

# **Quantibody® Human Angiogenesis Array 3**

**-Quantitative measurement of 30 Human Angiogenesis factors**

**Patent Pending Technology**

**User Manual (Version Jun08)**

**Cat # QAH-ANG-3**



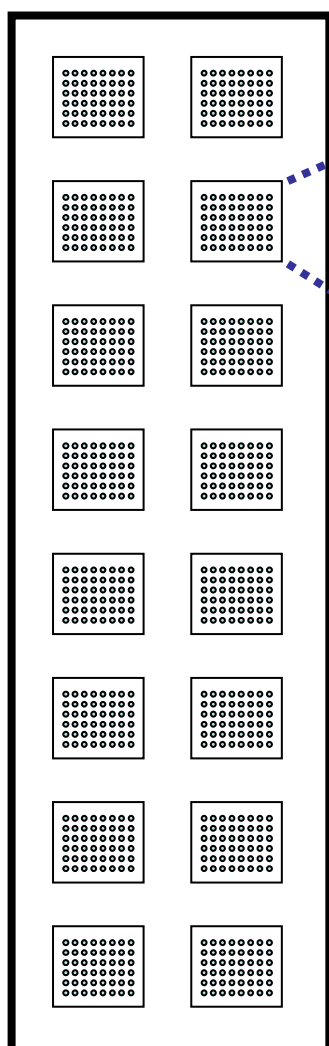
**We Provide You With Excellent  
Protein Array Systems And Service**

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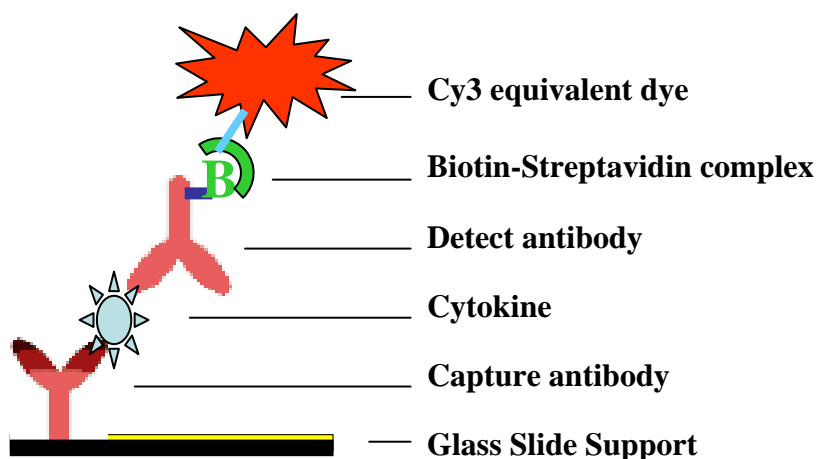
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# Quantibody® Human Angiogenesis Array 3

Cytokines Detected (30)	ANG-1, Angiostatin, CXCL16, EGF, FGF-4, Follistatin, G-CSF, GM-CSF, I-309, IL-1 $\beta$ , IL-4, IL-10, IL-12p40, IL-12p70, I-TAC, MCP-2, MCP-3, MCP-4, MMP-1, MMP-9, PECAM-1, TGF $\alpha$ , TGF $\beta$ 3, Tie-1, Tie-2, uPAR, VEGF, VEGF R2, VEGF R3, VEGF-D
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 equivalent dye
Sample Volume	50 – 100 $\mu$ l
Reproducibility	CV <20%
Assay duration	4 hrs



	1,2,3,4	5,6,7,8	9,10,11,12
a	POS1	POS2	NEG
b	ANG-1	Angiostatin	CXCL16
c	EGF	FGF-4	Follistatin
d	G-CSF	GM-CSF	I-309
e	IL-1b	IL-4	IL-10
f	IL-12p40	IL-12p70	I-TAC
g	MCP-2	MCP-3	MCP-4
h	MMP-1	MMP-9	PECAM-1
i	TGF $\alpha$	TGF $\beta$ 3	Tie-1
j	Tie-2	uPAR	VEGF
k	VEGF R2	VEGF R3	VEGF-D





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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<sup>®</sup> 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.

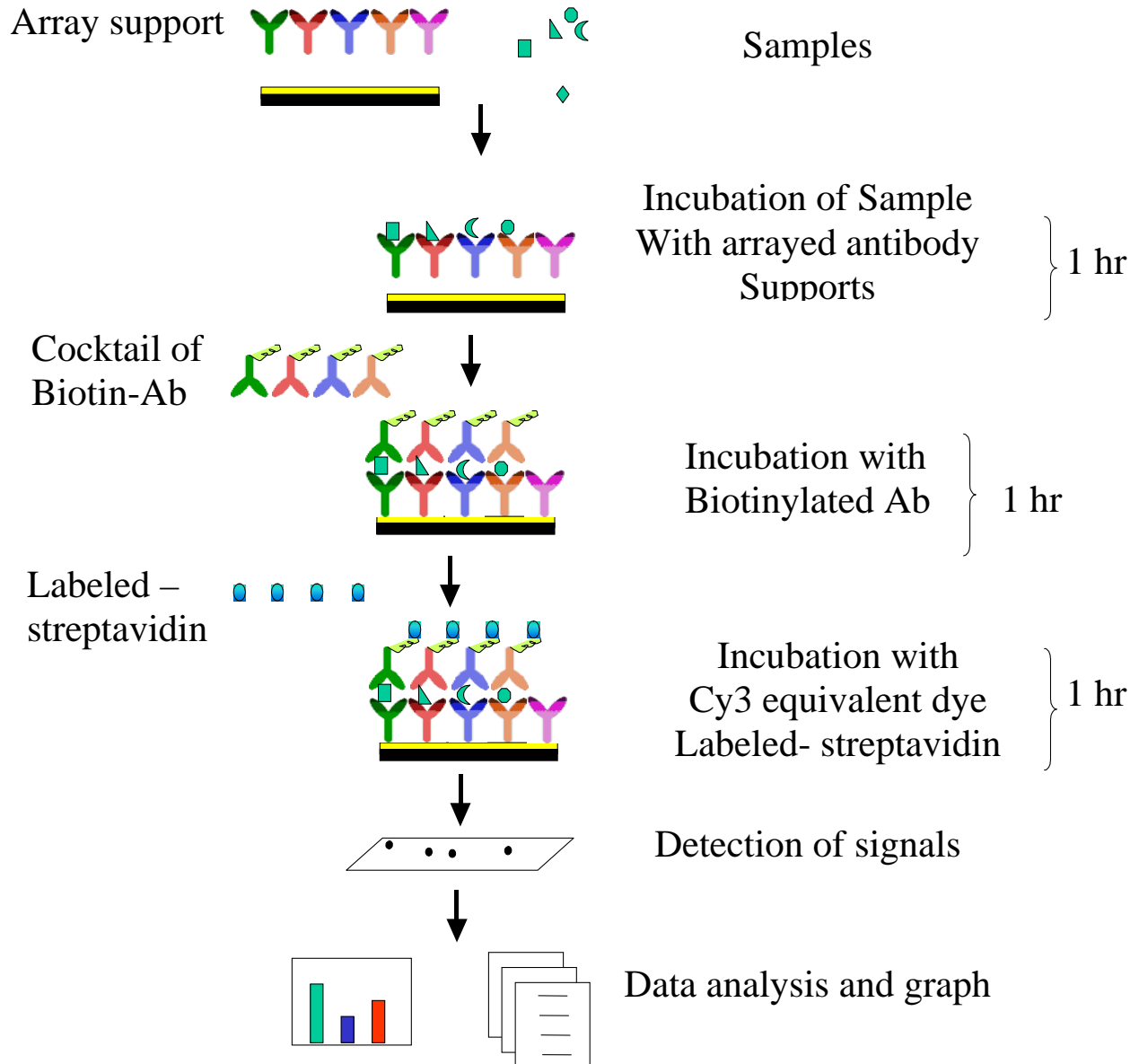
Our new multiplex Quantibody<sup>®</sup> Array is another quantum leap forward in protein microarray technology. This glass-chip-based multiplexed sandwich ELISA system enables researchers to accurately determine the concentration of 30 cytokines simultaneously. The system is relatively rapid and simple compared to the traditional ELISAs, which requires large sample volumes and significant processing time. Furthermore, with this system, 132 times more data can be obtained in four hours and with as little as 50  $\mu$ 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 30 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 30 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 30 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 30 cytokine expression levels from cell culture supernatant, patient's serum, tissue lysate and other sources. The sensitivity of each of the 30 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 the components of the Quantibody® Array 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 kit	2-Slide kit
1	Quantibody® Array 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 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 (ng)*

Antigen	ng/vial	Antigen	ng/vial	Antigen	ng/vial
ANG-1	25	IL-4	0.5	PECAM-1	10
Angiostatin	1000	IL-10	0.5	TGF $\alpha$	5
CXCL16	1	IL-12p40	5	TGF $\beta$ 3	20
EGF	0.05	IL-12p70	0.25	Tie-1	250
FGF-4	50	I-TAC	1	Tie-2	10
Follistatin	50	MCP-2	0.5	uPAR	20
G-CSF	5	MCP-3	2	VEGF	5
GM-CSF	1	MCP-4	20	VEGF R2	5
I-309	2	MMP-1	10	VEGF R3	10
IL-1 $\beta$	0.5	MMP-9	10	VEGF-D	20

### 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 100µl of original or diluted serum, plasma or cell culture supernatant or 20-500 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 4<sup>0</sup>C for overnight. Please make sure to cover the incubation chamber tightly to prevent evaporation.

## IV. Protocol

### A. Complete air dry the glass chip

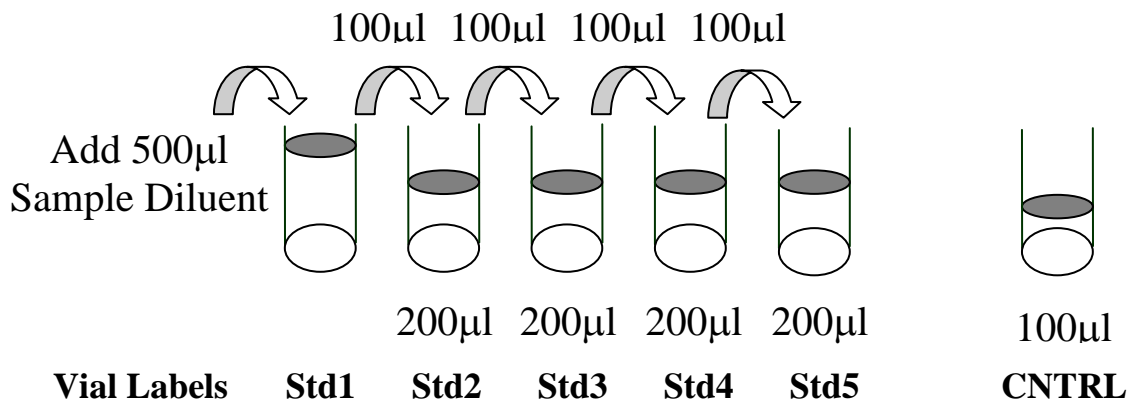
1. Take out the glass chip from the box; remove it from the plastic bag after equilibrating to the room temperature; then peel off the covering film, and let it air dry at room temperature for at least 1-2 hours.

*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  $-80^{\circ}\text{C}$ .*

#### Prepare serial dilution of cytokine standards



2. Reconstitute the Cytokine Standard Mix (lyophilized) by adding 500µl 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. Incubation may be done at 4<sup>0</sup>C for overnight.*

*Note: We recommend using 50 to 100 µl of original or diluted serum, plasma or conditioned media or 20-500 µg of protein for cell lysates and tissue lysates. **In order to minimize the matrix effects and to lower the background of the assay, we recommended that the samples at least diluted 2 folds with Sample Diluent. Dilute the lysate at least 5 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.*

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 4<sup>0</sup>C 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 4<sup>0</sup>C 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.

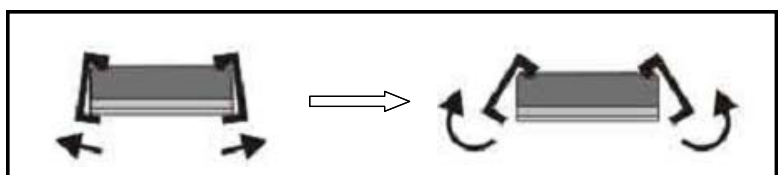
*Note: incubation may be done at 4<sup>0</sup>C for overnight.*

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 equipped with a cy3 wavelength such as Axon GenePix. The settings should be: Excitation: 555 nm; Emission: 565 nm; Resolution: 10 um. Make sure that the signal from the well containing the highest standard concentration (Std1) receives the highest possible reading, yet remains unsaturated. Saved the image as a high resolution (16-bit) .tif file.

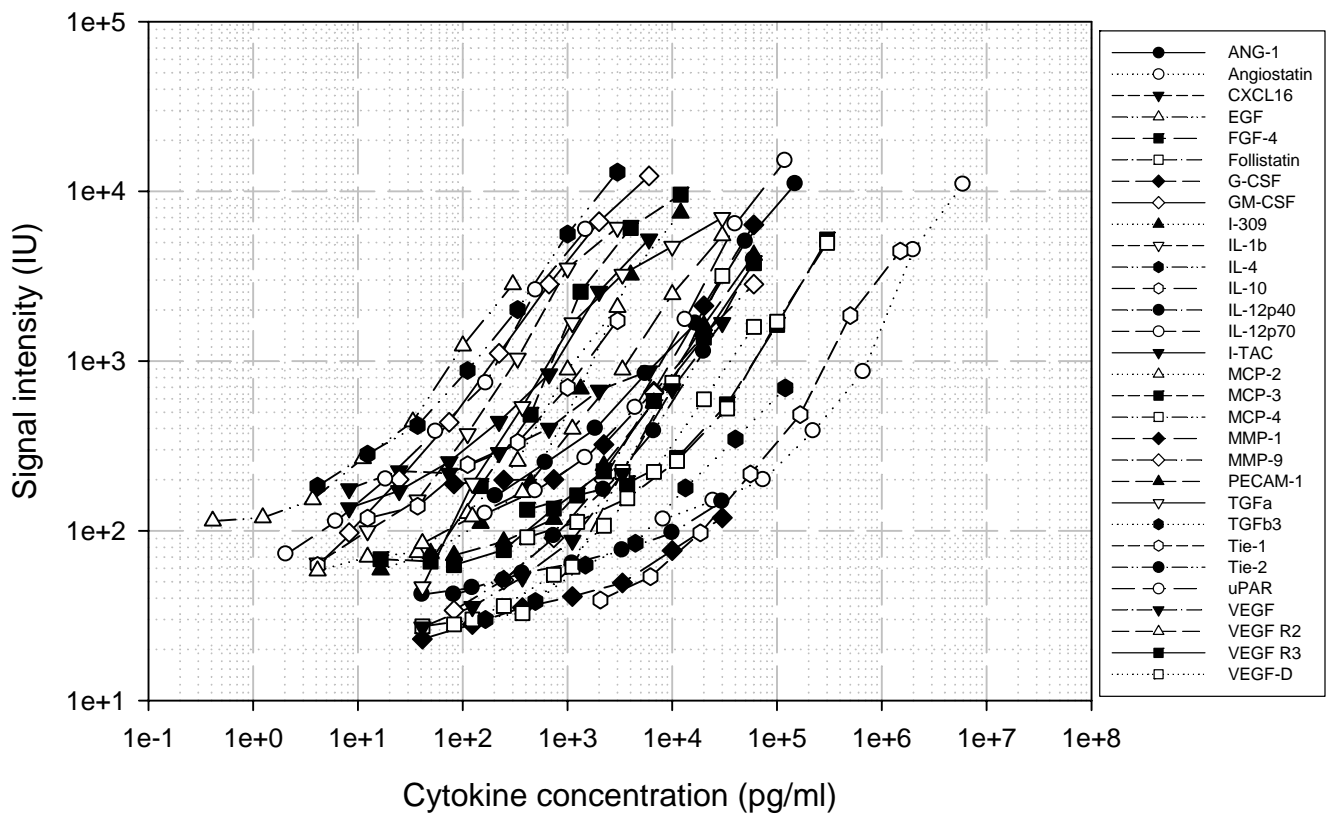
*Note: In case the signal intensity for different cytokine varies greatly in the same array, we recommend using multiple scans for the low signal ones. We recommend scanning slide right after experiment. You can also store the slide at 4<sup>0</sup>C in a dry dark container for several days. If you do 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<sup>®</sup> 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 for Individual Cytokines

### Quantibody Angiogenesis Array 3 Standard Curves



## VI. System Performance

The performance of Quantibody<sup>®</sup> Human Angiogenesis Array 3 Kit has been tested in the following areas: specificity, sensitivity, and spiking recovery.

### Specificity

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

## Sensitivity

The five-point cytokine concentration used for generating the standard curve of a given antigen was listed below. The sensitivity of each protein, which is defined as the corresponding concentration at two standard deviations above the average 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)	CNTRL	Std5	Std4	Std3	Std2	Std1	Average	Stdev	LOD (pg/ml)
ANG-1	0	617	1,852	5,556	16,667	50,000	198	11	228
Angiostatin	0	24,691	74,074	222,222	666,667	2,000,000	135	9	2,722
CXCL16	0	25	74	222	667	2,000	168	14	13
EGF	0	1	4	11	33	100	124	4	1
FGF-4	0	1,235	3,704	11,111	33,333	100,000	152	6	843
Follistatin	0	1,235	3,704	11,111	33,333	100,000	101	6	767
G-CSF	0	123	370	1,111	3,333	10,000	31	4	67
GM-CSF	0	25	74	222	667	2,000	70	11	7
I-309	0	49	148	444	1,333	4,000	28	5	13
IL-1 $\beta$	0	12	37	111	333	1,000	68	7	4
IL-4	0	12	37	111	333	1,000	171	17	6
IL-10	0	12	37	111	333	1,000	89	4	13
IL-12p40	0	123	370	1,111	3,333	10,000	46	6	54
IL-12p70	0	6	19	56	167	500	63	6	2
I-TAC	0	25	74	222	667	2,000	146	6	10
MCP-2	0	12	37	111	333	1,000	60	5	8
MCP-3	0	49	148	444	1,333	4,000	45	8	10
MCP-4	0	494	1,481	4,444	13,333	40,000	20	4	119
MMP-1	0	247	741	2,222	6,667	20,000	190	8	172
MMP-9	0	247	741	2,222	6,667	20,000	15	4	110
PECAM-1	0	247	741	2,222	6,667	20,000	62	4	103
TGF $\alpha$	0	123	370	1,111	3,333	10,000	7	7	25
TGF $\beta$ 3	0	494	1,481	4,444	13,333	40,000	31	2	482
Tie-1	0	6,173	18,519	55,556	166,667	500,000	40	5	2,842
Tie-2	0	247	741	2,222	6,667	20,000	33	4	145
uPAR	0	494	1,481	4,444	13,333	40,000	145	13	171
VEGF	0	123	370	1,111	3,333	10,000	37	4	126
VEGF R2	0	123	370	1,111	3,333	10,000	82	5	43
VEGF R3	0	247	741	2,222	6,667	20,000	41	5	146
VEGF-D	0	494	1,481	4,444	13,333	40,000	29	3	109

## Recovery

The recovery of the Human antigens by the kit was tested through spiking different levels of the recombinant proteins in 4x diluted Human serum H4522 and 4x diluted Human cell culture media (CM). The non-spiked serum sample and cell culture media were used as negative control. The recovery rate for each antigen was then determined by subtracting the endogenous antigen level from the observed value and divided by the spiking antigen concentration.

### *The spiking recovery rate for human culture media and serum*

ID	Spiking	SA	SA+Ag	SA%	CM	CM+Ag	CM%
<b>ANG-1</b>	25000	9154.8	31374.0	88.9%	6.0	27090.1	108.3%
<b>Angiostatin</b>	1000000	1970046.0	3112128.7	114.2%	16122.7	931280.5	91.5%
<b>CXCL16</b>	1000	3640.4	4727.8	108.7%	6.2	1088.9	108.3%
<b>EGF</b>	50	3.7	38.8	70.1%	0.0	44.0	88.1%
<b>FGF-4</b>	50000	6758.0	57752.2	102.0%	0.0	42143.7	84.3%
<b>Follistatin</b>	50000	6597.8	44620.6	76.0%	0.0	60538.6	121.1%
<b>G-CSF</b>	5000	141.9	3798.4	73.1%	18.1	5362.6	106.9%
<b>GM-CSF</b>	1000	14.8	852.6	83.8%	0.0	1080.0	108.0%
<b>I-309</b>	2000	88.2	2338.3	112.5%	32.1	1831.6	90.0%
<b>IL-1<math>\beta</math></b>	500	27.8	522.0	98.8%	6.1	419.3	82.7%
<b>IL-4</b>	500	44.9	586.3	108.3%	2.4	568.8	113.3%
<b>IL-10</b>	500	129.1	612.5	96.7%	0.0	556.6	111.3%
<b>IL-12p40</b>	5000	21.5	3916.8	77.9%	5.4	4698.1	93.9%
<b>IL-12p70</b>	250	16.5	284.7	107.3%	0.0	219.6	87.9%
<b>I-TAC</b>	1000	187.8	966.3	77.9%	0.0	1087.3	108.7%
<b>MCP-2</b>	500	17.5	508.4	98.2%	0.0	638.2	127.6%
<b>MCP-3</b>	2000	39.7	2273.7	111.7%	4.6	2237.3	111.6%
<b>MCP-4</b>	20000	14.9	22912.4	114.5%	6.2	20560.4	102.8%
<b>MMP-1</b>	10000	1050.6	9430.5	83.8%	0.0	10753.6	107.5%
<b>MMP-9</b>	5000	7898.5	12474.2	91.5%	60.3	4859.6	96.0%
<b>PECAM-1</b>	10000	974.5	9702.0	87.3%	2.9	8362.2	83.6%
<b>TGF<math>\alpha</math></b>	5000	7.6	6055.2	121.0%	13.6	5472.1	109.2%
<b>TGF<math>\beta</math>3</b>	20000	2309.6	22793.1	102.4%	434.7	21549.5	105.6%
<b>Tie-1</b>	250000	12900.5	256689.5	97.5%	4828.3	247573.7	97.1%
<b>Tie-2</b>	10000	1290.6	9622.7	83.3%	581.5	9920.5	93.4%
<b>uPAR</b>	20000	3157.3	17410.8	71.3%	828.1	18571.3	88.7%
<b>VEGF</b>	5000	4339.3	9168.1	96.6%	1751.6	7486.6	114.7%
<b>VEGF R2</b>	5000	3533.1	7232.9	74.0%	206.9	5057.3	97.0%
<b>VEGF R3</b>	10000	235.4	6914.8	66.8%	69.3	8616.7	85.5%
<b>VEGF-D</b>	20000	469.0	21507.9	105.2%	530.1	24198.6	118.3%

## **Calibration**

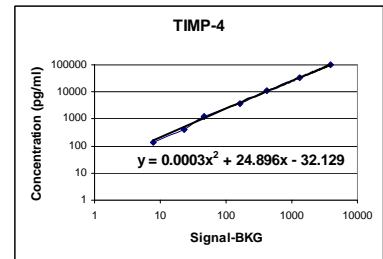
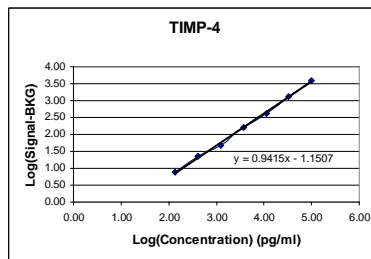
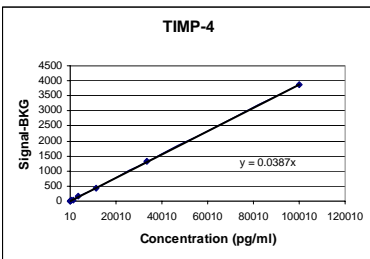
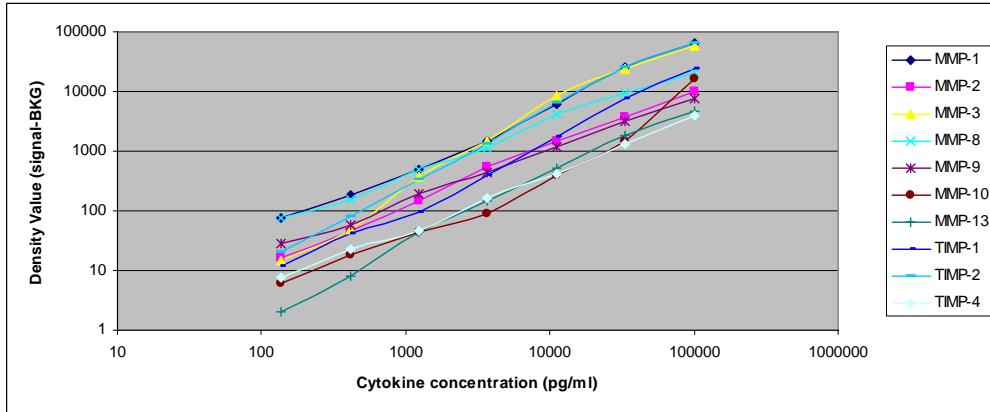
The RayBio® Cytokine standard concentration was determined by the internal cytokine standards.

## VII. Troubleshooting guide

<b>Problem</b>	<b>Cause</b>	<b>Recommendation</b>
<b>Weak Signal</b>	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.
<b>Uneven signal</b>	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
<b>Poor standard curve</b>	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.
	Use freeze-thawed cytokine standards	Always use new cytokine standard vial for new set of experiment. Discard any leftover.
<b>High background</b>	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, and users can choose either linear regression or log-log algorithms 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
MMP-1	0	538	3,626	8,202	43,812	96,822	551	2,122
MMP-2	0	69	7,066	7,479	26,460	46,335	1,496	2,802
MMP-3	0	6	1,362	3,573	21,670	25,902	30,364	966
MMP-8	0	234	917	2,029	9,986	18,793	1,409	678
MMP-9	0	2,891	9,740	6,013	14,510	27,976	37,079	19,933
MMP-10	0	10,952	16,428	37,660	116,437	406,305	320,779	56,489
MMP-13	0	1,021	1,293	4,167	10,277	18,553	1,056	1,797
TIMP-1	0	1,356	1,111	2,759	6,923	19,838	71,685	9,304
TIMP-2	0	234	131	1,876	4,139	20,897	133,539	5,705
TIMP-4	0	2,288	5,917	8,094	25,384	46,641	16,342	3,912

## IX. Reference List

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# X. Experiment Record Form

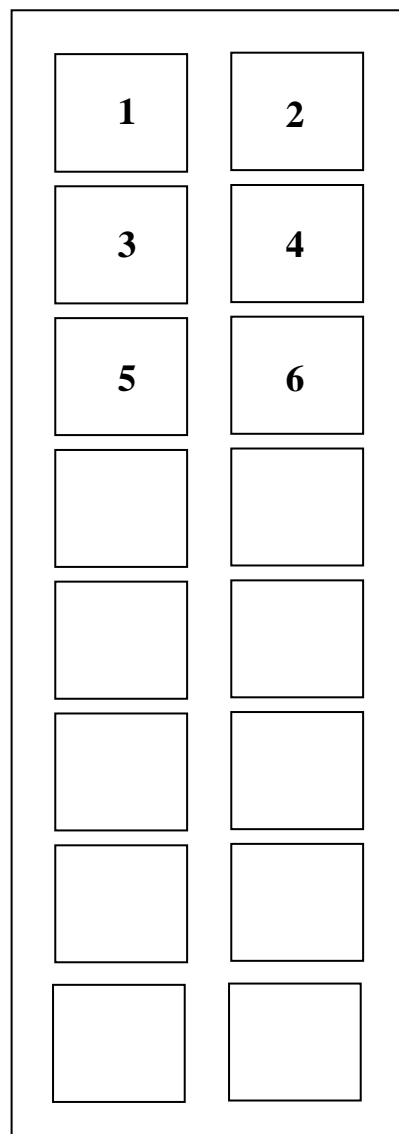
Date: \_\_\_\_\_

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		



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