

Quantibody[®] Human Angiogenesis Array 2

Quantitative measurement of 30 human cytokines

Catalog #: QAH-ANG-2

User Manual
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Caution:
Extraordinarily useful information enclosed



ISO 13485 Certified

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Please read the entire manual carefully before starting your experiment

I. Overview

Cytokines Detected (30)	Activin A, AgRP, Angiogenin, Angiopoietin-2, ANGPTL4, bFGF, ENA-78 (CXCL5), GRO alpha/beta/gamma, HB-EGF, HGF, IFN-gamma, IGF-1, IL-1 alpha (IL-1 F1), IL-2, IL-6, IL-8 (CXCL8), IL-17A, IP-10 (CXCL10), Leptin, LIF, MCP-1 (CCL2), PDGF-BB, PLGF, RANTES (CCL5), TGF beta 1, TIMP-1, TIMP-2, TNF alpha, TNF beta (TNFSF1B), Thrombopoietin (TPO) <i>See Section IX for Array Map</i>
Format	One standard glass slide is spotted with 16 wells of identical cytokine antibody arrays. Each antibody is arrayed in quadruplicate.
Detection Method	Fluorescence. Go to www.RayBiotech.com/Scanners for a list of compatible laser scanners.
Sample Volume	50 - 100 µl per array
Reproducibility	CV <20%
Assay Duration	6 hours

II. 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 assay (ELISA). In this method, target protein is 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 this traditional method works well for a single protein, the overall procedure is time consuming and requires a relatively high volume of sample. Thus, conservation of precious small sample quantities becomes a challenging task. Innovations in microarray technology over the last decade have addressed this problem. A long-standing leader in the field, Raybiotech, has pioneered the development of cytokine antibody arrays, which have now been widely applied in the research community with hundreds of peer

reviewed publications, including top-tier journals such as *Cell* and *Nature*.

The Quantibody[®] array, our multiplexed sandwich ELISA-based quantitative array platform, enables researchers to accurately determine the concentration of multiple cytokines simultaneously. It combines the advantages of the high detection sensitivity & specificity of ELISA and the high throughput of arrays. Like a traditional sandwich-based ELISA, it uses a pair of cytokine specific antibodies for detection. A capture antibody is first bound to the glass surface. After incubation with the sample, the target cytokine is trapped on the solid surface. A second biotin-labeled detection antibody is then added, which can recognize a different epitope of the target cytokine. The cytokine-antibody-biotin complex can then be visualized through the addition of the streptavidin-conjugated Cy3 equivalent dye, using a laser scanner. Unlike the traditional ELISA, Quantibody products use an array format. By arraying multiple cytokine specific capture antibodies onto a glass support, quantitative, multiplex detection of cytokines in one experiment is made possible.

In detail, one standard glass slide is divided into 16 wells of identical cytokine antibody arrays. Each antibody, together with the positive controls is arrayed in quadruplicate. The slide comes with a 16-well removable gasket which allows for the process of 16 samples on one slide. Four slides can be nested into a tray, which matches a standard microplate footprint and allows for automated robotic high throughput process of 64 arrays simultaneously. For cytokine quantification, the array specific cytokine standards, whose concentration has been predetermined, are provided to generate a standard curve for each cytokine. In a real experiment, standard cytokines and samples will be assayed in each array simultaneously through a sandwich ELISA procedure. By comparing signals from unknown samples to the standard curve, the cytokine concentration in the samples will be determined.

Quantibody[®] array kits have been confirmed to have similar detection sensitivity as traditional ELISA. Our current high density Quantibody kits allow scientists to quantitatively determine the concentration of 1000 human, 200 mouse, and 67 rat cytokines in a single experiment. This is not only one of the most efficient products on the market for cytokine quantification, but makes it more affordable for quantification of large number of proteins. Simultaneous detection of multiple cytokines undoubtedly provides a powerful tool for drug and biomarker discovery.

III. How It Works



IV. Materials Provided

	Catalog #	Component Name	1 Slide Box	2 Slide Box*
1	QAH-ANG-2 S	Human Angiogenesis Array 2 Glass Slide	1	2
2	QA-SDB	Quantibody [®] Sample Diluent	15 ml	
3	AA-WB1-30ML	20X Wash Buffer I	2 x 30 ml	3 x 30 ml
4	AA-WB2-30ML	20X Wash Buffer II	30 ml	
5	QAH-ANG-2 -STD	Human Angiogenesis Array 2 Lyophilized Standard Mix**	1 Vial	
6	QAH-ANG-2 B	Human Angiogenesis Array 2 Biotinylated Antibody Cocktail	1-25 µl	2 x 1-25 µl
7	QA-CY3E	Cy3 equivalent dye-conjugated Streptavidin	5 µl	2 x 5 µl
8	QA-SWD	Slide Washer/Dryer	1 x 30 ml Tube	
9	QA-ADH	Adhesive Film	1	2

* 4 slide kits are comprised of 2 separate 2 slide kits.

** See Section X for detailed cytokine concentrations after reconstitution.

V. Storage

Upon receipt, all components should be stored at -20°C. The kit will retain activity for up to 6 months. Once thawed, the glass slide, standard mix, antibody cocktail and dye-conjugated Streptavidin should be kept at -20°C. All other components may be stored at 4°C. The entire kit should be used within 6 months of purchase.

VI. Additional Materials Required

- Benchtop rocker or orbital rocker
- Laser scanner for fluorescence detection
- Aluminum foil
- Distilled water
- 1.5 ml Polypropylene microcentrifuge tubes

VII. 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.
- Each array needs 100 μ l of total sample volume. To avoid matrix effects, we recommend using a minimum of 2-fold sample dilution of culture media, body fluids, or 0.5-1mg/ml total protein for lysates, after a 5-fold to 10-fold dilution to minimize the effects of any detergent(s). Please be aware, more sample volume is required for combination arrays. For example, the minimum sample volume for a 10-array kit is 500 μ l, or 500 μ g lysate.
- The suggested serum/plasma dilution for this array is: 2x

B. Handling Glass Slides

- 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 powder free gloves.
- Handle glass slide/s in clean environment.
- Permanent marker ink can significantly interfere with fluorescent signal detection. To help distinguish one slide from another, you may make a small marking (such as a number or a star) along the top or bottom edge, using a green or blue ultra-fine point Sharpie[®] brand marker. This can also serve to orient the slide. For best results during scanning, please **DO NOT**:
 - Write anywhere on the front (arrayed) side of the slide
 - Write on the slide while it is wet
 - Use red or black colored ink anywhere on the slide
 - Write over the arrayed well areas of the slide, as this interferes with scanning.

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 rocking or rotation.
- Cover the incubation chamber with adhesive film during incubation, particularly when incubation is more than 2 hours or <70 μ l of sample or reagent is used.

- 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 overnight at 4°C. Please make sure to cover the incubation chamber tightly to prevent evaporation.

VIII. Protocol

A. Completely Air Dry The Glass Slide

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

Incomplete drying of slides before use may cause the formation of "comet tails," thin directional smearing of antibody spots.

B. Prepare Cytokine Standard Dilutions

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.



2. Reconstitute the Cytokine Standard Mix (lyophilized) by adding 500 µl Sample Diluent to the tube. For best recovery, always quick-spin vial prior to opening. Dissolve the powder thoroughly by a gentle mix. Labeled the tube as Std1.

3. Label 6 clean microcentrifuge tubes as Std2 to Std7. Add 200 μ l Sample Diluent to each of the tubes.
4. Pipette 100 μ l Std1 into tube Std2 and mix gently. Perform 5 more serial dilutions by adding 100 μ l Std2 to tube Std3 and so on.
5. Add 100 μ l Sample Diluent to another tube labeled as CNTRL. Do not add standard cytokines or samples to the CNTRL tube, which will be used as negative control. For best results, include a set of standards in each slide.

Since the starting concentration of each cytokine is different, the serial concentrations from Std1 to Std7 for each cytokine are varied which can be found in Section X.

C. Blocking & Incubation

6. Add 100 μ l Sample Diluent into each well and incubate at room temperature for 30 minutes to block slides.
7. Decant buffer from each well. Add 100 μ l standard cytokines or samples to each well. Incubate arrays at room temperature for 1-2 hour.

Longer incubation time is preferable for higher signals. This step may be done overnight at 4°C.

We recommend using 50 to 100 μ l of original or diluted serum, plasma, conditioned media, or other body fluid, or 250 μ g/ml-1 mg/ml of protein for cell and tissue lysates. Cover the incubation chamber with adhesive film during incubation, especially if less than 70 μ l of sample or reagent is used.

8. Wash:

- Decant the samples from each well, and wash 5 times (5 min each) with 150 μ l of 1X Wash Buffer I at room temperature with gentle rocking. Completely remove wash buffer in each wash step. Dilute 20x Wash Buffer I with H₂O.
- *(Optional for Cell and Tissue Lysates)* Put the glass slide with frame into a box with 1X Wash Buffer I (cover the whole glass slide and frame with Wash Buffer

l), and wash at room temperature with gentle rocking for 20 min.

- Decant the 1x Wash Buffer I from each well, wash 2 times (5 min each) with 150 μ l of 1X Wash Buffer II at room temperature with gentle rocking. Completely remove wash buffer in each wash step. Dilute 20X Wash Buffer II with H₂O.

Incomplete removal of the wash buffer in each wash step may cause "dark spots," the background signals higher than the spots.

D. Incubation with Biotinylated Antibody Cocktail & Wash

9. Reconstitute the detection antibody by adding 1.4 ml of Sample Diluent to the tube. Spin briefly.
10. Add 80 μ l of the detection antibody cocktail to each well. Incubate at room temperature for 1-2 hour.

Longer incubation time is preferable for higher signals and backgrounds

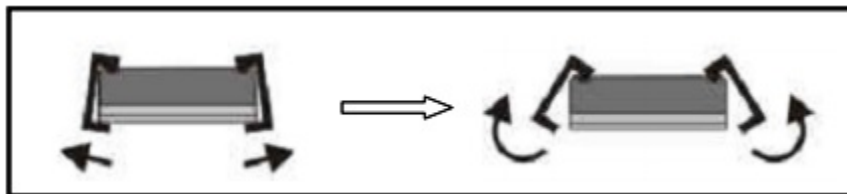
11. Decant the samples from each well, and wash 5 times (5 mins each) with 150 μ l of 1X Wash Buffer I and then 2 times with 150 μ l of 1x Wash Buffer II at room temperature with gentle rocking. Completely remove wash buffer in each wash step.

E. Incubation with Cy3 Equivalent Dye-Streptavidin & Wash

12. After briefly spinning down, add 1.4 ml of Sample Diluent to Cy3 equivalent dye-conjugated streptavidin tube. Mix gently.
13. Add 80 μ l 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.
Decant the samples from each well, and wash 5 times (5 mins each) with 150 μ l of 1X Wash Buffer I at room temperature with gentle rocking. Completely remove wash buffer in each wash step.
14. μ l of 1X Wash Buffer I at room temperature with gentle rocking. Completely remove wash buffer in each wash step.

F. Fluorescence Detection

15. Disassemble the device by pushing clips outward from the slide side. Carefully remove the slide from the gasket.



Be careful not to touch the surface of the array side.

16. Place the slide in the Slide Washer/Dryer (a 4-slide holder/centrifuge tube), add enough 1x Wash Buffer I (about 30 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 30 ml) and gently shake at room temperature for 5 minutes.

17. Remove water droplets completely by gently applying suction with a pipette to remove water droplets. Do not touch the array, only the sides.

You may also dry the glass slide by a compressed N₂ stream.

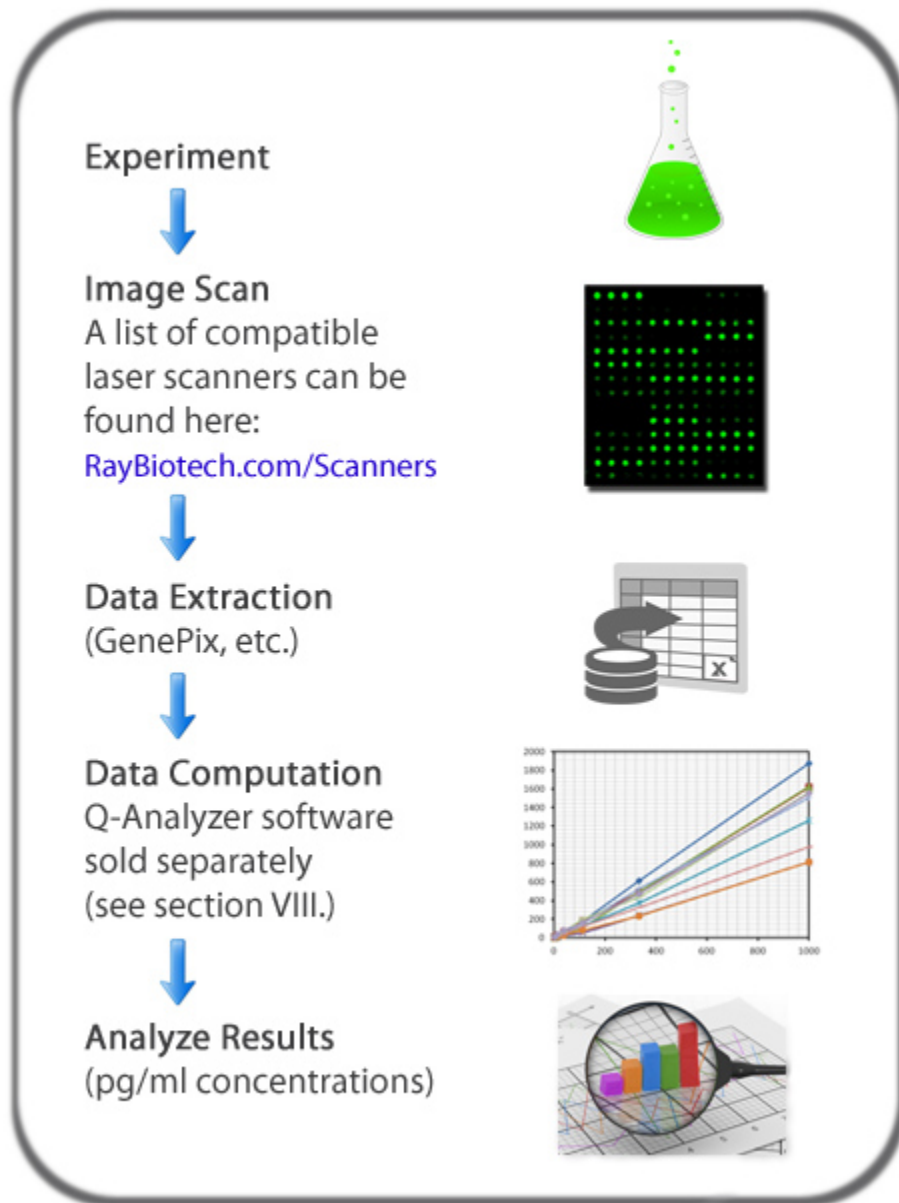
18. Imaging: The signals can be visualized through use of a laser scanner equipped with a Cy3 wavelength (green channel) such as Axon GenePix or Innopsys Innoscan. Make sure that the signal from the well containing the highest standard concentration (Std1) receives the highest possible reading, yet remains unsaturated.

In case the signal intensity for different cytokine varies greatly in the same array, we recommend using multiple scans, with a higher PMT for low signal cytokines, and a low PMT for high signal cytokines.

G. Data Analysis

19. Data extraction can be done using the GAL file that is specific for this array along with the microarray analysis software (GenePix, ScanArray Express, ArrayVision, MicroVigene, etc.). GAL files can be found here: www.RayBiotech.com/Gal-Files.html.

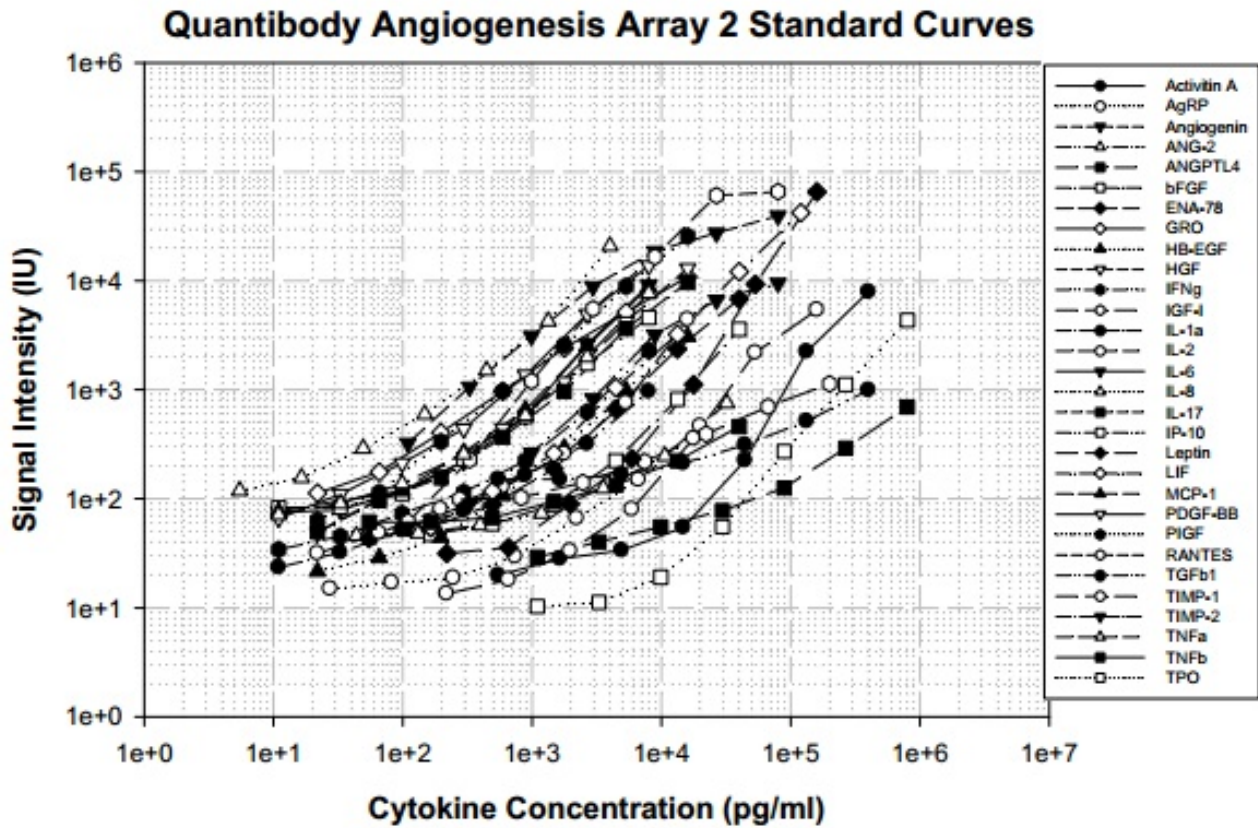
Need help analyzing all that data? Copy and paste your data into the Q-Analyzer Tool specific for this array, catalog number: **QAH-ANG-2-SW**. More information can be found in Section XII.



IX. Array Map & Standard Curves

Each antibody is printed in quadruplicate horizontally

	1	2	3	4	1	2	3	4	1	2	3	4
A	POS1				POS2				NEG			
B	Activin A				AgRP				Angiogenin			
C	Angiopoietin-2				ANGPTL4				bFGF			
D	ENA-78 (CXCL5)				GRO alpha/beta/gamma				HB-EGF			
E	HGF				IFN-gamma				IGF-1			
F	IL-1 alpha				IL-2				IL-6			
G	IL-8 (CXCL8)				IL-17A				IP-10 (CXCL10)			
H	Leptin				LIF				MCP-1 (CCL2)			
I	PDGF-BB				PLGF				RANTES (CCL5)			
J	TGF beta 1				TIMP-1				TIMP-2			
K	TNF alpha				TNF beta (TNFSF1B)				Thrombopoietin (TPO)			



X. Standard Concentrations

After reconstitution, the lyophilized cytokine standard mix contains the following concentrations for each antigen included.

Serial standard concentration (pg/ml)

(pg/ml)	Cntrl	Std7	Std6	Std5	Std4	Std3	Std2	Std1
Activin A	0	137	412	1,235	3,704	11,111	33,333	100,000
AgRP	0	7	21	62	185	556	1,667	5,000
Angiogenin	0	27	82	247	741	2,222	6,667	20,000
ANG-2	0	11	33	99	296	889	2,667	8,000
ANGPTL4	0	274	823	2,469	7,407	22,222	66,667	200,000
bFGF	0	14	41	123	370	1,111	3,333	10,000
ENA-78	0	14	41	123	370	1,111	3,333	10,000
GRO	0	5	16	49	148	444	1,333	4,000
HB-EGF	0	5	16	49	148	444	1,333	4,000
HGF	0	5	16	49	148	444	1,333	4,000
IFN γ	0	3	8	25	74	222	667	2,000
IGF-I	0	69	206	617	1,852	5,556	16,667	50,000
IL-1 α	0	3	8	25	74	222	667	2,000
IL-2	0	5	16	49	148	444	1,333	4,000
IL-6	0	3	8	25	74	222	667	2,000
IL-8	0	1	4	12	37	111	333	1,000
IL-17	0	5	16	49	148	444	1,333	4,000
IP-10	0	3	8	25	74	222	667	2,000
Leptin	0	55	165	494	1,481	4,444	13,333	40,000
LIF	0	41	123	370	1,111	3,333	10,000	30,000
MCP-1	0	3	8	25	74	222	667	2,000
PDGF-BB	0	3	8	25	74	222	667	2,000
PIGF	0	5	16	49	148	444	1,333	4,000
RANTES	0	27	82	247	741	2,222	6,667	20,000
TGF β 1	0	137	412	1,235	3,704	11,111	33,333	100,000
TIMP-1	0	55	165	494	1,481	4,444	13,333	40,000
TIMP-2	0	27	82	247	741	2,222	6,667	20,000
TNF α	0	3	8	25	74	222	667	2,000
TNF β	0	14	41	123	370	1,111	3,333	10,000
TPO	0	274	823	2,469	7,407	22,222	66,667	200,000

XI. Spiking & Recovery

The antibody pairs used in the kit have been tested to recognize their specific antigen. The spiking recovery rates of each protein in some common fluids are listed in the following tables.

The spiking recovery rate for human culture media and serum

ID	Spiking	SA	SA+Ag	SA%	CM	CM+Ag	CM%
Activin A	50,000	0	41082	82.2%	0	37715	75.4%
AgRP	2,500	125	2985	114.4%	0	2627	105.1%
Angiogenin	10,000	over	over	-	50	7700	76.5%
ANG-2	2,000	1800	3362	78.1%	0	1867	93.3%
ANGPTL4	100,000	19974	143712	123.7%	0	70907	70.9%
bFGF	5,000	0	3920	78.4%	0	4080	81.6%
ENA-78	2,500	14	2639	105.0%	0	2122	84.9%
GRO	2,000	179	1931	87.6%	10	2492	124.1%
HB-EGF	1,000	9	1096	108.7%	0	988	98.8%
HGF	2,000	34	1695	83.1%	3	1630	81.3%
IFN γ	2,000	18	1773	87.8%	7	1632	81.2%
IGF-I	25,000	948	21833	83.5%	0	17910	71.6%
IL-1 α	1,000	0	814	81.4%	0	897	89.7%
IL-2	2,000	0	1580	79.0%	0	1473	73.6%
IL-6	1,000	122	1181	105.9%	83	1214	113.1%
IL-8	500	1	363	72.4%	14	484	94.0%
IL-17	3,000	8	2943	97.8%	0	3313	110.4%
IP-10	1,000	44	961	91.7%	22	867	84.4%
Leptin	20,000	2013	28898	134.4%	0	19834	99.2%
LIF	15,000	48	17374	115.5%	0	13484	89.9%
MCP-1	1,000	129	856	72.6%	2441	3456	101.5%
PDGF-BB	2,000	304	1922	80.9%	1	1560	77.9%
PIGF	2,000	3	1662	83.0%	0	1519	75.9%
RANTES	20,000	500	18473	89.9%	0	18430	92.1%
TGF β 1	50,000	42761	86990	88.5%	0	34590	69.2%
TIMP-1	20,000	over	over	-	over	over	-
TIMP-2	10,000	over	over	-	13331	21154	78.2%
TNF α	1,000	31	1138	110.7%	0	1017	101.6%
TNF β	1,500	0	1306	87.1%	0	973	64.9%
TPO	100,000	1449	72128	70.7%	741	125311	124.6%

XII. Quantibody[®] Q-Analyzer

The Q-Analyzer is an array specific, Excel-based program. It is much more than a simple calculation macro; it performs sophisticated data analysis (see below for description).

The Q-Analyzer Tool specific for this array is catalog number: **QAH-ANG-2-SW**.

Key features:

- Simplicity: Easy to operate and requires no professional training. With a simple copy and paste process, the cytokine concentration is determined.
- Outlier Marking & Removing: The software can automatically mark and remove the outlier spots for more accurate data analysis
- Normalization: The program allows for intra- and inter-slide normalization for large numbers of samples.
- Two Positive Controls: The program utilizes the two positive controls in each array for normalization.
- Two Analytical Algorithms: Users can choose either linear regression or log-log algorithms to meet their analytical needs.
- Two Data Outputs: standard curves and digital concentration.
- User Intervention: The program allows for user manual handling of outliers and other analytical data.
- Lower and Upper Limits Determination: The program automatically marks out the values below or above the detection range.
- Standard Deviation: The program outputs the standard deviations of the quadruplicate spots for data accuracy.
- Analytical Tips: Q-Analyzer analysis tips are included in the program.

XIII. 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	Increase incubation time or change sample incubation step to overnight
	Too low protein concentration in sample	Lessen dilution or do not dilute sample. Concentrate sample if necessary.
	Improper storage of kit	Store kit as suggested temperature. Don't freeze/thaw the slide.
Uneven signal	Bubble formed during incubation	Decrease amount of rocking during incubations. check for bubble formation and remove bubbles.
	Arrays are not completely covered by reagent	Completely cover arrays with solution for all required steps.
	Reagent evaporation	Cover the incubation chamber with adhesive film during incubation
Poor standard curve	Cross-contamination from neighboring wells	Avoid overflowing wash buffer and other solutions into neighboring wells.
	Comet tail formation	Air dry the slide for at least 1 hour before usage
	Inadequate standard reconstitution or Improper dilution	Reconstitute the lyophilized standard well at the room temperature before making serial dilutions. Check pipettes and ensure proper serial dilutions.
	Inadequate detection	Increase laser power so 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 PMT or signal gain.
	Dark spots	Completely remove wash buffer in each wash step.
	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.

XIV. Publications Citing This Product

1. Das K., et al. Differential expression of steroid 5alpha-reductase isozymes and association with disease severity and angiogenic genes predict their biological role in prostate cancer. *Endocr Relat Cancer*. 2010 Aug 16;17(3):757-70. doi: 10.1677/ERC-10-0022.

Species: Human

Sample Type: Cell Lysate

More citations for this product may be available.

Contact techsupport@raybiotech.com.

XV. Experiment Record Form

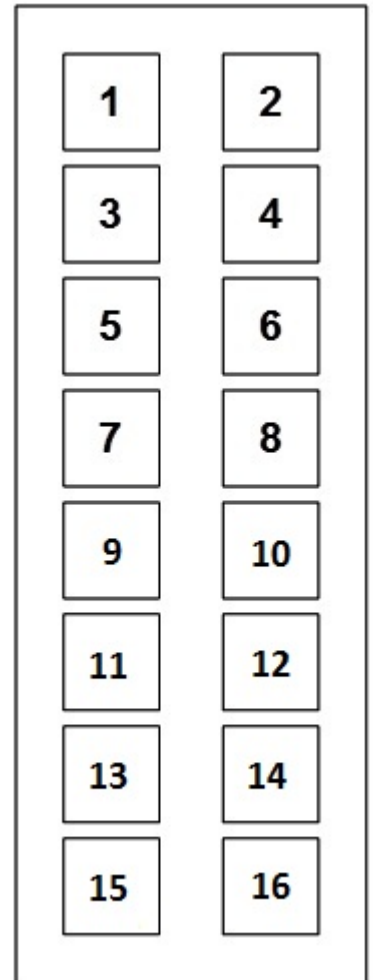
Date: _____

File Name: _____

Laser Power: _____

PMT: _____

Well No.	Sample Name	Dilution factor
1	CNTRL	
2	Std7	
3	Std6	
4	Std5	
5	Std4	
6	Std3	
7	Std2	
8	Std1	
9		
10		
11		
12		
13		
14		
15		
16		



XVI. How to Choose a Quantibody[®] Array?

Species-based selection:

Human (QAH-)	Mouse (QAM-)	Rat (QAR-)	Bovine (QAB-)	Canine (QAC-)
Equine (QAE-)	Feline (QAF-)	Primates (QAN-)	Porcine (QAP-)	Rabbit (QAL-)

Function-based selection:

Adhesion Molecule Arrays	Angiogenesis Arrays	Bone Metabolism Arrays	Chemokine Arrays
Custom Arrays	Cytokine Arrays	Growth Factor Arrays	IGF Signaling Arrays
IL-1 Family Arrays	Immune Response Arrays	Inflammation Arrays	Interleukin Arrays
Isotyping Arrays	MMP Arrays	Obesity Arrays	Ophthalmic Arrays
Periodontal Disease Arrays	Receptor Arrays	Th1/Th2/Th17 Arrays	

Cytokine Number-based selection:

Arrays are available in the Quantibody[®] platform to detect 1000 human, 200 mouse, or 67 rat proteins. GLP-Compliant testing services are also available.

To learn more about the Quantibody[®] Antibody Array, visit www.RayBiotech.com/Quantibody-Multiplex-Elisa-Array.html

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