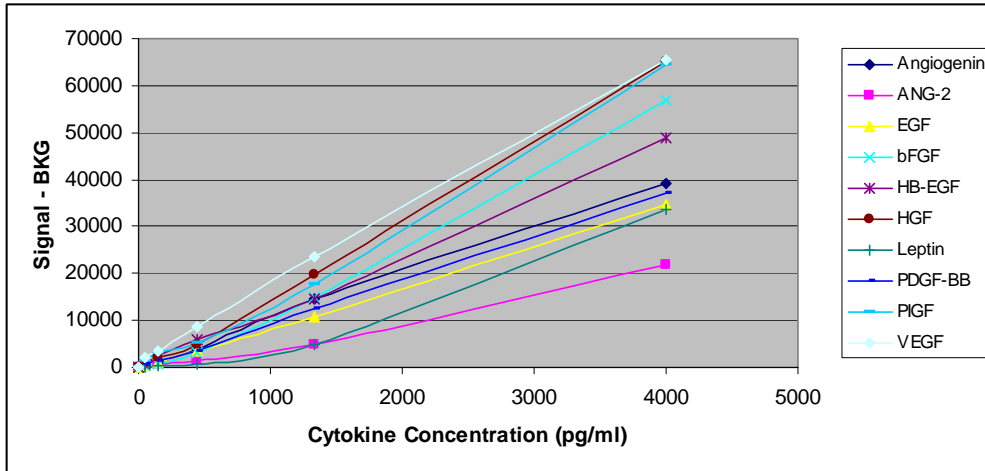
Cytokine	Angiogenin	ANG-2	EGF	bFGF	HB-EGF	HGF	Leptin	PDGF-BB	PIGF	VEGF
Standard	internal	internal	internal	internal	internal	NIBSC	internal	internal	internal	NIBSC
Lot						96/564				02/286

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	Don't make too low dilution or concentrate sample
	Improper storage of kit	Store kit as suggested temperature. Don't freeze/thaw the slide.
Uneven signal	Bubble formed during incubation	Avoid bubble formation during incubation
	Arrays are not completely covered by reagent	Completely cover arrays with solution
	Reagent evaporation	Cover the incubation chamber with adhesive film during incubation
Poor standard curve	Cross-contamination from neighboring wells	Avoid overflowing wash buffer
	Comet tail formation	Air dry the slide for at least 1 hour before usage
	Inadequate detection	Increase laser power that the highest standard concentration for each cytokine receives the highest possible reading yet remains unsaturated.
High background	Overexposure	Lower the laser power
	Insufficient wash	Increase wash time and use more wash buffer
	Dust	Work in clean environment
	Slide is allowed to dry out	Don't dry out slides during experiment.

VIII. Sample Raybio® Q Analyzer Output

Raybio® Q Analyzer greatly facilitates the data analysis. Instead of tedious calculation, user can now quickly figure out the unknown sample concentration through a simple copy and paste process. The program can automatically remove the outlier spots. Best of all, users can choose as many as three algorithms (linear regression, log-log, and 2-order polynomial) to best meet their analytical needs.



Sample Cytokine Concentration (pg/ml) (Base on Linear Regression)								
ID	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8
Angiogenin	0.0	0.0	4.3	23.9	54.7	159.2	685.6	1473.2
ANG-2	0.0	10.5	22.2	80.0	132.1	352.2	975.7	4129.6
EGF	0.0	0.4	0.6	2.1	4.1	10.1	33.6	100.0
bFGF	0.0	8.8	4.8	14.2	46.8	231.7	906.3	4170.2
HB-EGF	0.0	3.6	12.2	60.1	195.7	543.0	1219.5	1818.3
HGF	0.0	2.1	12.6	46.6	107.0	327.5	1147.8	4076.4
Leptin	0.0	0.7	2.4	3.9	12.9	108.0	712.4	4250.0
PDGF-BB	0.0	1.2	4.8	19.1	44.1	116.1	288.6	1014.0
PIGF	0.0	21.0	71.3	384.9	662.2	1619.5	2577.6	3431.2
VEGF	0.0	39.9	124.8	517.7	992.4	1883.2	2965.0	3258.7

IX. Reference List

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Note:

RayBio[®] is the trademark of RayBiotech, Inc.

Cytokine protein arrays are RayBiotech patent-pending technology.

This product is intended for research only and is not to be used for clinical diagnosis. Our products may not be resold, modified for resale, or used to manufacture commercial products without written approval by RayBiotech, Inc.

Under no circumstances shall RayBiotech be liable for any damages arising out of the use of the materials.

Products are guaranteed for three months from the date of purchase when handled and stored properly. In the event of any defect in quality or merchantability, RayBiotech's liability to buyer for any claim relating to products shall be limited to replacement or refund of the purchase price.

X. Experiment Record Form

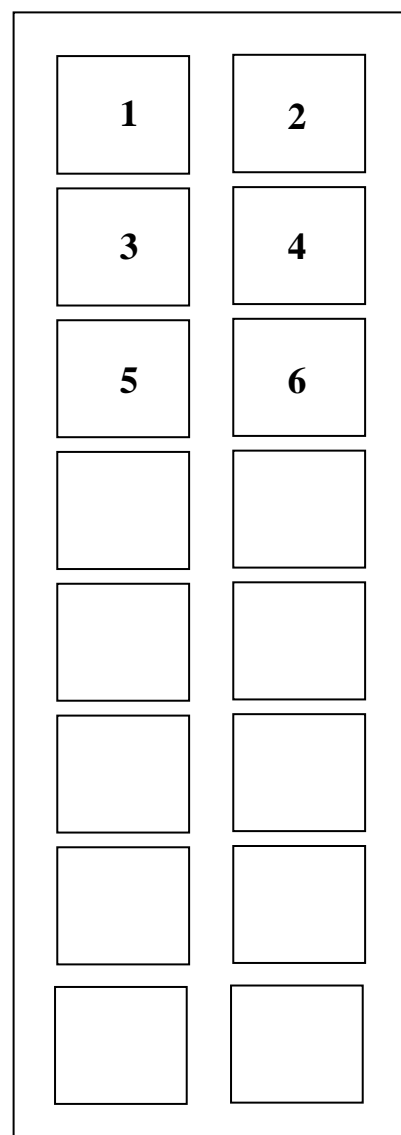
Date: _____

File Name: _____

Laser Power: _____

PMT: _____

Well No.	Sample Name	Dilution factor
1	CNTRL	
2	Std5	
3	Std4	
4	Std3	
5	Std2	
6	Std1	
7		
8		
9		
10		
11		
12		
13		
14		
15		
16		



This product is for research use only.



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