

Quantibody® Human Receptor Array 1

-Quantitative measurement of 40 Human Cytokines

Patent Pending Technology

User Manual (Version Dec08)

Cat # QAH-REC-1

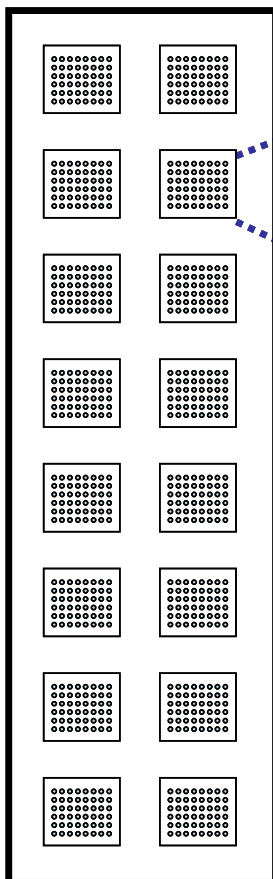


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Protein Array Systems And Service**

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Quantibody[®] Human Receptor Array 1

Cytokines Detected (40)	4-1BB, ALCAM, B7-1, BCMA, CD14, CD30, CD40L, CEACAM-1, DR6, Dtk, Endoglin, ErbB3, E-Selectin, Fas, Flt-3L, GTR, HVEM, ICAM-3, IL-1 R4, IL-1 RI, IL-10 R β , IL-17R, IL-2R γ , IL-21R, LIMPII, Lipocalin-2, L-Selectin, LYVE-1, MICA, MICB, NRG1- β 1, PDGF R β , PECAM-1, RAGE, TIM-1, TRAIL R3, Trappin-2, uPAR, VCAM-1, XEDAR
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 μ l
Reproducibility	CV <20%
Assay duration	4 hrs



	1,2,3,4	5,6,7,8	9,10,11,12
a	POS	NEG	4-1BB
b	ALCAM	B7-1	BCMA
c	CD14	CD30	CD40 L
d	CEACAM-1	DR6	Dtk
e	Endoglin	ErbB3	E-Selectin
f	Fas	Flt-3L	GTR
g	HVEM	ICAM-3	IL-1 R4
h	IL-1 RI	IL-2 R γ	IL-10 R β
i	IL-17R	IL-21R	LIMPII
j	Lipocalin-2	L-Selectin	LYVE-1
k	MICA	MICB	NRG1-b1
l	PDGF R β	PECAM-1	RAGE
m	TIM-1	TRAIL R3	Trappin-2
n	uPAR	VCAM-1	XEDAR

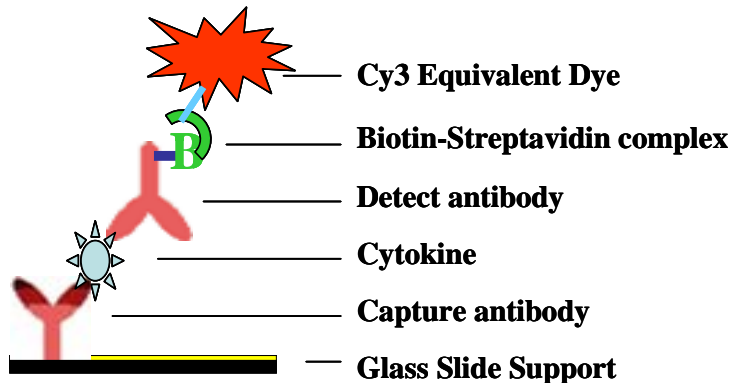




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I. Introduction

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.

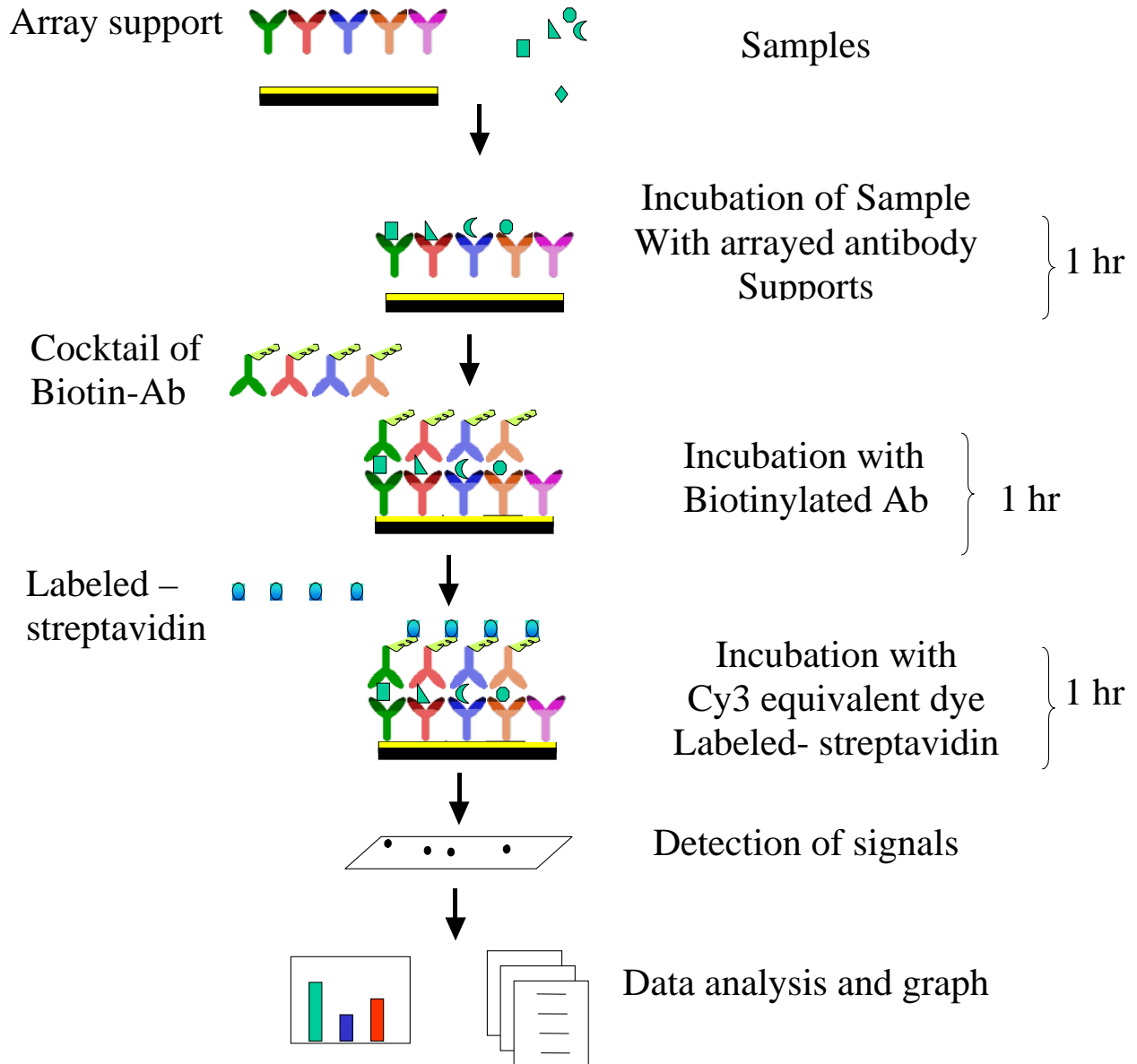
Our new multiplex Quantibody[®] Array is another quantum leap forward in protein microarray technology. This glass-chip-based multiplexed sandwich ELISA system enables researchers to accurately determine the concentration of 40 cytokines simultaneously. The system is relatively rapid and simple compared to the traditional ELISAs, which requires large sample volumes and significant processing time. Furthermore, with this system, 168 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 40 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 40 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 40 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 40 cytokine expression levels from cell culture supernatant, patient's serum, tissue lysate and other sources. The sensitivity of each of the 40 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	Antigen	ng/vial
4-1BB	5	Endoglin	2	IL-2 R γ	5	NRG1- β 1	7.5
ALCAM	5	ErbB3	5	IL-10 R β	2	PDGF R β	50
B7-1	5	E-Selectin	20	IL-17R	2	PECAM-1	10
BCMA	10	Fas	1	IL-21R	10	RAGE	5
CD14	5	Flt-3L	1	LIMPII	2	TIM-1	5
CD30	5	GITR	5	Lipocalin-2	0.5	TRAIL R3	2.5
CD40 L	5	HVEM	20	L-Selectin	20	Trappin-2	5
CEACAM-1	5	ICAM-3	50	LYVE-1	1	uPAR	20
DR6	2	IL-1 R4	2	MICA	5	VCAM-1	100
Dtk	10	IL-1 RI	2	MICB	7.5	XEDAR	5

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 4⁰C for overnight. Please make sure to cover the incubation chamber tightly to prevent evaporation.

IV. Protocol

A. Complete air dry the glass chip

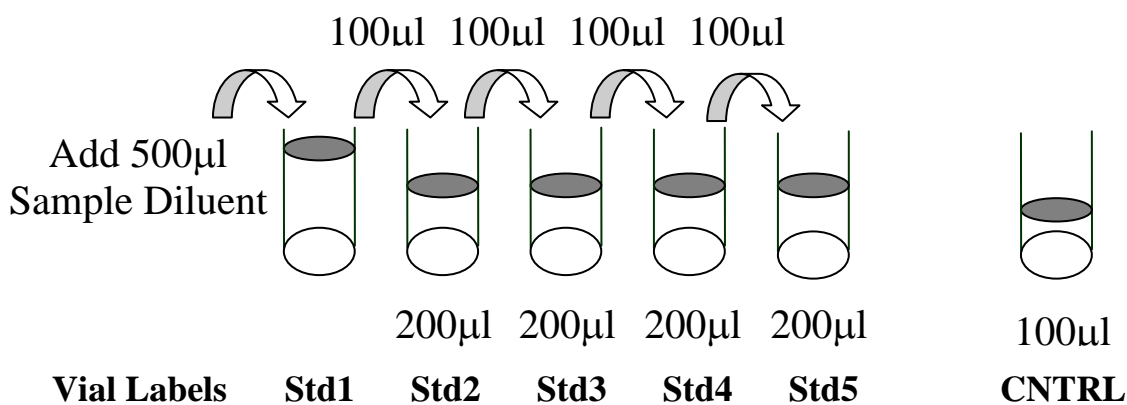
1. Take out the glass chip from the box, and let it equilibrate to room temperature inside the sealed plastic bag for 20-30 minutes. Remove it from the plastic bag; peel off the covering film, and let it air dry at room temperature for another 1-2 hours.

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 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 4⁰C 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 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.

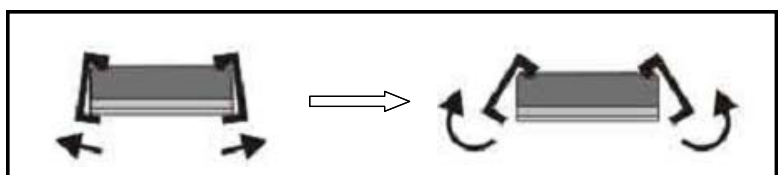
Note: incubation may be done at 4⁰C for overnight.

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 equipped with a cy3 wavelength 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 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.

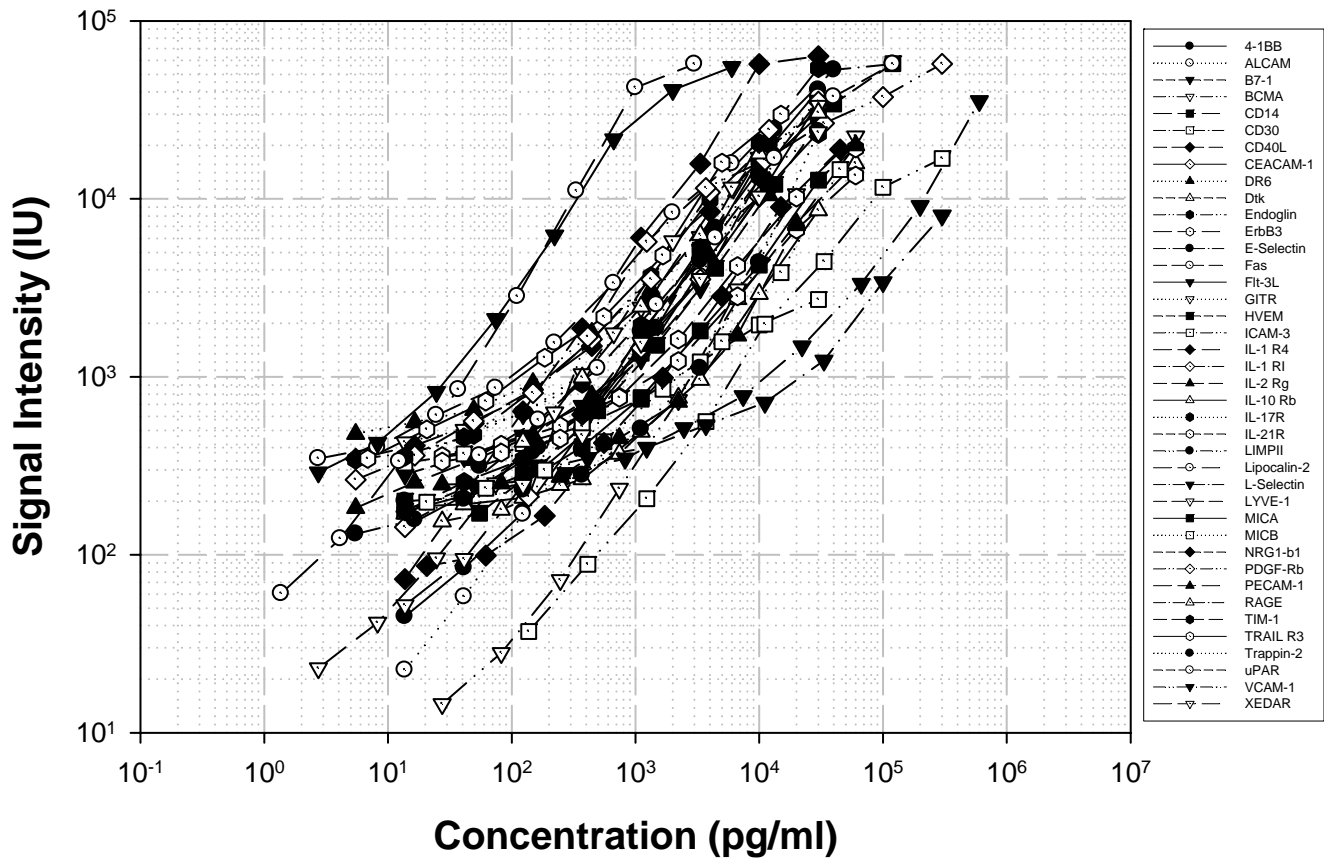
Note: In case the signal intensity for different cytokine varies greatly in the same array, we recommend using multiple scans for the low signal ones. We recommend scanning slide right after experiment. You can also store the slide at 4⁰C in a dry dark container for several days. If you do not have a 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 for Individual Cytokines

Quantibody Human Receptor Array 1



VI. System Performance

The performance of Quantibody[®] Human Receptor Array 1 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)	Control	Std5	Std4	Std3	Std2	Std1	Average (IU)	Stdev	Sensitivity
4-1BB	0	123	370	1,111	3,333	10,000	11	1	12
ALCAM	0	123	370	1,111	3,333	10,000	44	2	39
B7-1	0	123	370	1,111	3,333	10,000	258	65	135
BCMA	0	247	741	2,222	6,667	20,000	8	1	87
CD14	0	123	370	1,111	3,333	10,000	135	15	117
CD30	0	123	370	1,111	3,333	10,000	347	73	148
CD40 L	0	123	370	1,111	3,333	10,000	28	10	55
CEACAM-1	0	123	370	1,111	3,333	10,000	134	41	179
DR6	0	49	148	444	1,333	4,000	606	34	44
Dtk	0	247	741	2,222	6,667	20,000	106	11	70
Endoglin	0	49	148	444	1,333	4,000	214	13	35
ErbB3	0	123	370	1,111	3,333	10,000	423	27	107
E-Selectin	0	494	1,481	4,444	13,333	40,000	267	17	234
Fas	0	25	74	222	667	2,000	365	73	21
Flt-3L	0	25	74	222	667	2,000	247	74	27
GITR	0	123	370	1,111	3,333	10,000	331	83	97
HVEM	0	494	1,481	4,444	13,333	40,000	77	31	91
ICAM-3	0	1,235	3,704	11,111	33,333	100,000	26	13	358
IL-1 R4	0	49	148	444	1,333	4,000	477	47	39
IL-1 RI	0	49	148	444	1,333	4,000	246	16	52
IL-2 P γ	0	123	370	1,111	3,333	10,000	315	16	116
IL-10 R β	0	49	148	444	1,333	4,000	199	8	48
IL-17R	0	49	148	444	1,333	4,000	80	63	37
IL-21R	0	247	741	2,222	6,667	20,000	200	25	154
LIMPII	0	49	148	444	1,333	4,000	116	27	44
Lipocalin-2	0	12	37	111	333	1,000	16	13	7
L-Selectin	0	494	1,481	4,444	13,333	40,000	153	29	450
LYVE-1	0	25	74	222	667	2,000	5	1	10
MICA	0	123	370	1,111	3,333	10,000	125	37	62
MICB	0	185	556	1,667	5,000	15,000	209	55	163
NRG1- β 1	0	185	556	1,667	5,000	15,000	28	8	124
PDGF R β	0	1,235	3,704	11,111	33,333	100,000	381	34	370
PECAM-1	0	247	741	2,222	6,667	20,000	171	5	301
RAGE	0	123	370	1,111	3,333	10,000	98	12	13
TIM-1	0	123	370	1,111	3,333	10,000	101	16	107
TRAIL R3	0	62	185	556	1,667	5,000	166	22	89
Trappin-2	0	123	370	1,111	3,333	10,000	166	16	128
uPAR	0	494	1,481	4,444	13,333	40,000	538	67	465
VCAM-1	0	2,469	7,407	22,222	66,667	200,000	280	28	2,363
XEDAR	0	123	370	1,111	3,333	10,000	6	8	29

Recovery

The recovery of the Human antigens by the kit was tested through spiking different levels of the recombinant proteins in 10x diluted Human serum H4522 and 2x 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. (See next page)

Calibration

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

The spiking recovery rate for human culture media and serum

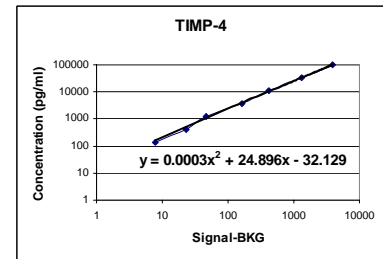
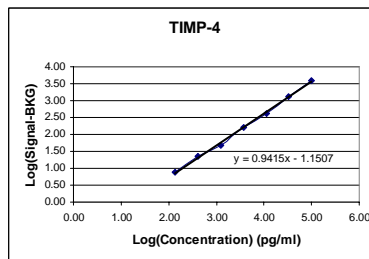
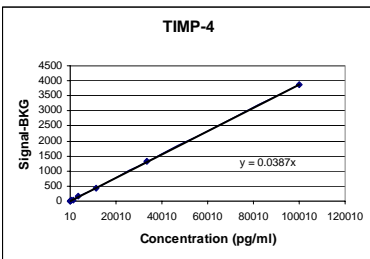
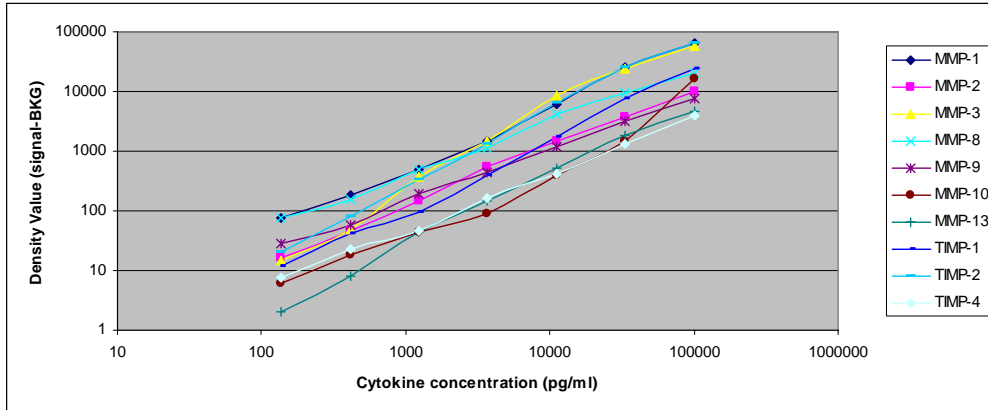
(pg/ml)	Spiking	H4522	H4522+Ag	H4522%	CM	CM+Ag	CM%
4-1BB	5,000	0	6,452	129%	0	4,992	100%
ALCAM	5,000	0	6,887	138%	0	6,823	136%
B7-1	10,000	0	6,986	70%	0	11,297	113%
BCMA	10,000	608	10,833	102%	0	12,026	120%
CD14	5,000	19,397	12,171	-	0	5,838	117%
CD30	5,000	0	4,827	97%	0	6,194	124%
CD40 L	5,000	0	4,746	95%	0	6,917	138%
CEACAM-1	5,000	1,324	5,744	88%	0	4,808	96%
DR6	2,000	0	2,248	112%	0	2,667	133%
Dtk	10,000	0	8,280	83%	0	13,417	134%
Endoglin	2,500	308	3,287	119%	0	2,924	117%
ErbB3	5,000	1,397	7,567	123%	0	6,089	122%
E-Selectin	20,000	2,800	20,043	86%	0	21,082	105%
Fas	1,000	118	1,040	92%	0	855	86%
Fit-3L	1,000	126	1,510	138%	257	1,374	112%
GITR	5,000	0	5,560	111%	0	6,529	131%
HVEM	20,000	0	23,546	118%	0	14,850	74%
ICAM-3	50,000	0	68,316	137%	0	55,961	112%
IL-1 R4	2,000	0	1,782	89%	0	2,123	106%
IL-1 RI	2,000	0	1,642	82%	0	1,528	76%
IL-2 R γ	5,000	0	5,149	103%	0	6,094	122%
IL-10 R β	2,000	0	2,346	117%	0	2,204	110%
IL-17R	2,000	0	2,575	129%	0	2,705	135%
IL-21R	10,000	1,567	9,777	82%	0	7,687	77%
LIMPII	2,000	0	2,239	112%	0	1,700	85%
Lipocalin-2	500	1,027	933	-	0	409	82%
L-Selectin	20,000	47,128	48,703	-	1,248	22,463	106%
LYVE-1	1,000	2,383	2,559	-	0	1,280	128%
MICA	5,000	571	5,310	95%	0	3,823	76%
MICB	7,500	0	6,836	91%	0	6,456	86%
NRG1- β 1	7,500	0	8,906	119%	0	9,013	120%
PDGF R β	50,000	0	60,513	121%	0	44,877	90%
PECAM-1	10,000	1,022	11,828	108%	530	9,915	94%
RAGE	5,000	0	6,772	135%	0	6,064	121%
TIM-1	5,000	0	4,514	90%	0	5,331	107%
TRAIL R3	2,500	0	2,897	116%	0	2,528	101%
Trappin-2	5,000	3,579	8,896	106%	0	5,640	113%
uPAR	20,000	0	24,255	121%	0	27,715	139%
VCAM-1	100,000	73,262	212,854	140%	0	72,652	73%
XEDAR	5,000	0	4,873	97%	0	5,070	101%

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.
	Use freeze-thawed cytokine standards	Always use new cytokine standard vial for new set of experiment. Discard any leftover.
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, 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

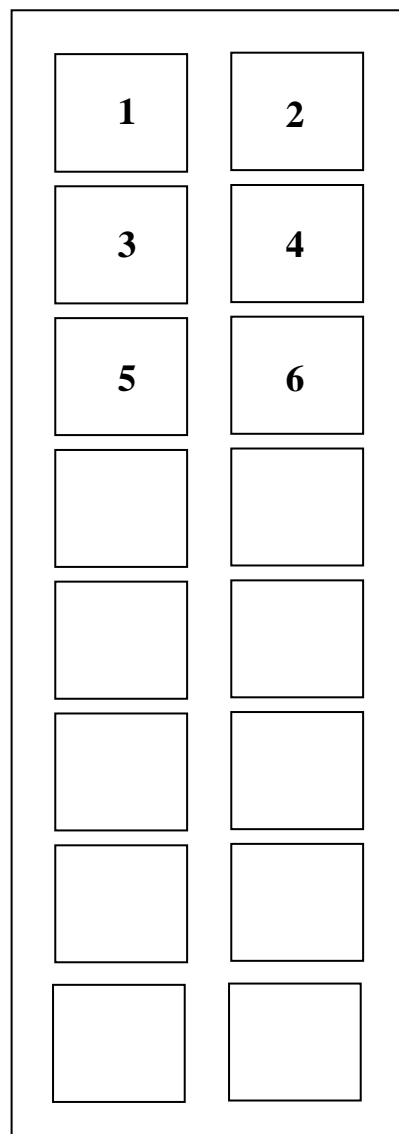
Date: _____

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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