RayBio® Human RTK Phosphorylation Antibody Array G-series 1

For Simultaneously Detecting the Relative Level of Tyrosin Phosphorylation of Human Receptor Tyrosine Kinases (RTKs)

User Manual

(Revised Mar 12, 2008)

(Cat#: AAH-PRTK-G1-4 and AAH-PRTK-G1-8)

RayBiotech, Inc.

We Provide You With Excellent Protein Array System And Service

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RayBiotech, Inc.

RayBio® Huamn RTK Phosphorylation Antibody Array G-series 1 Protocol

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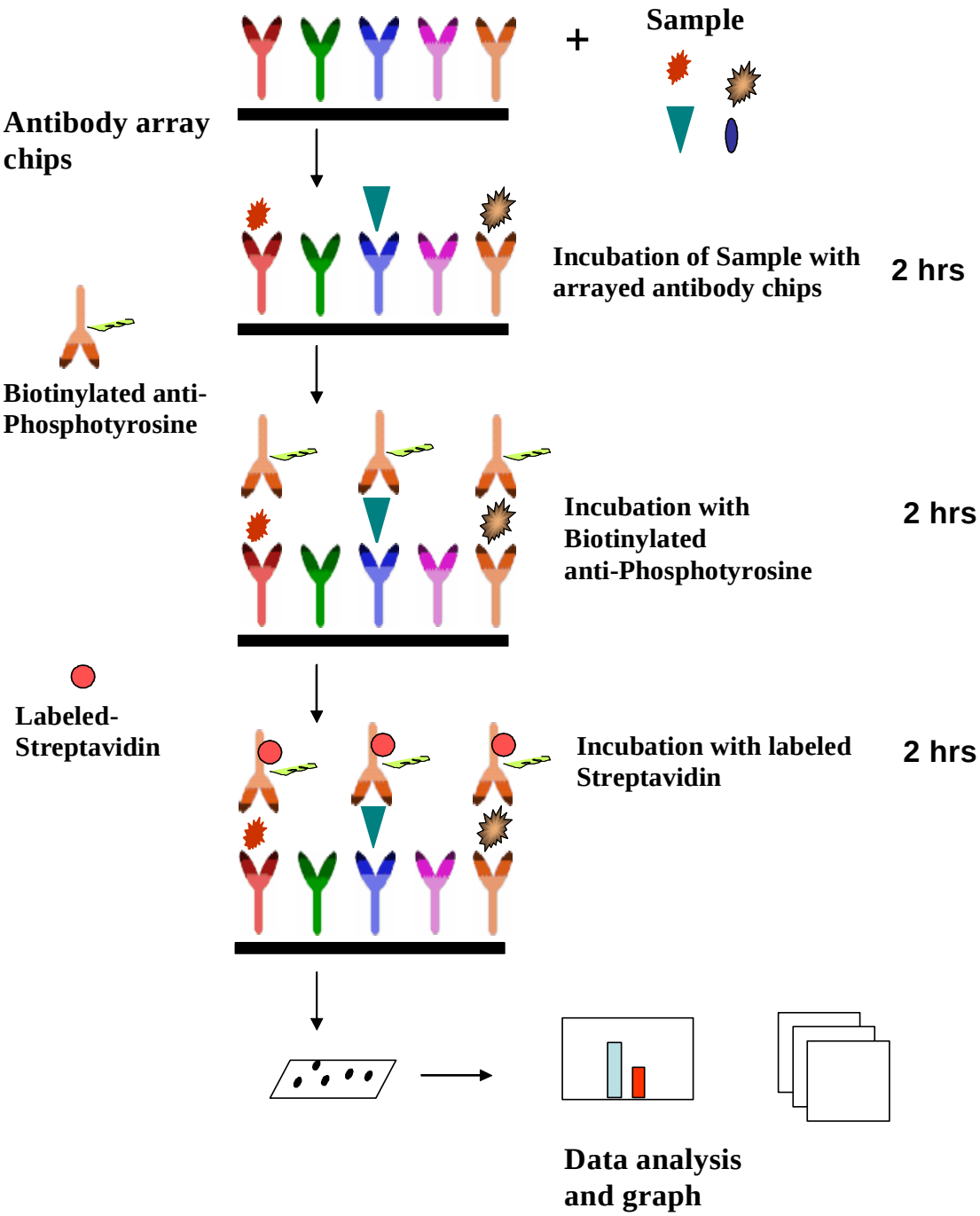
I. Introduction

Protein phosphorylation plays an unusually prominent role in cell signaling, development and growth. The RayBio® Human RTK Phosphorylation Antibody Array G-series 1 is a very rapid, convenient and sensitive assay to simultaneous detect multiple protein phosphorylations and can be used to monitor activation or function of important biological pathways.

RayBiotech is committed to developing a series of phosphorylation antibody arrays. Our first product in this series is RayBio® Human RTK Phosphorylation Antibody Array 1 which is specifically designed for simultaneously identifying the relative levels of phosphorylation of 71 different Human Receptor Tyrosine Kinases (RTKs) in cell lysate. By monitoring the changes in protein tyrosine phosphorylation in your experimental model system, you can verify pathway activation in your cell lines without spending excess time and effort in performing an analysis of immunoprecipitation and/or Western Blot.

To use the RayBio® Human RTK Phosphorylation Antibody Array G-series 1, treated or untreated cell lysate is added into antibody array glass chip. The antibody array chips are washed and biotinylated anti-phosphotyrosine antibodies are used to detect phosphorylated tyrosines on activated receptors. After incubation with Fluorescent dye-Conjugated Streptavidin (cy3 equivalent), image the signals using laser scanner, such as the Axon GenePix, using the cy3 channel.

Here's how it works



II. Materials Provided

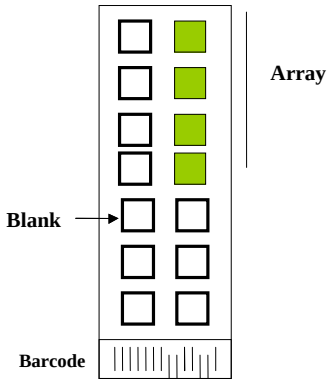
Upon receipt, the kit should be stored at –20 °C to -80 °C. Please use within 6 months from the date of shipment. After initial use 2X Cell Lysis Buffer, Blocking Buffer, 20X Wash Buffer I, 20X Wash Buffer II, Biotin-Conjugated Anti-phosphotyrosine and Fluorescent dye-Conjugated Streptavidin (cy3 equivalent) should be stored at 4 °C to avoid repeated freeze-thaw cycles. Array I Glass Chip, Protease Inhibitor Cocktail and Phosphatase Inhibitor Cocktail Set II should be kept at –20 ° to -80°C. Use within three months after initial use.

- RayBio® Human RTK Phosphorylation Antibody Array G-series 1 Glass Chip with Frame (each slide with 4 Subarrays, 1 slide for 4 subarray chips, and 2 for 8 subarray chips)
- 2X Cell Lysis Buffer (5 ml)
- Protease Inhibitor Cocktail (1/2 tubes, 1 tube for 4-subarrary chips, and 2 for 8-subarray chips)
- Phosphatase Inhibitor Cocktail Set II (1/2 tubes, 1 tube for 4-subarrary chips, and 2 for 8-subarray chips)
- Blocking Buffer (8 ml)
- 20X Wash Buffer I (30ml)
- 20X Wash Buffer II (30ml)
- Biotin-Conjugated Anti-phosphotyrosine (1/2 tubes, 1 tube for 4-subarrary chips, and 2 for 8-subarray chips)
- Fluorescent dye-Conjugated Streptavidin (cy3 equivalent) (1/2 tubes, 1 tube for 4-subarrary chips, and 2 for 8-subarray chips)
- Wash Buffer III (20 ml)
- Adhesive film

III. Additional Materials Required

- Shaker
- Laser scanner for fluorescence detection
- Aluminum foil
- Distilled water
- Plastic box
- 50 ml Centrifuge tube
- Isopropanol (2-propanol)

Layout of Array Glass Chip



4 arrays in one glass chip

IV. Reagent Preparation.

1. Protease Inhibitor Cocktail: Briefly spin down the Protease Inhibitor Cocktail tube before use. Add 60 µl of 1x Lysis Buffer into the vial to prepare a 100X Protease Inhibitor Cocktail.

2. Phosphatase Inhibitor Cocktail Set II: Briefly spin down the Phosphatase Inhibitor Cocktail Set II tube before use. Add 180 µl of 1X Lysis Buffer into the vial to prepare 25X Phosphatase Inhibitor Cocktail Set II Concentrate**. Dissolve the powder thoroughly by a gentle mix.**

3. 2X Cell Lysis Buffer: Cell lysis buffer should be diluted 2-fold with deionized or distilled water. Add 20 µl of Protease Inhibitor Cocktail Concentrate and 80 µl of Phosphatase Inhibitor Cocktail Set II Concentrate into 1.9 ml of 1X Lysis Buffer before use. Mix well.

4. 20X Washing Buffer I or II : If the 20X Wash Concentrate contains visible crystals, warm to room temperature and mix gently until dissolved. Dilute 25 ml of Wash Buffer Concentrate into deionized or distilled water to yield 500 ml of 1X Wash Buffer.

5. Biotinylated anti-Phosphotyrosine: Briefly spin the Detection Antibody tube before use. Add 65 µl of Blocking Buffer into the tube to prepare a Biotinylated Anti-phosphotyrosine Concentrate. Pipette up and down to mix gently (the concentrate can be stored at 4^0C for 5 days). Add 30 µl of Detection Antibody Concentrate into a tube with 570 µl of Blocking Buffer. Mix gently to prepare 1X Biotinylated Anti-phosphotyrosine.

6. Fluorescent dye-Conjugated Streptavidin (cy3 equivalent): briefly spin the Fluorescent dye-Conjugated Streptavidin before use. Add 50 µl of Blocking Buffer into the tube to prepare a Streptavidin Concentrate. Pipette up and down to mix gently. Add 10 µl of Streptavidin Concentrate into a tube with 1 ml of Blocking Buffer. Mix gently to prepare 1X Streptavidin solution.

V. Overview and General Considerations

A. Preparation of Samples

The cell can be prepared using following conventional way.

For attached cells, remove supernatant from cell culture, wash cells twice with cold 1X PBS (for suspension cells, pellet the cells by spinning down the cells at 1500 rpm for 10 min) making sure to remove any remaining PBS before adding Lysis Buffer containing Protease Inhibitor Cocktail and Phosphatase Inhibitor Cocktail Set II. Solubilize the cells at $2x10^7$ cells/ml in 1X Lysis Buffer. Pipette up and down to resuspend the cells and rock the lysates gently at 2–8 °C for 30 minutes. Transfer extracts to microcentrifuge tubes and centrifuge at 14,000 x g for 5 min.

It is recommended that sample protein concentrations be determined using a total protein assay. For incubation with the Phosphorylation Antibody Array I, use cell lysates in 50–1000 µg/ml of concentration (as starting point, we recommended to use 400 µg/ml of cell lysate, dilute the lysate at least 5-folds with Blocking Buffer).

Lysates should be used immediately or aliquoted and stored at –70 °C. Thawed lysates should be kept on ice prior to use.

If you experience high background, you may further dilute your sample. If signals are too week, the cell lysates can be pretreated by immunoprecipitations before incubation with array membranes. Immunoprecipitations can be done using anti-phosphotyrosine and protein A.

B. Handling glass chips

- The microarray slides are sensitive, do not touch the array surface by tips, forceps or hand. Hold the slides by the edges only.
- Handle all buffers and slides with latex free gloves.
- Avoid breaking glass slide.
- Clean environment

C. Incubation

- Completely cover array area with sample or buffer during incubation, and cover the incubation chamber with adhesive film or plastic sheet protector to avoid drying.
- 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 less than 50 µl of sample or reagent is used.
- Avoid cross-contamination from overflowing solution to neighboring wells.
- Several incubation steps such as step 3 (sample incubation), step 8 (biotin-Ab incubation) or step 11 (Fluorescent dye-Conjugated Streptavidin incubation) may be done at 4 °C for overnight. Please make sure to cover the incubation chamber tightly to prevent evaporation.
- Avoid array slide to exposure to light since step 9 in page 11.

VI. Protocol

A. Dry the Glass Chip

Open the box containing glass chip with frame and take it out, and then let it air dry for 1 hour in clean environment before use.

Note: *Protect the chip from dust or others contaminants.*

B. Blocking and Incubation

1. Add 100 µl of 1 X Blocking Buffer into each well and incubate at room temperature with gentle shaking for 30 min to block slides. Make sure no bubbles are in the well.

   Note: Only add reagents to wells printed with antibodies.

2. Decant Blocking Buffer from each well (make sure to remove all of buffer). Add 100 µl of each sample into appropriate wells. Incubate arrays with sample at room temperature with gentle shaking for 2 hours or 4 °C for overnight.

   Note: We recommended using 100 µl of cell lysates in 50–1000 ug/ml of concentration (as starting point, we recommended to use 400 µg/ml of concentration of cell lysate). ***Dilute the lysate at least 5 folds with blocking buffer. Make sure there is no bubble in the wells.***

   Note: The amount of sample used depends on the abundance of target proteins. More sample 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 °C overnight.*

Note: *The cell lysates can be pretreated by immunoprecipitations before incubation with array membranes if signals are too week. Immunoprecipitations can be done using anti-phosphotyrosine and protein A. The elution samples from protein A can be diluted with Blocking Buffer and then incubate with array membranes.*

3. Decant the samples from each well, and wash 3 times with 100 µl of 1X Wash Buffer I at room temperature with gentle shaking. 5 min per wash.

   Note: *avoid solution flowing into neighboring wells.*

4. Put the glass chip with frame into a box with Wash Buffer I (cover the whole glass slide and frame with Wash Buffer I), and wash at room temperature with gentle shaking for 20 min.

5. Decant the Wash Buffer I from each well, Put the glass chip with frame into the box with Wash Buffer II (cover the whole glass slide and frame with Wash Buffer II), and wash 2 times at room temperature with gentle shaking for 5 min.

6. Remove all of Wash Buffer II in the well. Add 100 µl of 1x Biotin-conjugated Anti-phosphotyrosine to each corresponding well. Incubate at room temperature with gentle shaking for 2 hours.

7. Decant the antibody solution and wash as directed in steps 4, Wash 3 times.

8. Wash as directed in steps 5.

9. Remove all of Wash Buffer II in the well. Add 100 µl of 1X Fluorescent dye-Conjugated Streptavidin to each subarray. Cover the incubation chamber with Adhesive film. Cover the plate with aluminum foil to avoid exposure to light or incubate in dark room (the array slide also needs to avoid exposure to light in the following step 12, 13 and 14).

10. Incubate at room temperature with gentle shaking for 2 hours.

Note: *Incubation may be done at 4 °C for overnight.*

11. Decant the streptavidin solution and disassemble the slide out of the incubation frame and chamber.

12. Gently put the glass chip into a 50 ml centrifuge tube or a plastic box with 40 ml of 1X Wash Buffer I. Gently roll over the tube or shake for 5 min. Remove the wash buffer. Repeat 2 times for a total of 3 washes.

13. Wash the glass chip with 40 ml of Wash Buffer II. Repeat one time for a total of two washes. 5 min per wash.

14. Finally wash the glass chip with 40 ml of deionized or distilled water.

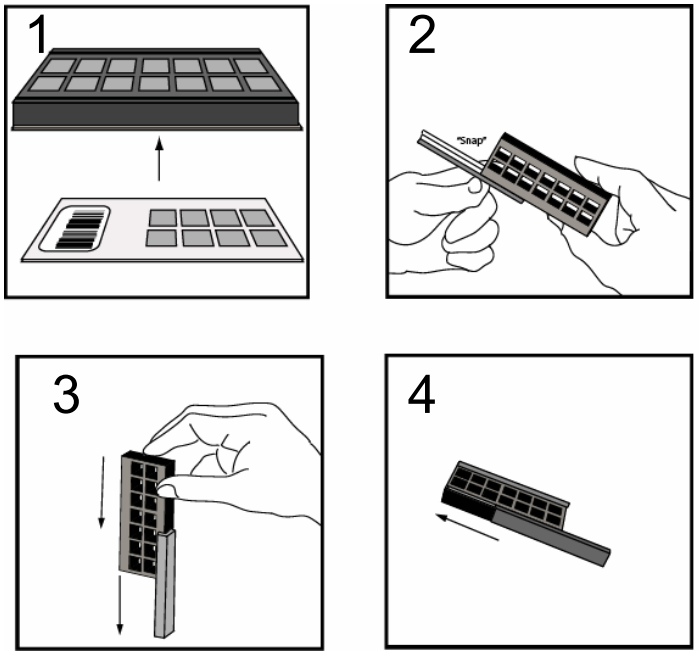
Note: You may assemble the glass chip into an incubation chamber by following step. You may want to practice assembling the device with a blank glass slide.

1. Apply slide to incubation chamber barcode facing upward as in step 1.

2. Gently snap one edge of a snap-on side as shown in step 2.

3. Gently press other of side against lab bench and push in Direction shown in step 3.

4. Repeat with the other side.



C. Fluorescence Detection

1. Put the glass chip into a 50 ml centrifuge tube, dry the glass chip by centrifuge at 1,000 rpm for 3 minutes. Or dry the glass chip by a compressed N_2 stream. Or let glass chip dry completely in clean air condition (protect from light). Make sure the slides are absolutely dry before the scanning procedure.

   Image the signals using laser scanner, such as the Axon GenePix, using the cy3 channel.

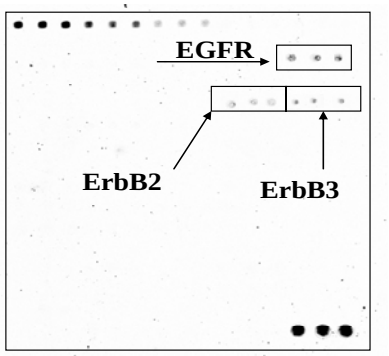
   Note: *We recommend scanning slides right after experiment.You also can store the slide at –20 °C in dark for several days .If you do not have a laser scanner, we can provide service for you. Just simply send your slide to us and we will take care of it.*

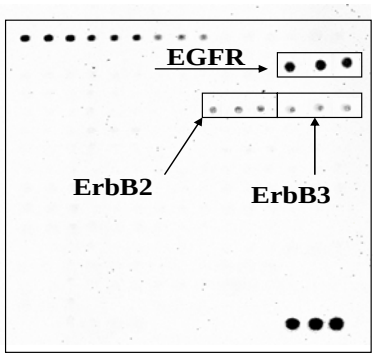
   Note: *Put the glass chip into a tube with 40 ml of 30% Wash Buffer III in isopropanol (add 15 ml of Wash Buffer III into a tube with 35 ml of isopropanol, mix well) and incubate for 10 min at room temperature if the background is not even or too high (cover the tube with aluminum foil to avoid exposure to light or incubate in dark room). Dry the slide and re-scan the slide.*

VII. Interpretation of Results:

The following figure shows the RayBio® Human RTK Phosphorylation Antibody Array 1 probed with different cell lysates. The images were captured using laser scanner. A biotinylated protein produces positive control signals, which can be used to identify the orientation and to normalize the results from different wells being compared.

Antibody affinity to its target varies significantly between antibodies. The fluorescence intensity detected on the array with each antibody depends on this affinity; therefore, signal intensity comparison can be performed only within the same antibody/antigen system and not between different antibodies. Certain proteins containing phosphorylated tyrosine may not be recognized by biotinylated anti-phosphotyrosine because of steric hindrance of the recognition site.



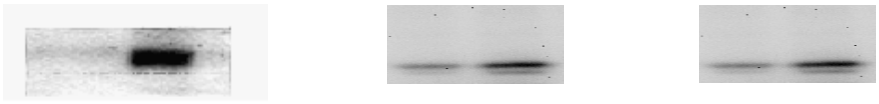


Untreated A431 cells (Cell lysate: 400 µg/ml)

EGF treated A431 cells (Cell lysate: 400 µg/ml)

Fig. 1. Human epidermoid carcinoma cell line, A431 cells that were 80-90% confluent were serum starved overnight, then exposed to 100 ng/ml EGF for 10 minutes at 37 °C. Control cells were serum starved without the subsequent stimulation with EGF. Cell

lysates were prepared following the "Sample Preparation" portion of our protocol IV. To use the RayBio®Human RTK Phosphorylation Antibody Array G-series 1, treated or untreated cell lysate was added into antibody array glass chips. The antibody array chips were washed and biotinylated anti-phosphotyrosine antibody was used to detect phosphorylated tyrosines on activated receptors. After incubation with Fluorescent dye-Conjugated Streptavidin, the signals were visualized by laser scanner using the cy3 channel.



Untreated Treated Untreated Treated Untreated Treated
EGFR **ErbB2** **ErbB3**

Fig. 2. Immunoprecipitations were done using anti-EGFR, ErbB2 and ErbB3 monoclonal antibodies and Protein A. Immunoblots were incubated with a biotinylated anti-phosphotyrosine monoclonal antibody to detect phosphorylated target protein receptors. Bands were visualized with Streptavidin-HRP followed by chemiluminescent detection substrate.

RayBio® Human RTK Phosphorylation Antibody Array G-series 1 Map

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	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1	POS 1	POS 1	POS 1	POS 2	POS 2	POS 2	POS3	POS3	POS3	ABL1	ABL1	ABL1	ACK1	ACK1	ACK1
2	NEG	NEG	NEG	NEG	NEG	NEG	ALK	ALK	ALK	Axl	Axl	Axl	Blk	Blk	Blk
3	BMX	BMX	BMX	Btk	Btk	Btk	Csk	Csk	Csk	Dtk	Dtk	Dtk	EGFR	EGFR	EGFR
4	EphA1	EphA1	EphA1	EphA2	EphA2	EphA2	EphA3	EphA3	EphA3	EphA4	EphA4	EphA4	EphA5	EphA5	EphA5
5	EphA6	EphA6	EphA6	EphA7	EphA7	EphA7	EphA8	EphA8	EphA8	EphB1	EphB1	EphB1	EphB2	EphB2	EphB2
6	EphB3	EphB3	EphB3	EphB4	EphB4	EphB4	EphB6	EphB6	EphB6	ErbB2	ErbB2	ErbB2	ErbB3	ErbB3	ErbB3
7	ErbB4	ErbB4	ErbB4	FAK	FAK	FAK	FER	FER	FER	FGFR1	FGFR1	FGFR1	FGFR2	FGFR2	FGFR2
8	FGFR2 (α isoform)	FGFR2 (α isoform)	FGFR2 (α isoform)	Fgr	Fgr	Fgr	FRK	FRK	FRK	Fyn	Fyn	Fyn	Hck	Hck	Hck
9	HGFR	HGFR	HGFR	IGF-I R	IGF-I R	IGF-I R	Insulin R	Insulin R	Insulin R	Itk	Itk	Itk	JAK1	JAK1	JAK1
10	JAK2	JAK2	JAK2	JAK3	JAK3	JAK3	LCK	LCK	LCK	LTK	LTK	LTK	Lyn	Lyn	Lyn
11	MATK	MATK	MATK	M-CSFR	M-CSFR	M-CSFR	MUSK	MUSK	MUSK	NGFR	NGFR	NGFR	PDGFR-α	PDGFR-α	PDGFR-α
12	PDGFR-β	PDGFR-β	PDGFR-β	PYK2	PYK2	PYK2	RET	RET	RET	ROR1	ROR1	ROR1	ROR2	ROR2	ROR2
13	ROS	ROS	ROS	RYK	RYK	RYK	SCFR	SCFR	SCFR	SRMS	SRMS	SRMS	SYK	SYK	SYK
14	Tec	Tec	Tec	Tie-1	Tie-1	Tie-1	Tie-2	Tie-2	Tie-2	TNK1	TNK1	TNK1	TRKB	TRKB	TRKB
15	TXK	TXK	TXK	Tyk2	Tyk2	Tyk2	TYRO10	TYRO10	TYRO10	VEGFR2	VEGFR2	VEGFR2	NEG	NEG	NEG
16	VEGFR3	VEGFR3	VEGFR3	ZAP70	ZAP70	ZAP70	NEG	NEG	NEG	NEG	NEG	NEG	POS4	POS4	POS4

VIII. Troubleshooting Guide

Problem	Cause	Recommendation
Weak signal	Inadequate detection	Check laser power and PMT parameters
	Inadequate reagent volumes or improper dilution	Check pipettors and ensure correct preparation
	Short incubation times	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 at suggested temperature
High background	Excess of biotinylated antibodies	Make sure to use the correct amount of antibodies
	Excess of streptavidin	Make sure to use the correct amount of streptavidin
	Inadequate detection	Check laser power and PMT parameters
	Inadequate wash	Increase the volume of wash buffer and incubation time
Uneven signal	Bubbles formed during incubation	Avoid bubble formation during incubation
	Arrays are not completely covered by reagent	Completely cover arrays with solution

IX. Reference List

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RayBiotech, Inc., the protein array pioneer company, strives to research and develop new products to meet demands of the biomedical community. RayBio's patent-pending technology allows detection of over 180 cytokines, chemokines and other proteins in a single experiment. Our format is simple, sensitive, reliable and cost effective. Products include: Cytokine Arrays, Chemokine Arrays, ELISA kits, Phosphotyrosine kits, Recombinant Proteins, Antibodies, and custom services.

Antibody Array

Cytokine Antibody Array: Simultaneous detection up to 200 proteins (cytokine, chemokine, growth factor, adipokine, angiogenic factor, protease) in one experiment

Phosphorylation Antibody Array

- **RTK antibody array**
- **EGFR phosphorylation antibody arrays**

Label based antibody array: Simultaneous detection more than 500 proteins in one experiment

Quantibody Array: Quantitative measurement of multiple protein levels

Protein Array

ELISA

Cell-Based Phosphorylation ELISA

Tissue MicroArray

Protein: Cytokine, Chemokine, Adiplokine, Angiogenic factor, Virus, bacteria and infectious disease protein, hormone, Enzyme, other

Peptide

Antibody: Cytokine, Adipokine, Angiogenic factor, Signal transduction, Transcription factor, Receptor, Adhesion molecule, Virus, bacteria and other infectious agents, Secondary antibody, Tag antibody, Immunoglobulin, Hormone, Cell surface, Protease, other

Assay service: just simply send your samples and get data in 1 to 2 weeks. Antibody array, Protein array, ELISA, Quantibody array

Antibody production: highest quality with very competitive price Monoclonal antibody, Recombinant antibody, Polyclonal antibody, Phase display, Antibody angineering, Antibody conjugation

Recombinant protein production

Assay development

Antibody array, Protein array, Peptide array, ELISA, Phosphorylation assay Tissue array

Array printing

Contact and non-contact arrayers. All kinds of substrates of your choice including glass slides, membranes and plates.

Note:

This product is for research use only.