Quantibody® Human Inflammation Array 2

-Quantitative measurement of 10 Inflammation cytokines

Patent Pending Technology

User Manual (Version Sept06)

Cat # QAH-INF-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® Human Inflammation Array 2

Cytokines (10)	IL-1ra, IL-6 sR, IL-12p40, IL-12p70, IL-16, OSM, RANTES, SAA, sTNF RI, sTNF RII
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 wavelengths equivalent dye
Sample Volume	50 – 100 ul
Reproducibility	CV <20%
Assay duration	4 hrs

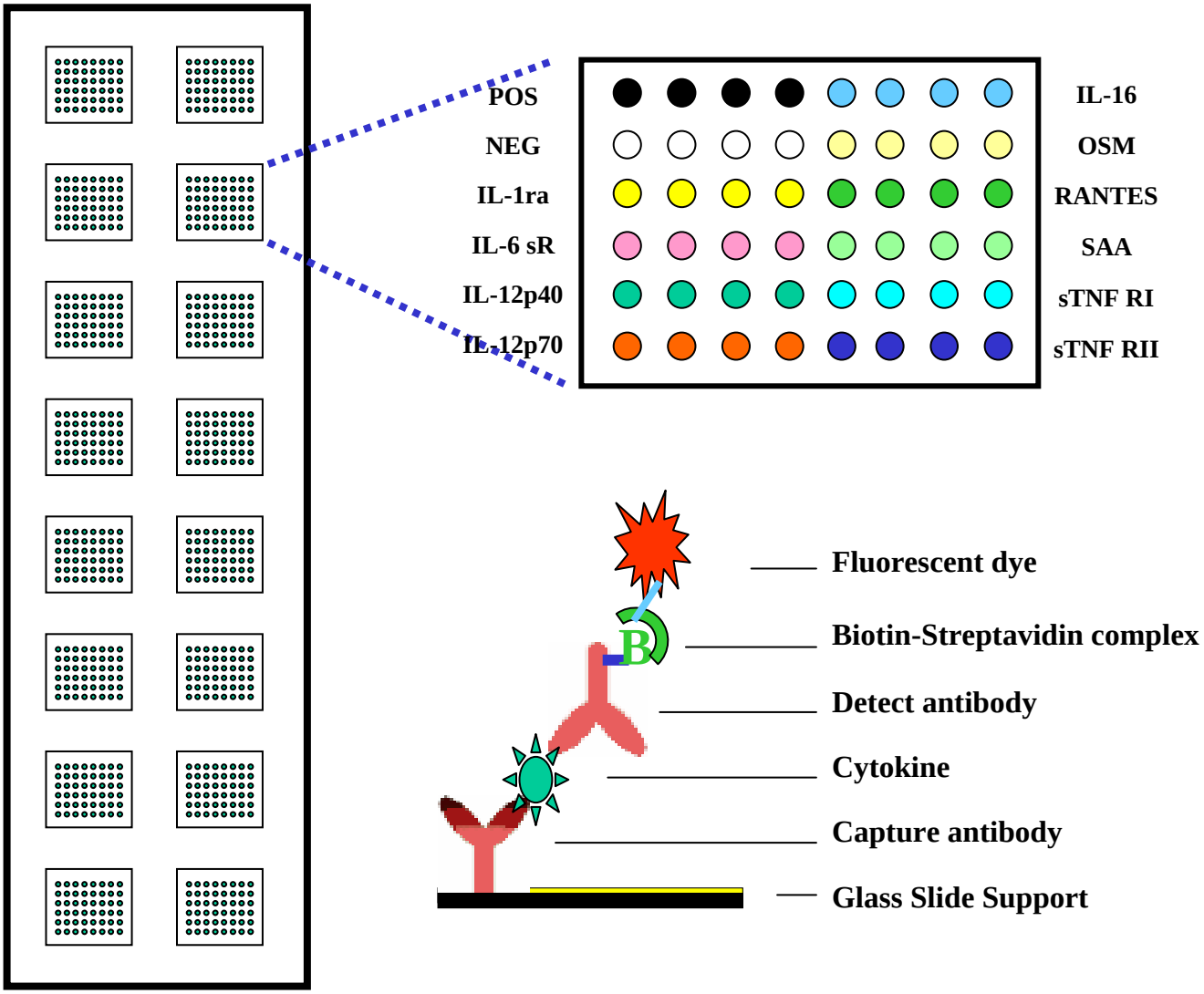


TABLE OF CONTENTS

I. Introduction

Cytokines play an important role in innate immunity, apoptosis, Inflammation, 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.

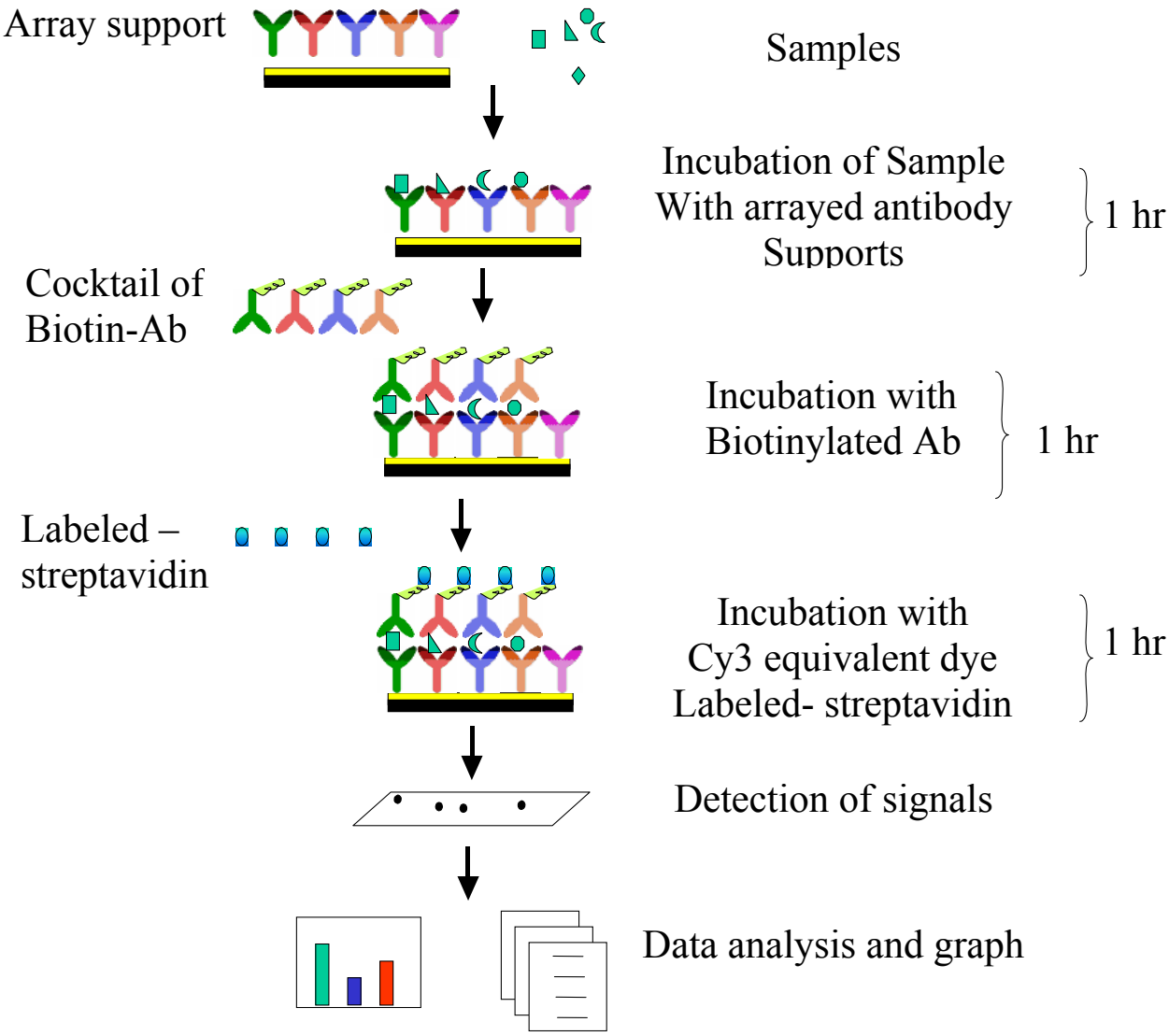
Our new multiplex Quantibody® Array is another quantum leap forward in protein microarray technology. This glass-chip-based multiplexed sandwich ELISA system enables researchers to accurately determine the concentration of 10 cytokines simultaneously. The system is relatively rapid and simple compared 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 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 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.

In a real experiment, standard cytokines and samples are assayed in each well simultaneously through a sandwich like ELISA procedure. The signals will be detected using 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


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 components of the Quantibody® Human Inflammation Array 2 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. Please use within six months of purchase.

Components:

Item	Description	1 Slide	2 slides
1	Quantibody® Human Inflammation 2 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 (ng)*

Cytokine	IL-1ra	IL-6 sR	IL-12p40	IL-12p70	IL-16	OSM	RANTES	SAA	sTNF RI	sTNF RII
Amount (ng)	20	2	4	0.4	4	4	2	20	1	1

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 the complete medium be used as control since many types of sera contains cytokines.
- We recommended the following usage of your samples:
  50 to 100 µl of cell culture supernatant
  or 50 to 100 µl of 10-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, you may further dilute your sample.

B. Handling glass chips

- Do not touch the surface of the slide, 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

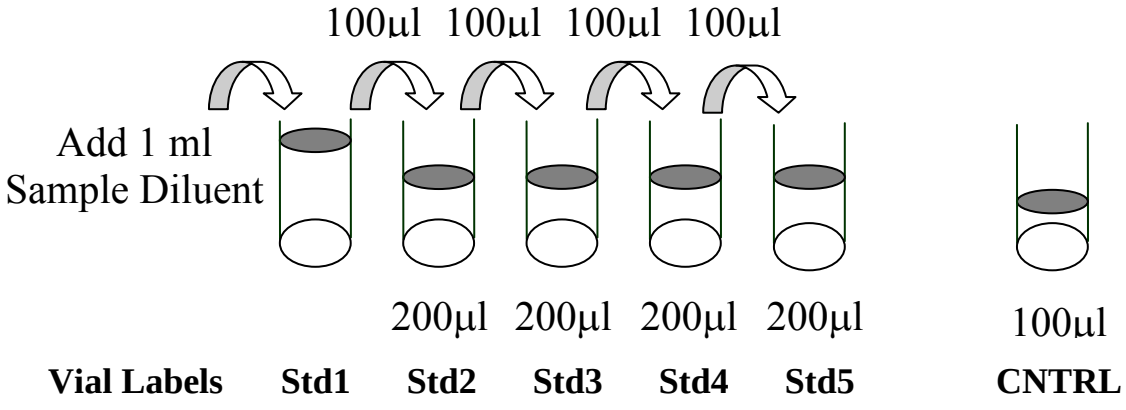
1. Take out the glass chip from the box; remove it from the plastic bag; peel off the covering film, and let it air dry at room temperature for at least 1-2 hours.

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 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 70µl, 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 10- 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 4^0C 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 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.

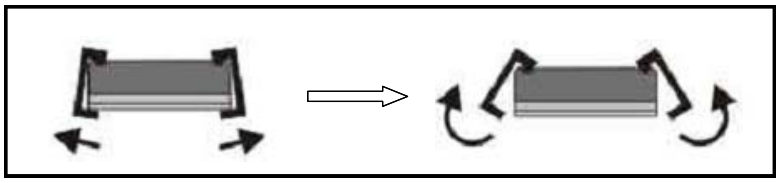
Note: incubation may be done at 4^0C for overnight.

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), 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 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.

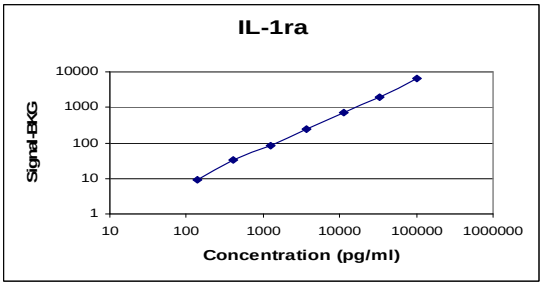
Note: In case the signal intensity for different cytokine varies greatly in the same array, we recommend using multiple scans for the low signal ones.
Note: we recommend scanning slide right after experiment. You can also store the slide at 4^0C in a dry dark container for several days. If you do not have a laser scanner, RayBiotech can provide service for you. Just simply send your slide to us and we will take care of it.

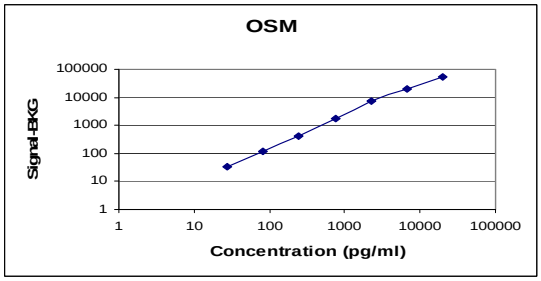
E. Data Analysis

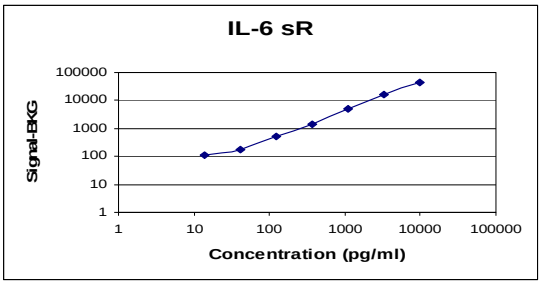
19. Data extraction can be done with most of the microarray analysis software (GenePix, ScanArray Express, ArrayVision, or MicroVigene). For quantitative data analysis, our RayBio® Q Analyzer software is available. It gives visual output as well as digital value. More information can be found in section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

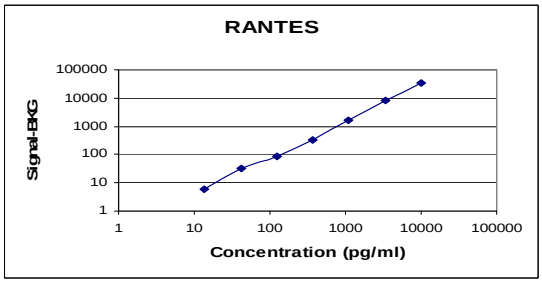
V. Standard Curve Range for Individual Cytokines

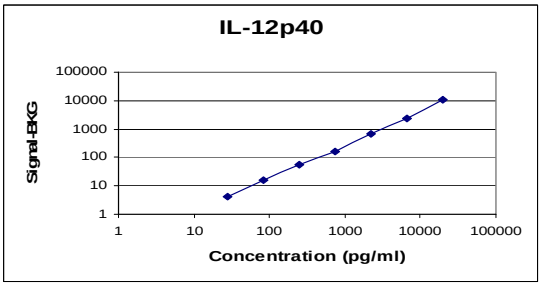
Log-log plot of Signal Intensity vs. Cytokine Concentration (pg/ml)

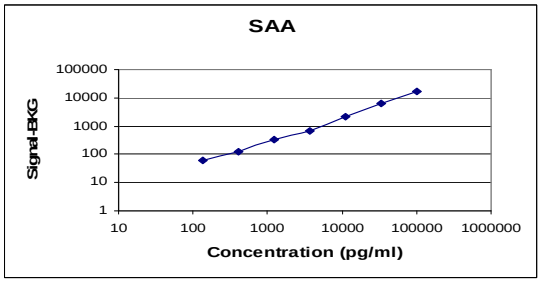


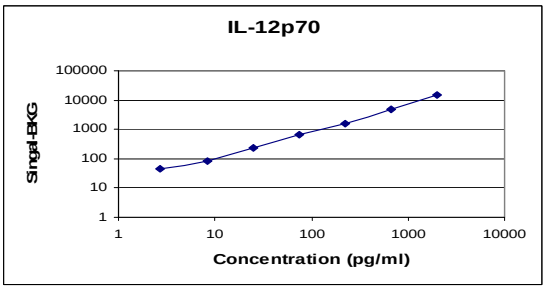


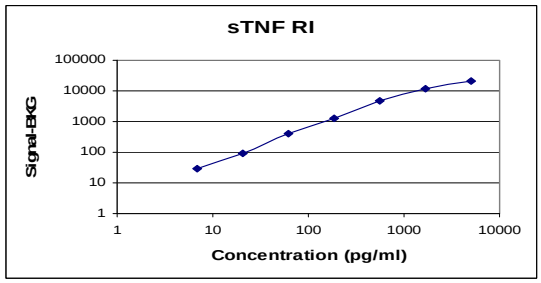


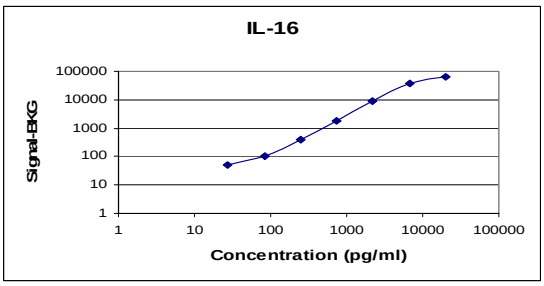


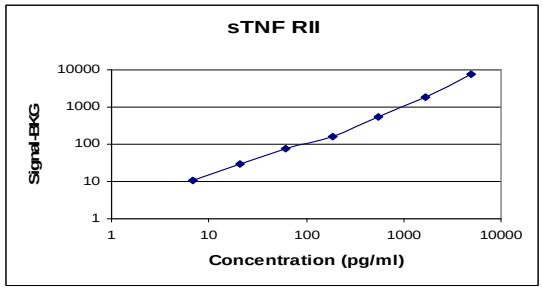












VI. System Performance

The Quantibody® Human Inflammation Array 2 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 cytokine standard curve range of a given cytokine were given in the following table, the quantifiable range for each cytokine can be extended to five times 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 dilution (pg/ml) and assay sensitivity

(pg/ml)	Std1	Std2	Std3	Std4	Std5	Control	Median Fluorescence	Standard Deviation	Sensitivity (pg/ml)
IL-1ra	20000	6667	2222	741	247	0	37	5	64.0
IL-6 sR	2000	667	222	74	25	0	65	13	2.4
IL-12p40	4000	1333	444	148	49	0	103	18	13.5
IL-12p70	400	133	44	15	5	0	40	9	1.0
IL-16	4000	1333	444	148	49	0	49	6	1.5
OSM	4000	1333	444	148	49	0	35	7	2.1
RANTES	2000	667	222	74	25	0	53	8	1.9
SAA	20000	6667	2222	741	247	0	45	8	39.0
sTNF RI	1000	333	111	37	12	0	81	8	1.4
sTNF RII	1000	333	111	37	12	0	43	7	3.9

Reproducibility

Four replicates of serial dilutions of recombinant human cytokines were assayed in the Sample Diluent. The cytokine concentrations in each sample were then determined through the RayBio® Q Analyzer. The CVs for the same dilution were then calculated by dividing the standard deviation by the average concentration and listed in the following table. The overall well-to-well CV was found to be <20%.

Cytokine	Starting Ag (ng/ml)	System CVs in serial antigen dilutions							
		Neat	1:3	1:9	1:27	1:81	1:243	1:729	Control
IL-1ra	100	4%	12%	2%	8%	9%	12%	17%	11%
IL-6 sR	10	13%	13%	4%	7%	4%	7%	10%	14%
IL-12p40	20	4%	3%	4%	9%	24%	18%	9%	9%
IL-12p70	2	7%	13%	12%	13%	12%	19%	21%	8%
IL-16	20	5%	2%	10%	3%	3%	8%	11%	7%
OSM	20	12%	6%	13%	6%	4%	12%	7%	19%
RANTES	10	6%	5%	16%	5%	5%	9%	12%	6%
SAA	100	6%	2%	10%	12%	10%	11%	5%	5%
sTNF RI	5	12%	4%	9%	6%	16%	10%	13%	6%
sTNF RII	5	17%	7%	12%	4%	15%	10%	10%	0%

Recovery

Different levels of recombinant human cytokines were spiked into the 20x diluted human serum (and 2x diluted cell culture media). All samples together with the non-spiked serum (and cell culture media) for negative control were assayed along side the standards. The recovery rate for each cytokine was then determined by subtracting the endogenous cytokine level from the observed cytokine and divided by the spiking cytokine concentration.

IL-1ra	20x diluted human serum				2x diluted cell culture media			
Spiking Ag (pg/ml)	20000	4000	800	0	20000	4000	800	0
Observed Ag (pg/ml)	15898	4313	1139	588	15885	3003	642	123
Net Ag (pg/ml)	15310	3725	551	0	15762	2880	519	0
Recovery Rate	77%	93%	69%		79%	72%	65%	

IL-6 sR	20x diluted human serum				2x diluted cell culture media			
Spiking Ag (pg/ml)	2000	400	80	0	2000	400	80	0
Observed Ag (pg/ml)	3513	1455	1256	1189	1986	287	68	8
Net Ag (pg/ml)	2324	266	67	0	1978	279	60	0
Recovery Rate	116%	67%	84%		99%	70%	75%	

IL-12p40	20x diluted human serum				2x diluted cell culture media			
Spiking Ag (pg/ml)	4000	800	160	0	4000	800	160	0
Observed Ag (pg/ml)	2733	400	70	0	4528	587	161	68
Net Ag (pg/ml)	2733	400	70	0	4460	519	93	0
Recovery Rate	68%	50%	44%		112%	65%	58%	

IL-12p70	20x diluted human serum				2x diluted cell culture media			
Spiking Ag (pg/ml)	400	80	16	0	400	80	16	0
Observed Ag (pg/ml)	274	36	5	0	514	101	14	1
Net Ag (pg/ml)	274	36	5	0	513	100	13	0
Recovery Rate	69%	45%	31%		128%	125%	81%	

IL-16	20x diluted human serum				2x diluted cell culture media			
Spiking Ag (pg/ml)	4000	800	160	0	4000	800	160	0
Observed Ag (pg/ml)	4956	524	98	9	4963	645	87	4
Net Ag (pg/ml)	4947	515	89	0	4959	641	83	0
Recovery Rate	124%	64%	56%		124%	80%	52%	

OSM	20x diluted human serum				2x diluted cell culture media			
Spiking Ag (pg/ml)	4000	800	160	0	4000	800	160	0
Observed Ag (pg/ml)	2965	495	106	18	2963	645	87	4
Net Ag (pg/ml)	2947	477	88	0	2959	641	83	0
Recovery Rate	74%	60%	55%		74%	80%	52%	

RANTES	20x diluted human serum				2x diluted cell culture media			
Spiking Ag (pg/ml)	2000	400	80	0	2000	400	80	0
Observed Ag (pg/ml)	2433	501	176	108	2564	512	94	35
Net Ag (pg/ml)	2325	393	68	0	2529	477	59	0
Recovery Rate	116%	98%	85%		126%	119%	74%	

SAA	20x diluted human serum				2x diluted cell culture media			
Spiking Ag (pg/ml)	20000	4000	800	0	20000	4000	800	0
Observed Ag (pg/ml)	40814	31123	29473	29077	24284	3454	423	0
Net Ag (pg/ml)	11737	2046	396	0	24284	3454	423	0
Recovery Rate	59%	51%	50%		121%	86%	53%	

sTNF RI	20x diluted human serum				2x diluted cell culture media			
Spiking Ag (pg/ml)	1000	200	40	0	1000	200	40	0
Observed Ag (pg/ml)	1153	215	63	27	1345	355	165	134
Net Ag (pg/ml)	1126	188	36	0	1211	221	31	0
Recovery Rate	113%	94%	90%		121%	111%	78%	

sTNF RII	20x diluted human serum				2x diluted cell culture media			
Spiking Ag (pg/ml)	1000	200	40	0	1000	200	40	0
Observed Ag (pg/ml)	1487	670	468	434	1105	127	27	1
Net Ag (pg/ml)	1053	236	34	0	1104	126	26	0
Recovery Rate	105%	118%	85%		110%	63%	65%	

Calibration

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

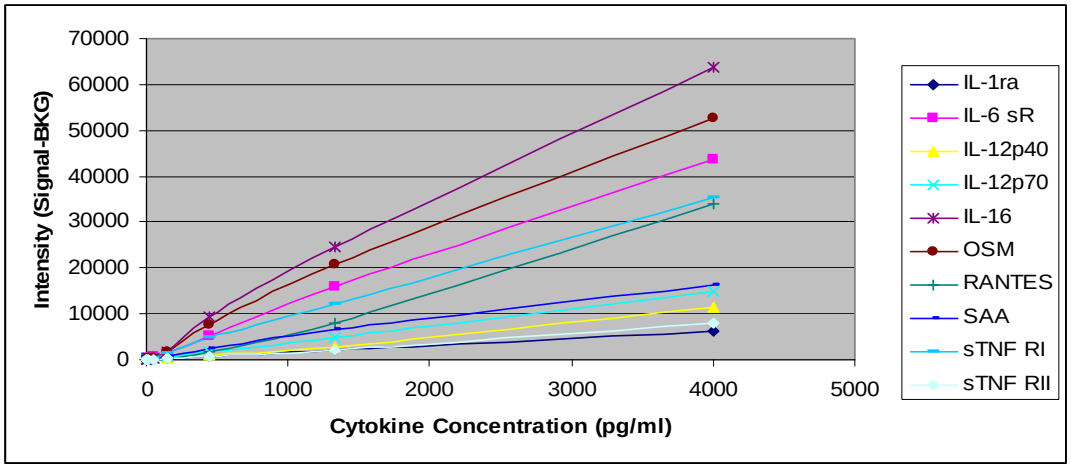
Cytokine	IL-1ra	IL-6 sR	IL-12p40	IL-12p70	IL-16	OSM	RANTES	SAA	sTNF RI	sTNF RII
Standard Lot	Internal	Internal	Internal	NIBSC 95/544	Internal	Internal	Internal	Internal	NIBSC 93/524	Internal

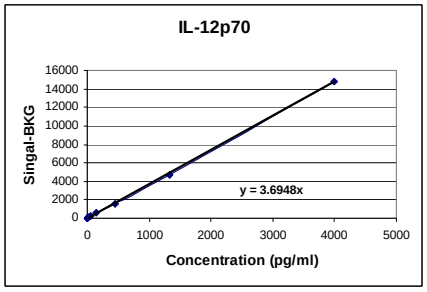
VII. Troubleshooting guide

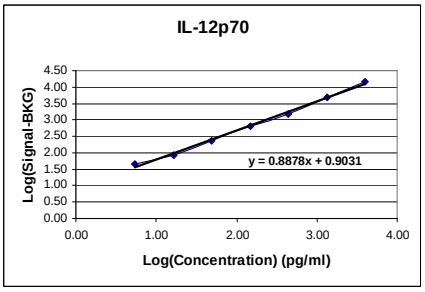
Problem	Cause	Recommendation
Weak signal	Inadequate detection	Increase laser power and PMT parameters
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
	Short incubation time	Ensure sufficient incubation time and change sample incubation step to overnight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

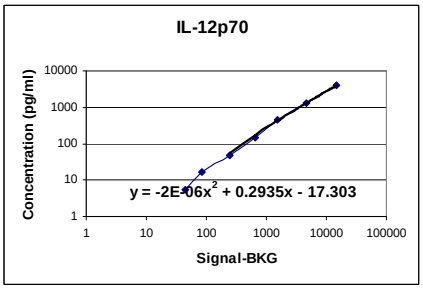
VIII. Sample Raybio® Q Analyzer Output

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) based on linear regression algorithm

ID	Sample 1	Sample 2	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8
IL-1ra	*0.0*	7.2	259.3	781.0	2350.2	6021.6	20199.0
IL-6 sR	*0.0*	5.0	23.8	64.2	239.1	724.7	1979.1
IL-12p40	*0.0*	*0.4*	21.6	45.0	246.7	907.2	4167.6
IL-12p70	*0.0*	1.7	6.5	17.6	42.0	127.7	402.0
IL-16	*0.0*	2.8	22.1	101.4	527.4	2243.3	3689.6
OSM	*0.0*	2.5	30.9	126.0	553.2	1542.0	3919.5
RANTES	*0.0*	1.8	5.2	19.9	104.2	486.5	2075.5
SAA	*0.0*	70.5	393.1	830.3	2692.4	7697.1	19598.8
sTNF RI	*0.0*	1.2	17.3	54.1	209.9	515.6	927.6
sTNF RII	*0.0*	1.4	12.3	21.9	72.8	252.1	1031.9

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

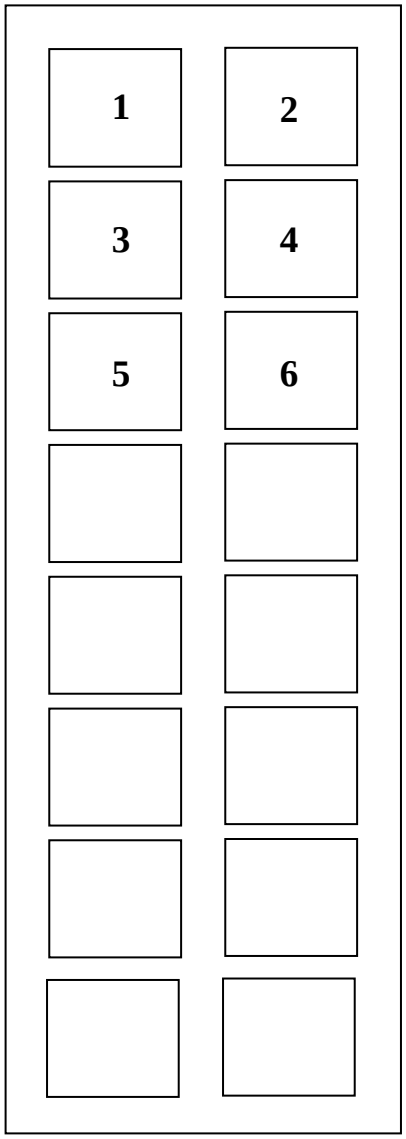
Date: _____

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		



This product is for research use only.