Quantibody® Human Th1/Th2 Array I

-Quantitative measurement of 10 Th1/Th2 cytokines

Patent Pending Technology

User Manual (Version Sept06)

Cat # QAH-TH-1

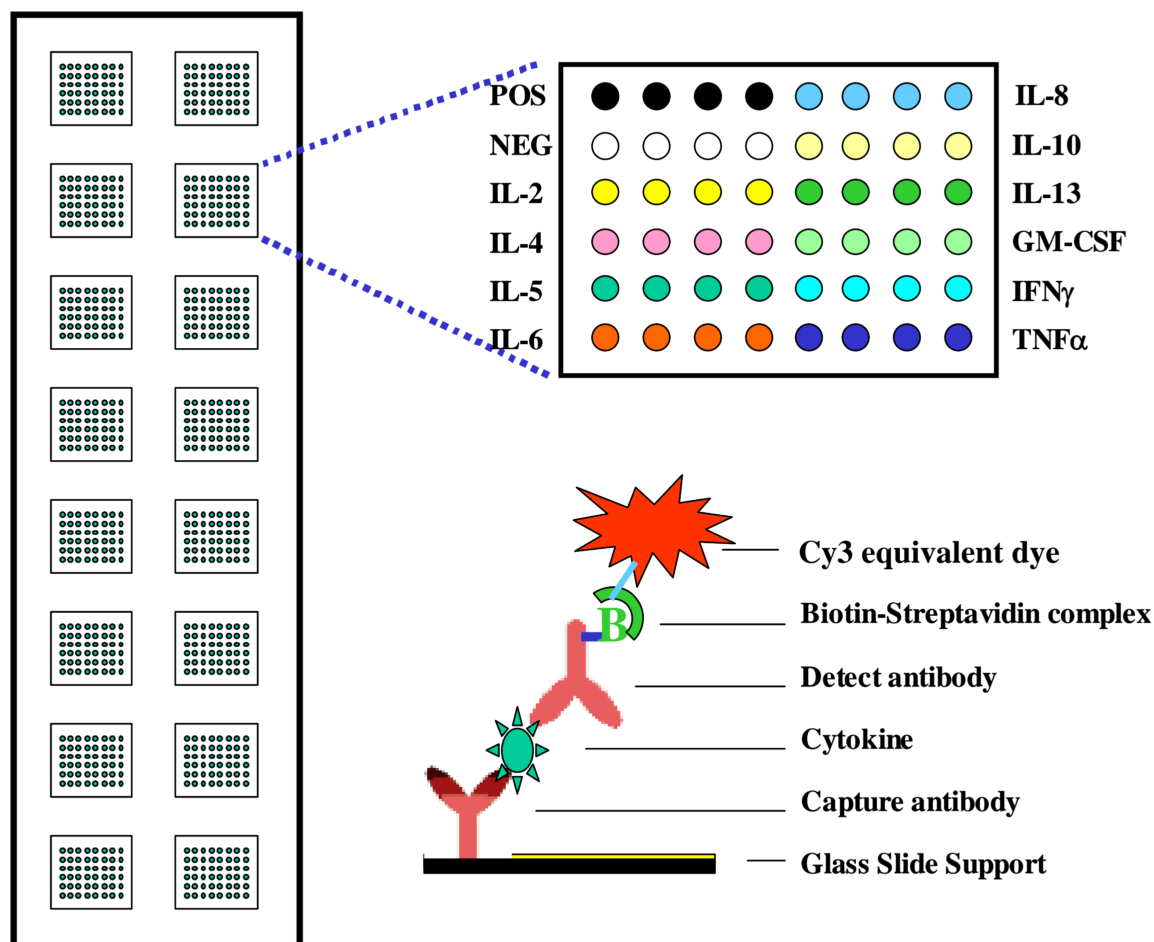
We Provide You With Excellent Protein Array Systems And Service

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 Th1/Th2 Array I

Description

Cytokines (10)	IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, GM-CSF, IFNγ, TNFα
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 ul
Detection range	1- 3000 pg/ml
Standard curve range	5 – 800 pg/ml
Reproducibility	CV <20%
Assay duration	4 hrs



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I. Introduction

Cytokines play an important role in innate immunity, apoptosis, angiogenesis, cell growth and differentiation. They are involved in interactions between different cell types, cellular responses to environmental conditions, and maintenance of homeostasis. Besides, 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.

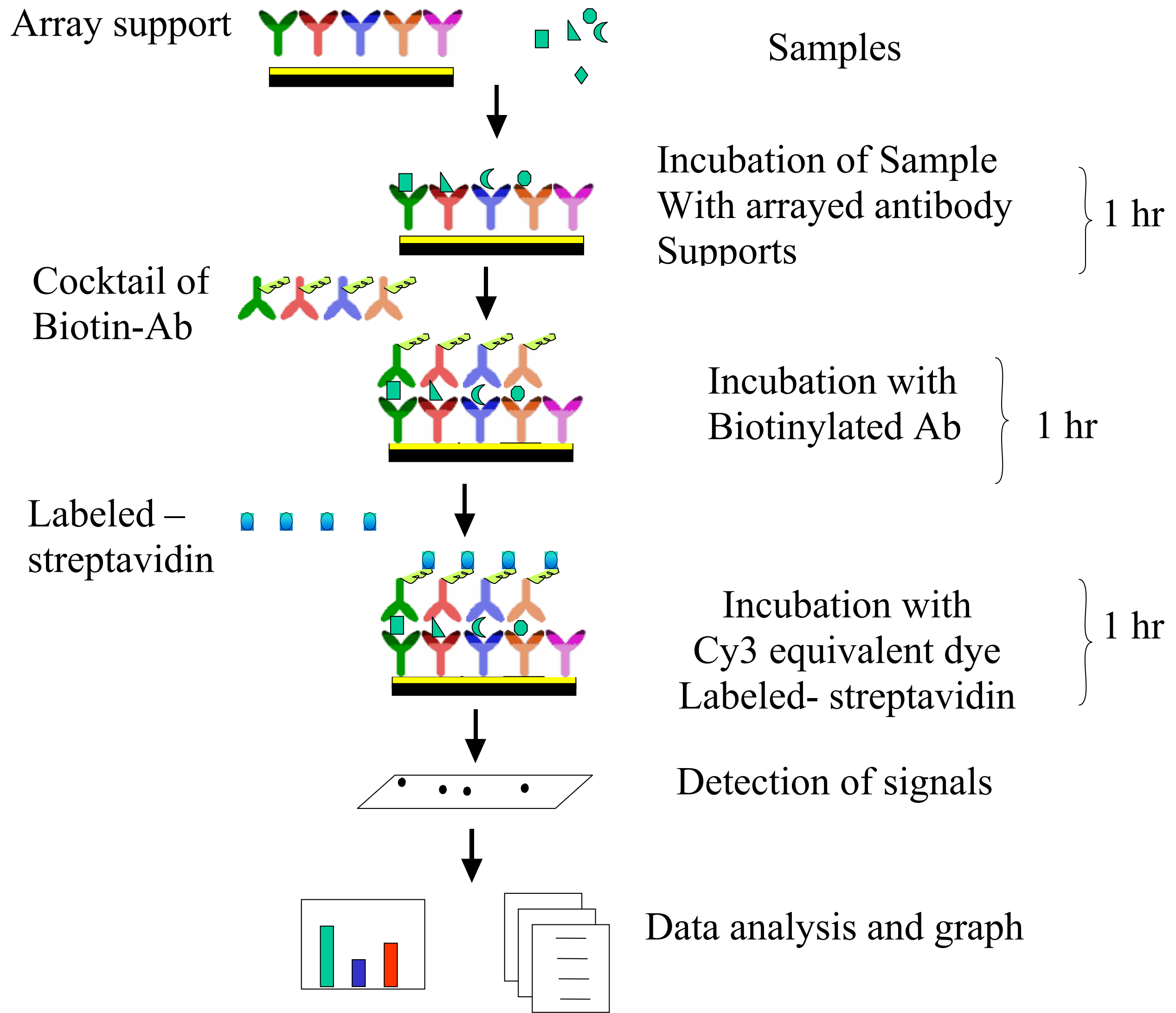
Our new multiplex Quantibody® Array is another quantum leap forward in protein microarray technology. This glass-chip-based multiplexed sandwich ELISA system enables researchers to accurately determine the concentration of 10 cytokines simultaneously. The system is relatively rapid and simple compared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



II. Materials Provided

Upon receipt, all components of the Quantibody® Human Th1/Th2 Array 1 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 Th1/Th2 Array 1 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 800pg each of IL-2, IL-4, IL-5,* IFNγ, *and* TNFα
400pg each of IL-6, IL-8, IL-10, IL-13 and GM-CSF

Additional Materials Required

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

III. General Considerations

A. Preparation of Samples

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

If you experience high background or the readings exceed the detection range, you may further dilute your sample.

B. Handling glass chips

- The microarray slides are sensitive, do not touch the surface. 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^{0}C for overnight. Please make sure to cover the incubation chamber tightly to prevent evaporation.

IV. Protocol

A. <u>Complete air dry the glass chip</u>

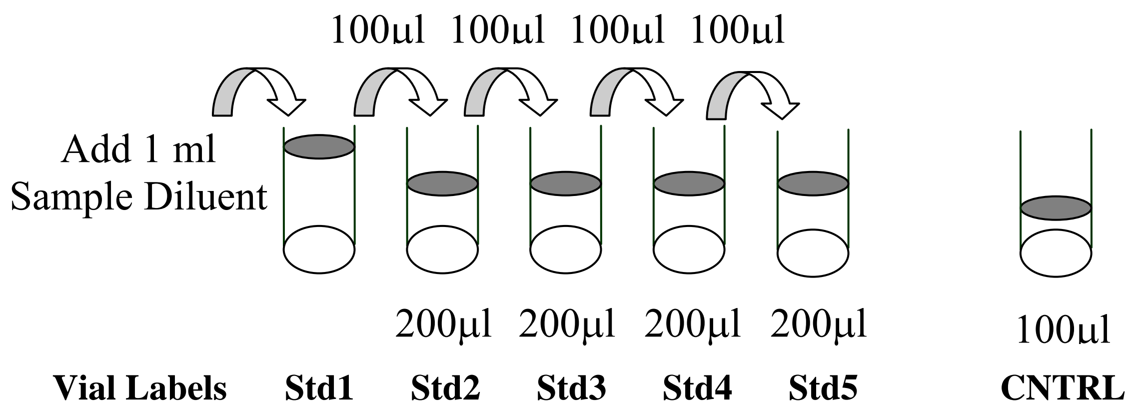
1. Take out the glass chip from the box; remove it from the plastic bag; peel off the covering film, and let it air dry at room temperature for at least 1-2 hours.

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

B. <u>Prepare Cytokine Standard Dilutions</u>

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.

<u>Prepare serial dilution of cytokine standards</u>



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. Label 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 100 µl 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 Th1/Th2 cytokines are:

- *IL-2, IL-4, IL-5,* IFNγ,,TNFα*: 800, 267, 89, 30, and 10 pg/ml*
- *IL-6, IL-8, IL-10, IL-13, GM-CSF: 400, 133, 44, 15, and 5 pg/ml*

C. Blocking and Incubation

6. Add 100 µl 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 in long time incubation.

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

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

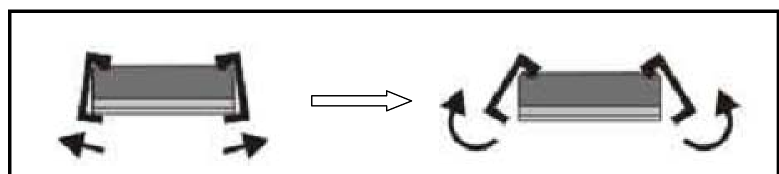
Note: incubation may be done at 4^0C for overnight.

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

D. <u>Fluorescence Detection</u>

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 gently shake at room temperature for 15 minutes. Decant Wash Buffer I. Wash with 1x Wash Buffer II (about 40 ml) with gentle 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. Image the signals using laser scanner such as Axon GenePix. The settings should be: Excitation: 555 nm; Emission: 565 nm; Resolution: 10 um. Make sure that the signals from the highest standard concentration well (Std1) gets the highest possible reading while remains unsaturated. Saved the image as a high resolution (16-bit) .tif file.

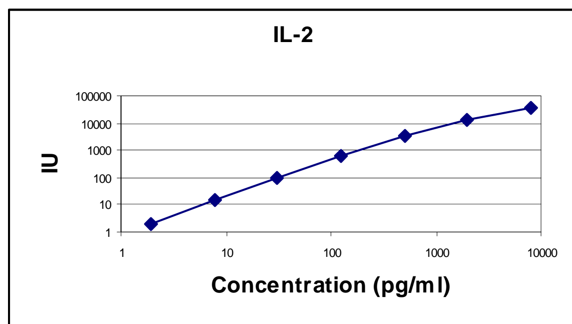
Note: we recommend scanning slide right after experiment. You can also store the slide at $4^{0}C$ in a dry dark container for several days. If you do not have a laser scanner, RayBiotech can provide service for you. Just simply send your slide to us and we will take care of it.

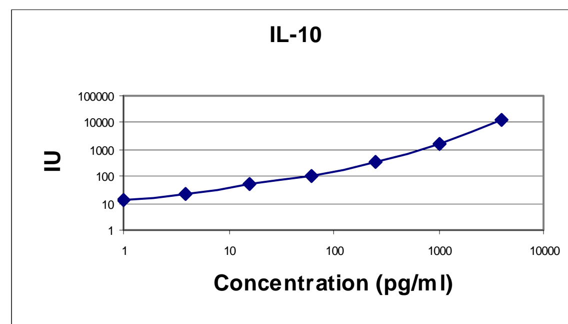
E. <u>Data Analysis</u>

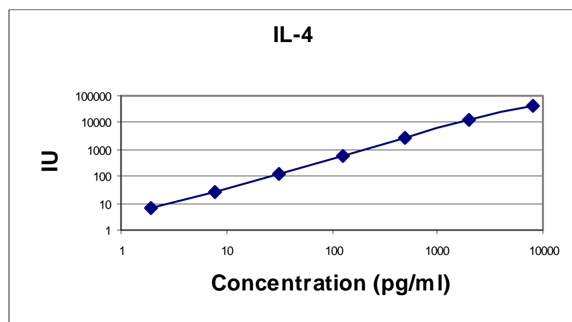
19. Data extraction can be done with most of the microarray analysis software (GenePix, ScanArray Express, ArrayVision, or MicroVigene). For quantitative data analysis, our RayBio® Q Analyzer software is available. It gives visual output as well as digital value. More information can be found in section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

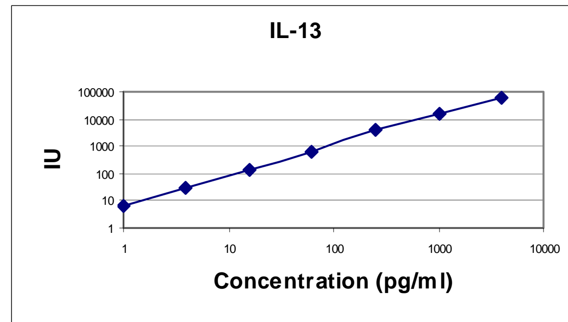
V. Standard Curve Range for Individual Cytokines

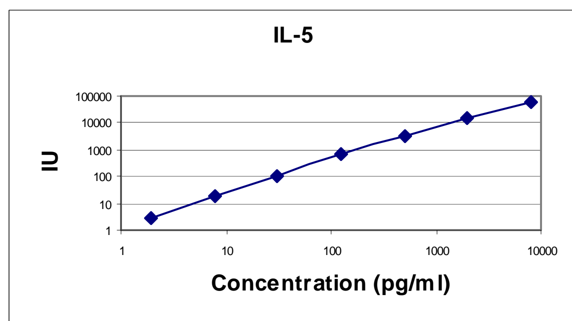
Log-log plot of Signal Intensity (IU) vs. Cytokine Concentration

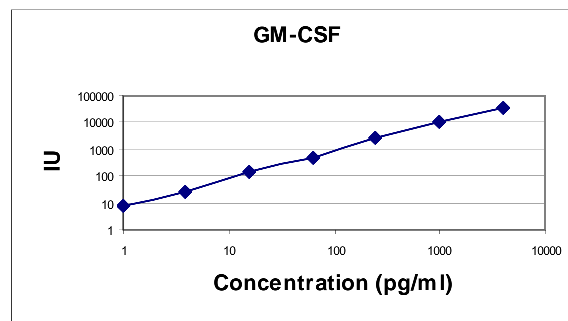


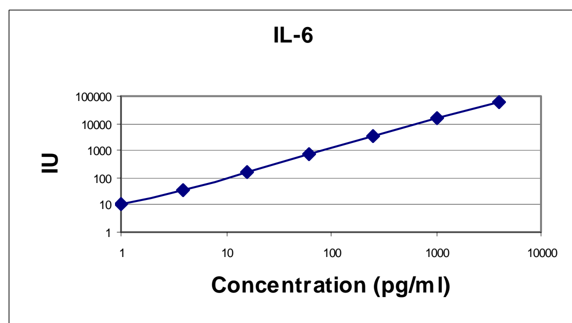


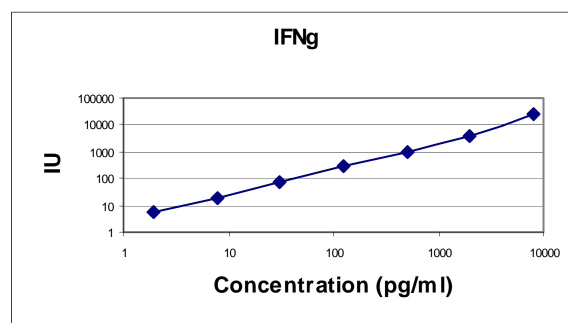


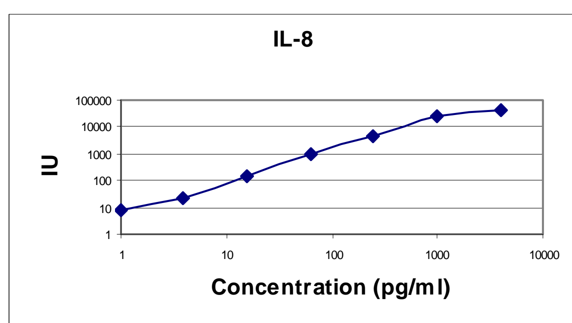


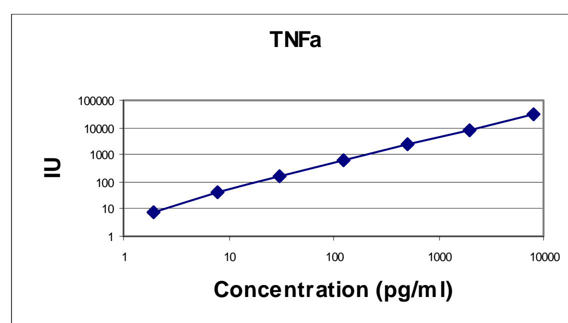












VI. System Performance

The Quantibody® Human TH1/Th2 Array Kit has been rigorously tested for its performance including: specificity, sensitivity, linearity, spiking recovery, and reproducibility.

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.

Linearity

Human serum sample was spiked with recombinant cytokines and mixed with equal volume of Sample Diluent. The sample cytokine mixture was then serially diluted in the same 1:1 serum: Sample Diluent. The observed cytokine concentration and the slope were listed as following:

Dilution	Observed IL-2 (pg/ml)	Observed IL-4 (pg/ml)	Observed IL-5 (pg/ml)	Observed IL-6 (pg/ml)	Observed IL-8 (pg/ml)	Observed IL-10 (pg/ml)	Observed IL-13 (pg/ml)	Observed GM-CSF (pg/ml)	Observed IFNg (pg/ml)	Observed TNFa (pg/ml)
neat	1574	2138	2178	813	1654	812	1051	1186	2313	2164
1:3	589	618	649	201	502	270	346	400	665	769
1:9	184	185	141	44	157	86	107	134	212	241
1:27	55	67	37	15	46	28	26	46	67	81
1:81	19	24	12	5	14	10	8	17	20	31
1:243	6	8	5	2	5	3	3	6	6	12
1:729	2	3	3	1	3	1	1	2	3	5
Slope	1.02	0.92	1.04	1.02	1.00	0.92	0.99	0.89	0.95	0.88

Sensitivity

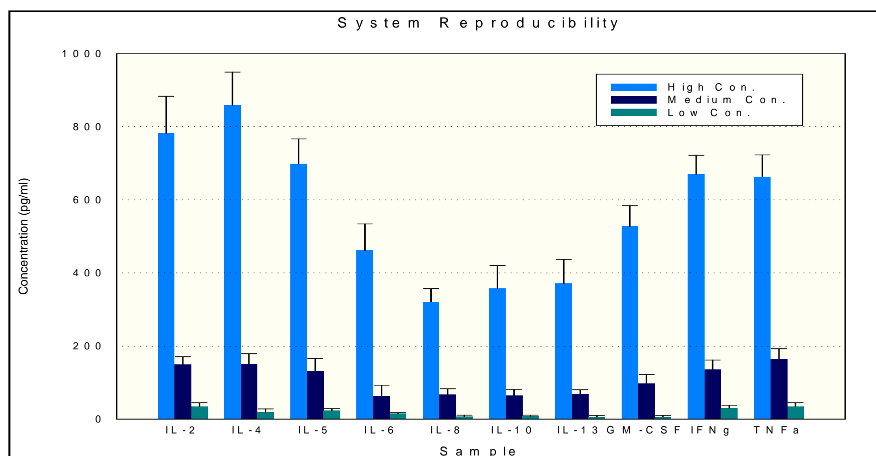
While the standard curve for a given cytokine ranges from 5 to 800 pg/ml, the quantifiable range for each cytokine can be extended up to 1-4000 pg/ml (see section V). The sensitivity of each cytokine, which is defined as the corresponding concentration at two standard deviations above the median fluorescence of 20 replicates of the negative control (0 pg/ml), is tested to be as low as 1 picogram.

Serial standard dilution and assay sensitivity (pg/ml)

(pg/ml)	Std1	Std2	Std3	Std4	Std5	Control	Median Fluorescence	Standard Deviation	Sensitivity (pg/ml)
IL-2	800	267	89	30	10	0	33	6	2.0
IL-4	800	267	89	30	10	0	30	6	2.2
IL-5	800	267	89	30	10	0	19	4	1.1
IL-6	400	133	44	15	5	0	23	5	0.8
IL-8	400	133	44	15	5	0	80	7	0.6
IL-10	400	133	44	15	5	0	13	4	1.2
IL-13	400	133	44	15	5	0	27	6	0.5
GM-CSF	400	133	44	15	5	0	52	6	1.2
IFNg	800	267	89	30	10	0	38	8	2.8
TNFa	800	267	89	30	10	0	19	4	0.9

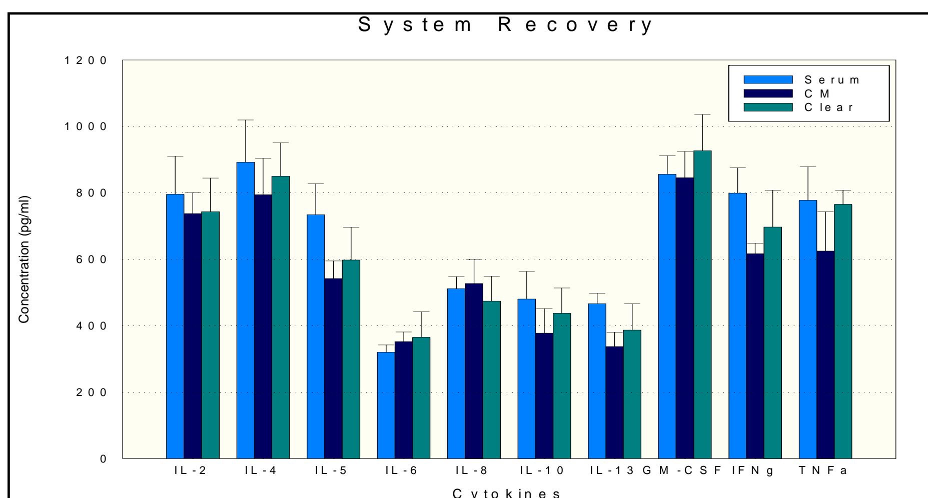
Reproducibility

Eight replicates of samples containing high, medium, and low levels of recombinant human cytokines were assayed along side the standards. The cytokine concentrations in each sample were then determined through the RayBio® Q Analyzer. The well-to-well CV was found to be <20%.



Recovery

Three sets of spiking experiments were done of spiking recombinant human cytokines in serum (5 x dilutions), condition media (20 x dilutions) and sample diluent respectively. The spiking cytokine concentration is 800 pg/ml for IL-2, IL-4, IL-5, GM-CSF, IFNγ, TNFα and 400 pg/ml for IL-6, IL-8, IL-10, and IL-13. Every sample was assayed in sextuplet along side the standards.



Recovery of samples spiked with recombinant human cytokines

Cytokine	Number of samples	Serum	Condition Media
IL-2	**6**	**99%**	**92%**
IL-4	**6**	**112%**	**99%**
IL-5	**6**	**92%**	**88%**
IL-6	**6**	**80%**	**88%**
IL-8	**6**	**128%**	**132%**
IL-10	**6**	**120%**	**94%**
IL-13	**6**	**117%**	**84%**
GM-CSF	**6**	**107%**	**106%**
IFNγ	**6**	**100%**	**87%**
TNFα	**6**	**97%**	**88%**

Calibration

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

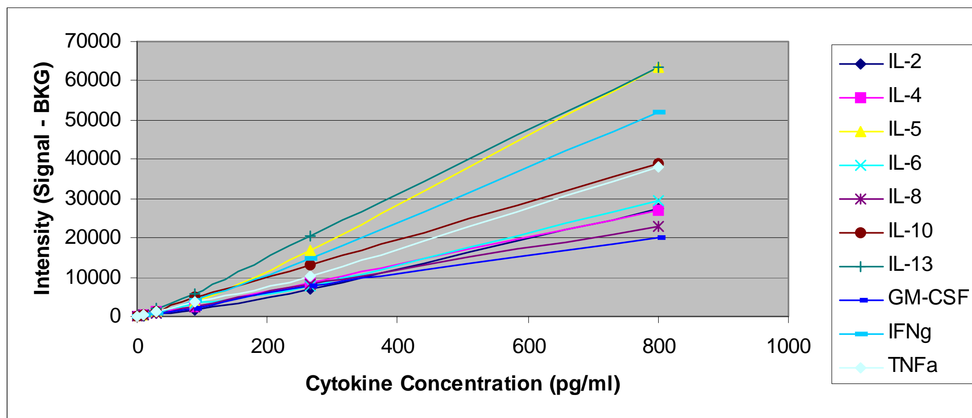
Cytokine	IL-2	IL-4	IL-5	IL-6	IL-8	IL-10	IL-13	GM-CSF	IFNg	TNFa
Standard	BRMP	NIBSC	NIBSC	NIBSC	Internal	NIBSC	NIBSC	NIBSC	NIAID	NIBSC
Lot	ISDP-841	88/656	90/586	89/548	Internal	92/516	94/622	88/646	G01-902-535	88/786

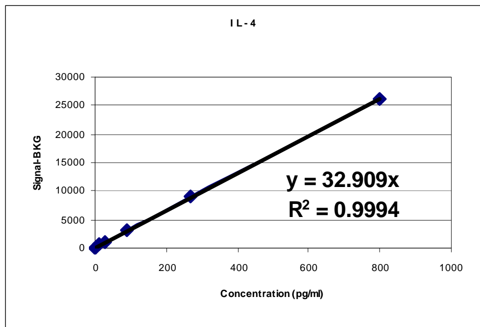
VII. Troubleshooting guide

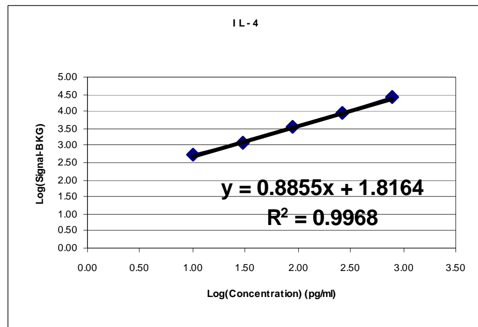
Problem	Cause	Recommendation
Weak Signal	Inadequate detection	Increase laser power and PMT parameters
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
	Short incubation time	Ensure sufficient incubation time and change sample incubation step to overnight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

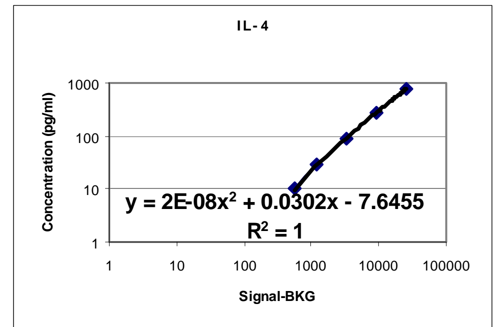
VIII. Sample Raybio© Q Analyzer Output

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)										
Cytokines	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10
IL-2	36.1	46.4	60.1	62.7	114.6	31.4	31.4	86.5	222.9	814.9
IL-4	22.1	116.4	186.8	304.6	660.8	0.3	17.7	107.1	295.0	789.1
IL-5	39.3	64.5	89.5	149.4	209.8	23.1	32.2	65.8	242.3	810.4
IL-6	61.7	95.2	88.7	143.3	136.8	42.9	45.9	73.7	199.2	823.6
IL-8	46.3	152.8	269.8	328.1	1379.2	23.9	22.1	96.7	222.6	813.9
IL-10	17.3	161.7	314.0	507.7	1258.1	15.3	24.8	112.5	219.5	813.2
IL-13	28.4	133.2	242.1	484.9	1044.8	19.5	18.0	87.5	227.3	813.1
GM-CSF	25.3	234.9	431.7	848.5	1881.3	10.4	17.1	104.2	259.3	801.2
IFNγ	17.0	39.1	55.5	98.7	175.1	10.5	20.3	84.5	271.2	799.3
TNFα	272.9	284.4	434.5	1139.0	836.9	38.4	38.4	174.9	481.0	1617.8

IX. Reference List

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X. Experiment Record Form

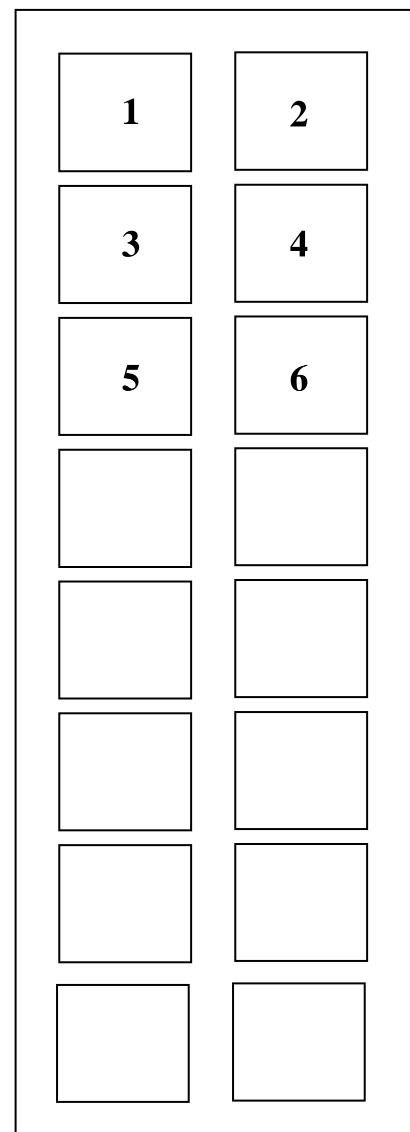
Date: ______________________________

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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