Quantibody® Mouse Cytokine Array 2

-Quantitative measurement of 20 Mouse cytokines

Patent Pending Technology

User Manual (Version Aug 07)

Cat # QAM-CYT-2

RayBiotech, Inc.

We Provide You With Excellent
Protein Array Systems And Service

Tel:(Toll Free) 1-888-494-8555 or 770-729-2992; Fax: 1-888-547-0580;
Website:www.raybiotech.com Email: info@raybiotech.com

Quantibody® Mouse Cytokine Array 2

Cytokines (20)	Axl, BLC, CD30, CD40, CXCL16, Eotaxin-2, Fas Ligand, IGFBP-3, IGFBP-5, LIX, L-Selectin, MIG, MIP-1α, MIP-1γ, PF-4, P-Selectin, SDF-1α, TCA-3, sTNF RII, VCAM-1
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 µl
Reproducibility	CV <20%
Assay duration	4 hrs

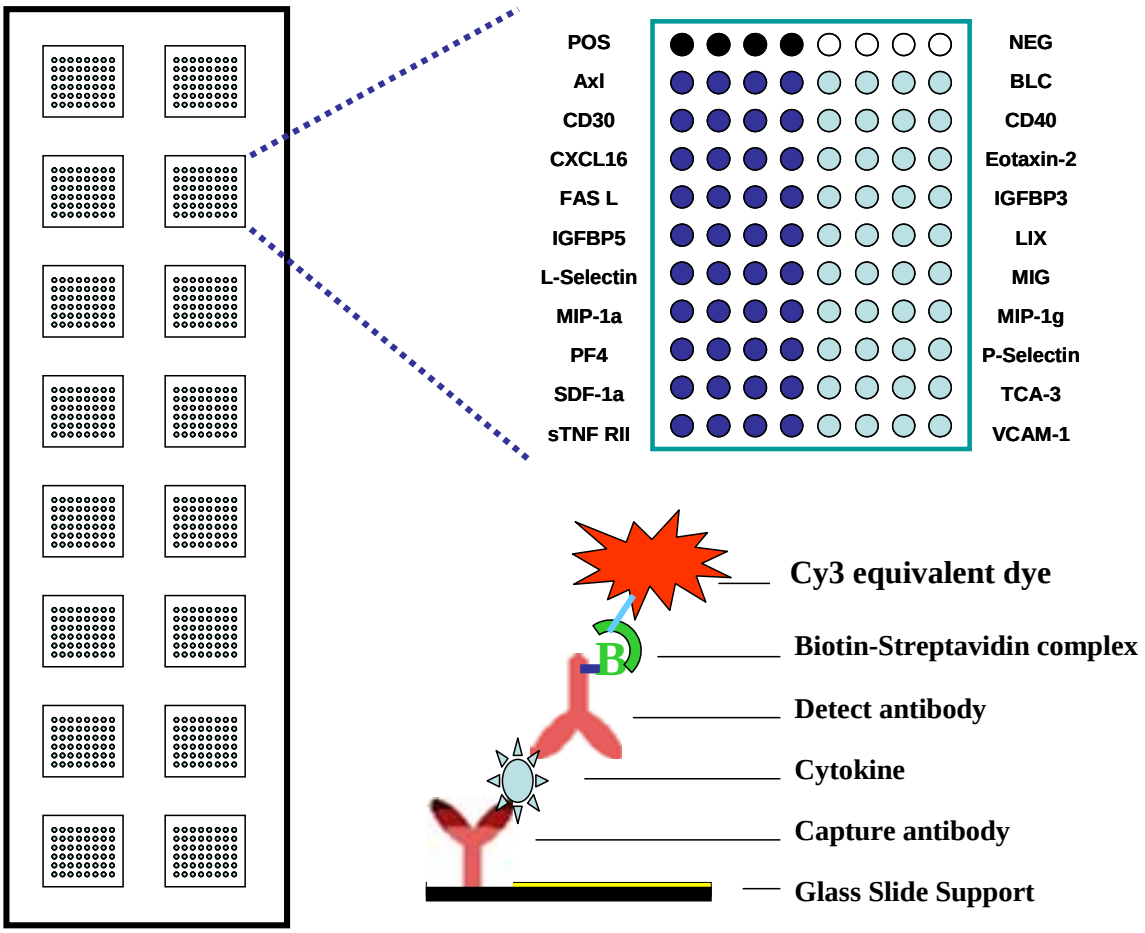


TABLE OF CONTENTS

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.

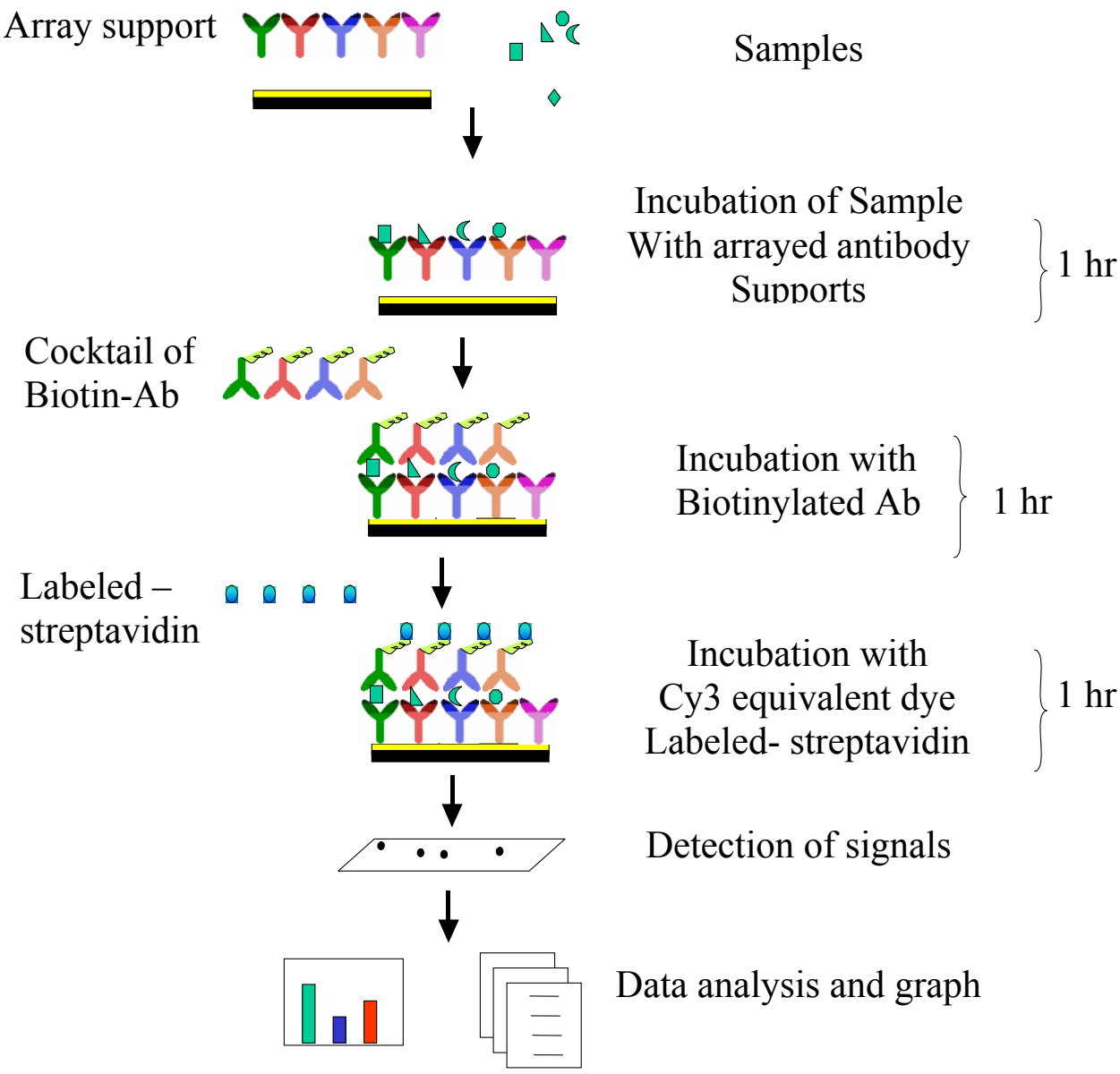
Our new multiplex Quantibody® Array is another quantum leap forward in protein microarray technology. This glass-chip-based multiplexed sandwich ELISA system enables researchers to accurately determine the concentration of 20 cytokines simultaneously. The system is relatively rapid and simple compared to the traditional ELISAs, which requires large sample volumes and significant processing time. Furthermore, with this system, 96 times more data can be obtained in four hours and with as little as 50 µl 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 20 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 20 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 20 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 20 cytokine expression levels from cell culture supernatant, patient's serum, tissue lysate and other sources. The sensitivity of each of the 20 cytokines is within Pico gram range. The experimental procedure is simple and can be performed in any laboratory.

How it works


Array support
Samples
Incubation of Sample
With arrayed antibody
Supports
1 hr
Cocktail of
Biotin-Ab
Incubation with
Biotinylated Ab
1 hr
Labeled –
streptavidin
Incubation with
Cy3 equivalent dye
Labeled- streptavidin
1 hr
Detection of signals
Data analysis and graph

II. Materials Provided

Upon receipt, all the components of the Quantibody® Array kit should be stored at -20^{0}C. At -20^{0}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^{0}C and all other components should be stored at 4^{0}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)*

Cytokine	Axl	BLC	CD30	CD40	CXCL16	Eotaxtin-2	Fas L	IGFBP3	IGFBP5	LIX
(ng/vial)	2	5	2	1	0.25	0.5	2	10	10	10
Cytokine	L-Selectin	MIG	MIP-1α	MIP-1γ	PF-4	P-Selectin	SDF-1α	TCA-3	sTNF RII	VCAM-1
(ng/vial)	20	5	5	0.5	20	10	20	0.5	0.5	4

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 20-200 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 4^0C for overnight. Please make sure to cover the incubation chamber tightly to prevent evaporation.

IV. Protocol

A. Complete air dry the glass chip

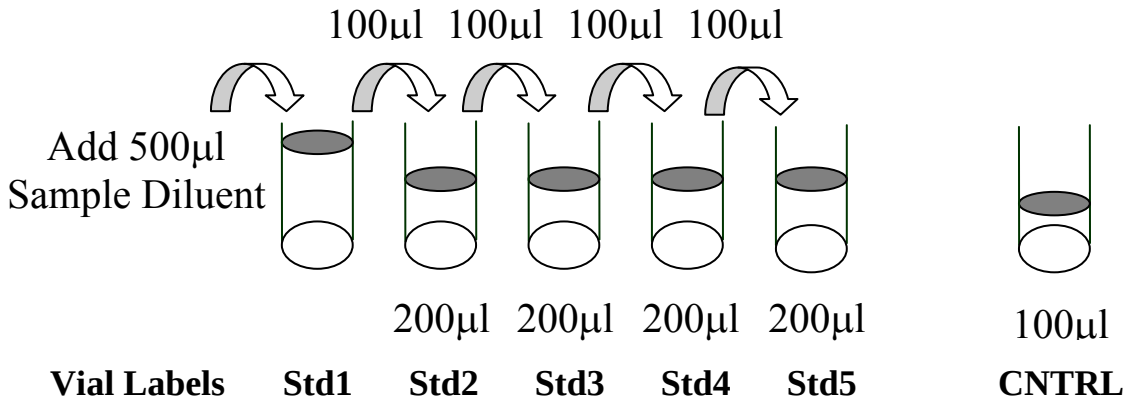
1. Take out the glass chip from the box; remove it from the plastic bag; peel off the covering film, and let it air dry at room temperature for at least 1-2 hours.

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 ^{0}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 4^0C for overnight.

Note: We recommend using 50 to 100 µl of original or diluted serum, plasma or conditioned media or 20-200 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 4^0C 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 4^0C 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.

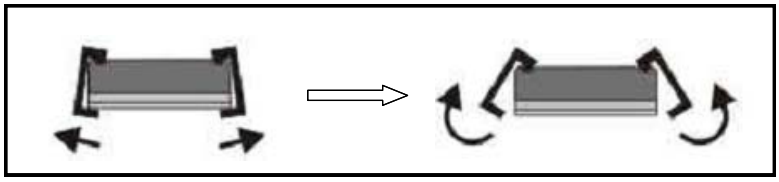
Note: incubation may be done at 4^0C for overnight.

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.

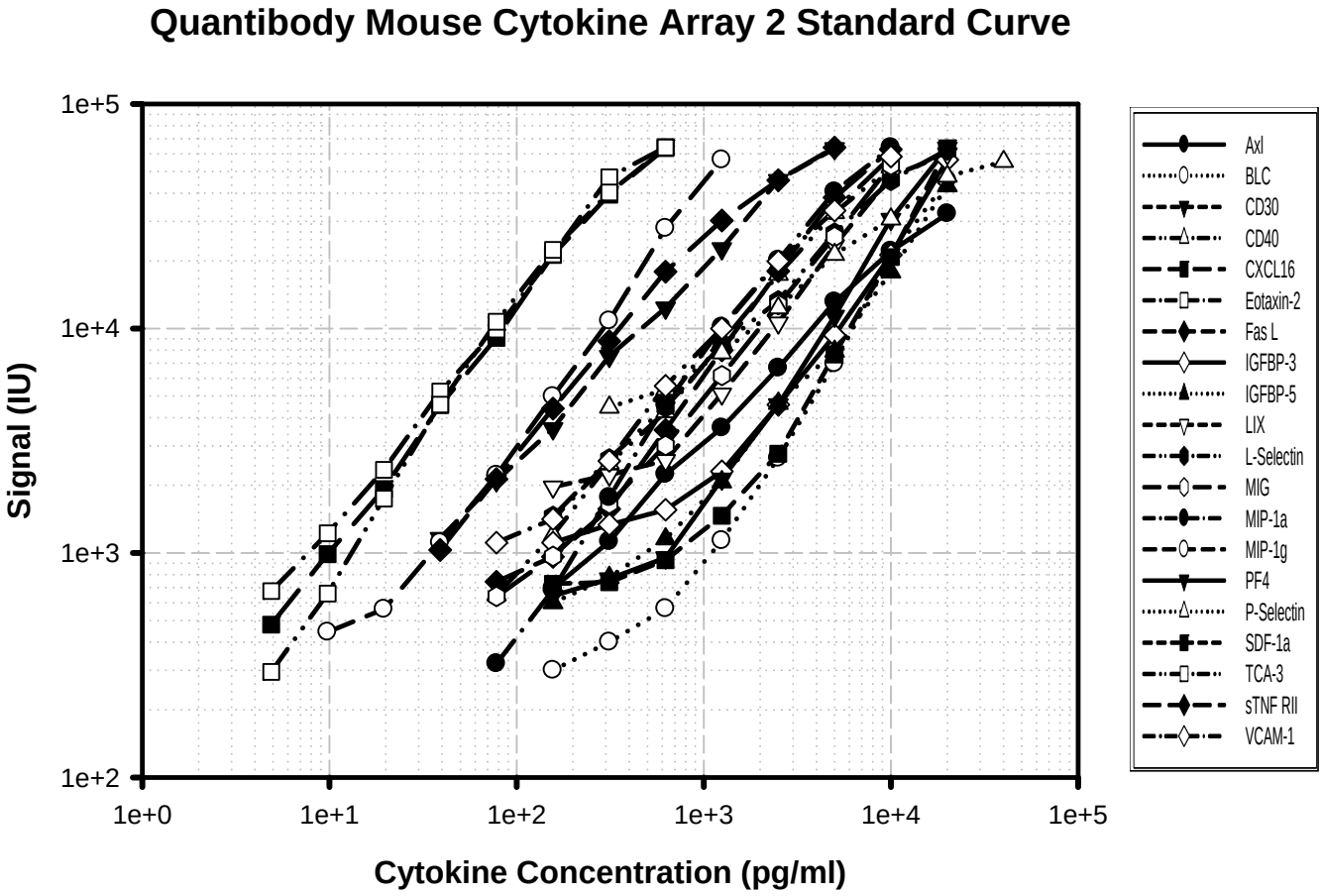
18. The signals can be visualized through use of a laser scanner equipped with a cy3 wavelength such as Axon GenePix. The settings should be: Excitation: 555 nm; Emission: 565 nm; Resolution: 10 um. Make sure that the signal from the standard well containing the highest concentration (Std1) receives 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.
Note: we recommend scanning slide right after experiment. You can also store the slide at 4^0C 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



VI. System Performance

The performance of Quantibody® Mouse Cytokine Array 2 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 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

Cytokines	Std1	Std2	Std3	Std4	Std5	Control	Median Fluorescence	Standard Deviation	Sensitivity (pg/ml)
Axl	4,000	1333	444	148	49	0	74	7	5.7
BLC	10,000	3333	1111	370	123	0	77	5	11.2
CD30	4,000	1333	444	148	49	0	32	3	1.0
CD40	2,000	667	222	74	25	0	128	11	6.1
CXCL16	500	167	56	19	6	0	81	12	0.3
Eotaxin-2	1,000	333	111	37	12	0	130	23	1.2
Fas Ligand	4,000	1333	444	148	49	0	73	39	47.8
IGFBP-3	20,000	6667	2222	741	247	0	134	18	70.3
IGFBP-5	20,000	6667	2222	741	247	0	69	22	54.0
LIX	20,000	6667	2222	741	247	0	152	57	62.6
L-Selectin	40,000	13333	4444	1481	494	0	74	27	310.1
MIG	10,000	3333	1111	370	123	0	69	33	13.0
MIP-1α	10,000	3333	1111	370	123	0	121	15	6.2
MIP-1γ	1,000	333	111	37	12	0	264	48	3.7
PF-4	40,000	13333	4444	1481	494	0	175	16	91.7
P-Selectin	20,000	6667	2222	741	247	0	2020	118	11.9
SDF-1α	40,000	13333	4444	1481	494	0	173	16	117.2
TCA-3	1,000	333	111	37	12	0	107	27	1.8
sTNF RII	1,000	333	111	37	12	0	55	2	1.0
VCAM-1	8,000	2667	889	296	99	0	95	24	7.4

Recovery

The recovery of the mouse antigens by the kit was tested through spiking different levels of the recombinant proteins in both the 10x diluted mouse serum and 10x diluted mouse cell culture media NIH 3T3 (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.

Cytokine	Spiking (pg/ml)	CM	CM+Ag	CM%	Serum	Serum+Ag	Serum%
Axl	2000	11	1861	92.5%	251	2079	91.4%
BLC	5000	3	5202	104.0%	135	5041	98.1%
CD30	2000	0	1891	94.6%	14	2084	103.5%
CD40	1000	2	1017	101.6%	8	1000	99.2%
CXCL16	250	0	261	104.4%	13	247	93.7%
Eotaxin-2	500	1	507	101.3%	5	433	85.6%
Fas L	2000	1	2076	103.8%	1	1547	77.3%
IGFBP3	10000	5	9453	94.5%	over	over	-
IGFBP5	10000	2	7863	78.6%	1928	9646	77.2%
LIX	10000	11	10381	103.7%	490	8452	79.6%
L-Selectin	10000	9	10352	103.4%	over	over	-
MIG	7000	0	7351	105.0%	150	8054	112.9%
MIP-1α	5000	0	4441	88.8%	3	4012	80.2%
MIP-1γ	500	4	464	92.0%	476	1023	109.4%
PF4	15000	0	14322	95.5%	over	over	-
P-Selectin	8000	0	7987	99.8%	over	over	-
SDF-1α	20000	13	17891	89.4%	2	17695	88.5%
TCA-3	500	0	530	105.9%	5	497	98.4%
sTNFRII	500	8	439	86.3%	877	1326	89.7%
VCAM-1	4000	525	4515	99.7%	over	over	-

Calibration

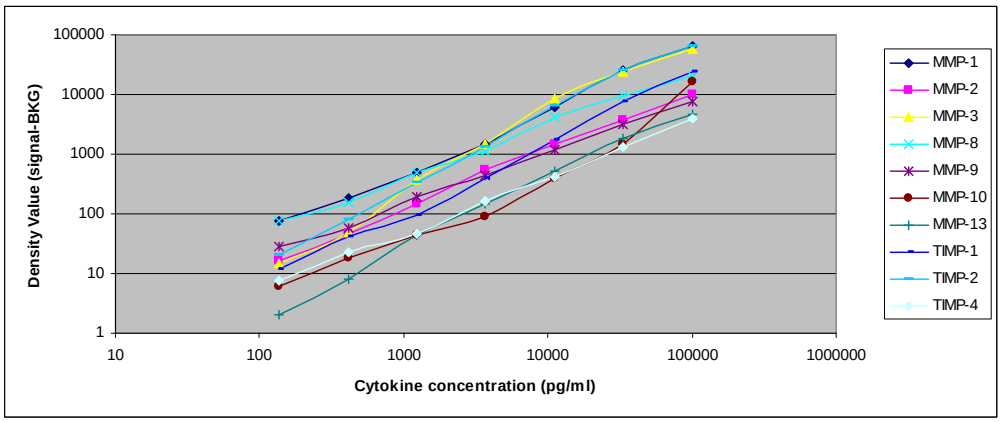
The RayBio® Cytokine standard concentration was determined by the internal cytokine standards.

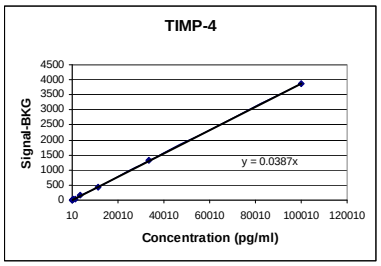
VII. Troubleshooting guide

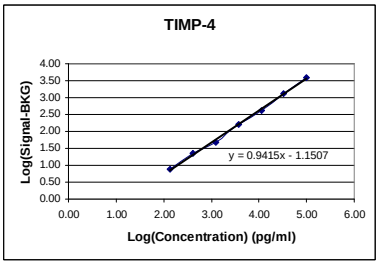
Problem	Cause	Recommendation
Weak Signal	Inadequate detection	Increase laser power and PMT parameters
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
	Short incubation time	Ensure sufficient incubation time and change sample incubation step to overnight
	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 completed 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.

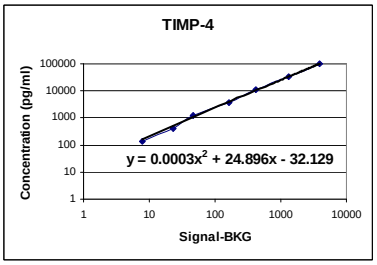
VIII. Sample Raybio® Q Analyzer Output

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

1. Thorpe, R.C. A.R. Mire-sluis, and M. Wadhwa. 2001. Cytokine Standardization. In Cytokine Reference Volume 1: Ligands. Oppenheim, J.J., Feldmann, M., Durum, S.K., Hirano, T., Vilcek, J., and Nicola, N.A. eds. Academic Press, San Diego, CA, pp83-91

2. Biological Reference Materials 2000. National Institute for Biological Standards and Control

3. Harlow, E. and Lane, D., 1999. Using Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory.

4. Barry, R. and Soloviev, M. 2004. Quantitative protein profiling using antibody arrays. Proteomics 4: 3717-3726

5. Huang, R.P., W. Yang, D. Yang, L. Flowers, I. R. Horowitz, X. Cao and R. Huang. 2005. The promise of cytokine antibody arrays in drug discovery process. Expert opinion on drug discovery. 9:601-615.

6. Berlier, J.E., Rothe, A., Buller, G. et al. 2003. Quantitative Comparison of Long-wavelength Alexa Fluor Dyes to Cy Dyes: Fluorescence of the Dyes and Their Bioconjugates. J. Histochem & Cytochem. 51(12): 1699-1712

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 produces 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

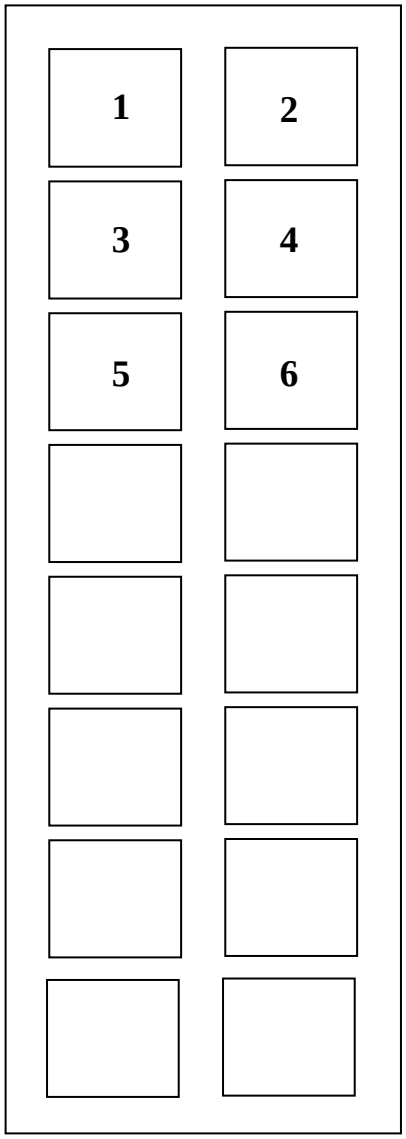
Date: _____

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		



NOTE

This product is for research use only.