RayBio® Human EGFR Phosphorylation Antibody Array 1

User Manual

(Revised May 14, 2010)

For Simultaneously Detecting the Relative Levels of Phosphorylation of EGF Receptors at 17 Different Phosphorylation Sites.

(Cat# AAH-PER-1-2; AAH-PER-1-4; AAH-PER-1-8)

Please read manual carefully before starting experiment

RayBiotech, Inc.

We Provide You with Excellent Protein Array Systems and Service

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RayBiotech, Inc.

RayBio® Human EGFR Phosphorylation Antibody Array 1 Protocol

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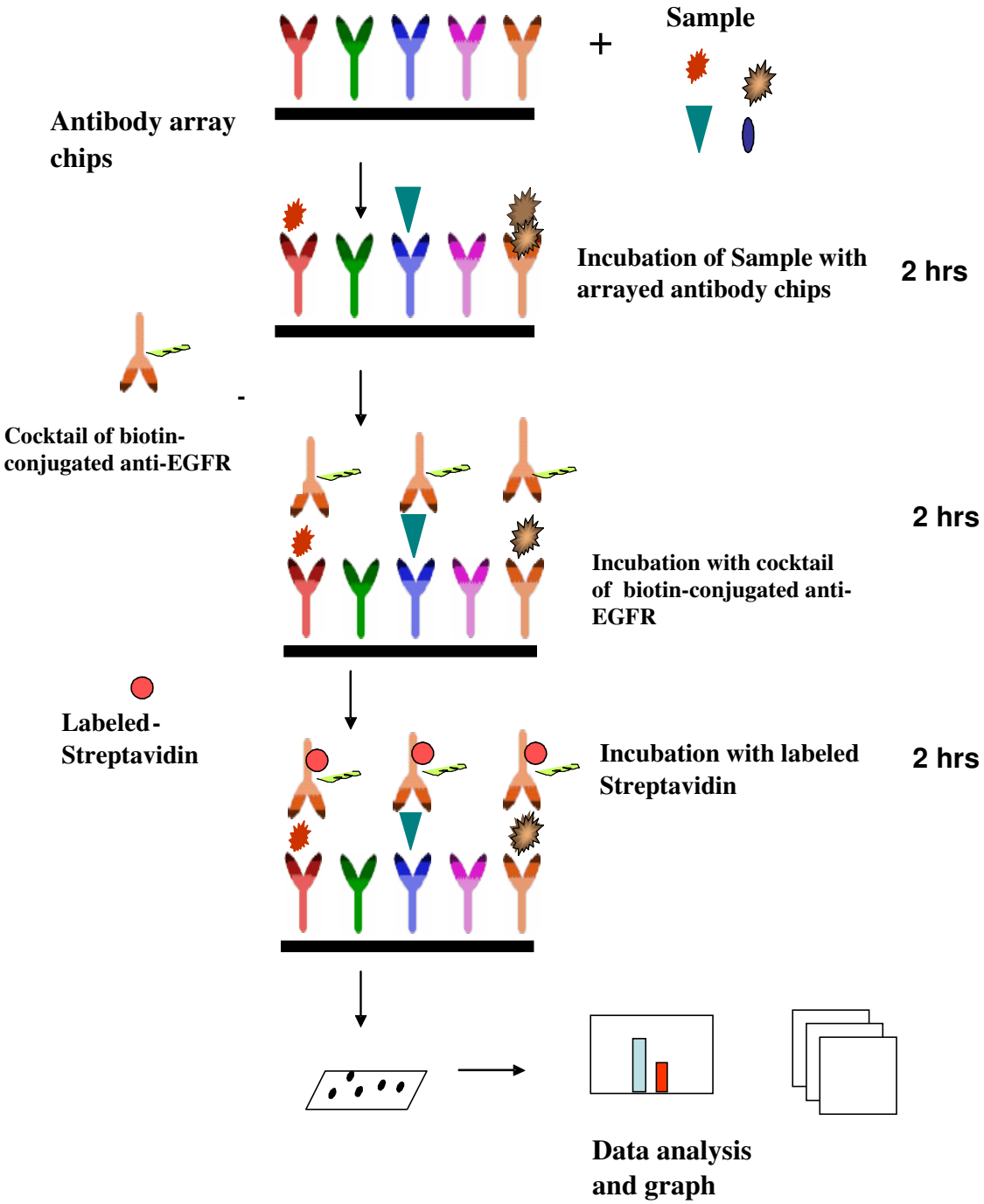
I. Introduction

The EGFR family of membrane receptors consists of four different proteins called EGFR/ErbB1/HER1, ErbB2/HER2, ErbB3/HER3, and ErbB4/HER4. Under normal physiological conditions, the ErbB receptors play crucial roles in propagating signals regulating cell proliferation, differentiation, motility, and apoptosis. EGF receptor family shows clear differences between individual receptors, and also a large overlap. ErbB1 is the family member with most interaction partners and the highest percentage of tyrosine residues with more than one binding partner. ErbB3 is characterized by a large number of binding sites for phosphatidylinositol-3-kinase (PI3K), while ErbB2 has only few interaction partners with Shc as the most frequent one. ErbB1 and ErbB4 have a variety of phosphotyrosines that bind Grb2, or Grb2 and Shc. The ErbB1 and ErbB4 have a greater diversity of interaction partners than ErbB2 and ErbB3. ErbB1 and ErbB2 are often over-expressed or amplified in cancers, making them important targets for drugs currently in use or under development.

With the RayBio® Human EGFR Phosphorylation Antibody Array 1, researchers can now simultaneously detect the relative level of phosphorylation of 17 different specific sites for Human EGFR family in cell lysate. By monitoring the changes in protein 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.

By using RayBio® Human EGFR Phosphorylation antibody Array 1, treated or untreated cell lysate is added into antibody array membranes. The antibody array membranes are washed and cocktail of biotin-congujated anti-EGFR is used to detect phosphorylated ErbB1~B4 and pan ErbB1~B4 . After incubation with HRP-streptavidin, the signals are visualized by chemiluminescence.

Here's how it works



II. Materials Provided

Upon receipt, the kit should be stored at –20 °C. After initial use 2X Cell Lysis Buffer, Blocking Buffer, 20X Wash Buffer I, 20X Wash Buffer II, Cocktail of Biotin-Conjugated Anti-EGFR and HRP-Conjugated Streptavidin should be stored at 4 °C to avoid repeated freeze-thaw cycles. Array membrane, Protease Inhibitor Cocktail and Phosphatase Inhibitor Cocktail should be kept at –20 ° C. Please use within 6 months from the date of shipment.

- RayBio® Human EGFR Phosphorylation Antibody Array 1 membrane (2, 4, or 8 membranes)
- 2X Cell Lysis Buffer (5 ml)
- Protease Inhibitor Cocktail (1 tube for 2-4 membranes, and 2 for 8 membranes)
- 100X Phosphatase Inhibitor Cocktail Set I Concentrate (1 tube for 2–4 membranes, and 2 for 8 membranes)
- Phosphatase Inhibitor Cocktail Set II (1 tube for 2–4 membranes, and 2 for 8 membranes)
- Blocking Buffer (25 ml for less 4 membranes and 50 ml for 8 membranes)
- 20X Wash Buffer I (30 ml)
- 20X Wash Buffer II (30 ml)
- Cocktail of Biotin-Conjugated Anti-EGFR (1 tube for 2 membranes, 2 for 4 membranes, and 4 for 8 membranes)
- 1,000X HRP-Conjugated Streptavidin (18 µl).
- Detection Buffer C (1.5 ml for 2~4 membranes, 2.5 ml for 8 membranes)
- Detection Buffer D (1.5 ml for 2~4 membranes, 2.5 ml for 8 membranes)
- Eight-Well Tray (1 each)

- Plastic sheets

III. Additional Materials Required

- Small plastic boxes or containers
- Shaker
- Plastic sheet protector or Saran Wrap
- Kodak X-Omat™ AR film (REF 165 1454) and film processor or Chemiluminescence imaging system

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 Concentrate.

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 each 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 before use. Add 20 µl of prepared 100X Protease Inhibitor Cocktail Concentrate and 20 µl of 100X Phosphatase Inhibitor Cocktail Set I Concentrate **(bring Set I concentrate tube to room temperature to thaw the solution before use)** , and 80 µl of 25X Phosphatase Inhibitor Cocktail Set II into 1.9 ml 1X Lysis Buffer before use. Mix well.

4. 20X Washing Buffer I or II: If the Wash Buffer Concentrate (20X) 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. Cocktail of Biotin-Conjugated Anti-EGFR: Briefly spin the Detection Antibody tube before use. Add 100 µl of Blocking Buffer to the tube. Mix gently and transfer all mixture to a tube containing 2.1 ml of Blocking Buffer to prepare 1X Cocktail of Biotin-Conjugated Anti-EGFR.

6. 1000X HRP-Conjugated Streptavidin: briefly spin down the HRP-Streptavidin Concentrate and pipette up and down to mix gently before use. E.g. add 5 µl of HRP-Conjugated Streptavidin concentrate into a tube with 5 ml Blocking Buffer. Mix gently to prepare 1X HRP-Conjugated Streptavidin (don't store the diluted Streptavidin for next day use).

   Note: mix tube containing 1,000X HRP-Conjugated Streptavidin well before use since precipitation may form during storage.

V. Overview and General Considerations

A. Preparation of Samples

The cell lysate can be prepared as follows.

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. Solubilize the cells at $2x10^7$ cells/ml in 1X Lysis Buffer containing Protease Inhibitor Cocktail and Phosphatase Inhibitor Cocktail Set I and Set II. Pipette up and down to resuspend cells and rock the lysates gently at 2–8 °C for 30 minutes. Transfer extracts to microfuge tubes and centrifuge at 14,000 x g for 10 min.

It is recommended that sample protein concentrations be determined using a total protein assay. For incubation with the EGFR Phosphorylation Antibody Array 1, use at a protein concentration of 50-1000 µg/ml for cell lysates.

Lysates should be used immediately or aliquot 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 samples.

B. Handling Array Membranes

- Always use forceps to handle membranes, and grip the membranes by the edges only.
- Never allow array membranes to dry during experiments.
- Avoid touch Array membrane by hand, tips or any sharp tools.

C. Incubation

- Completely cover membranes with sample or buffer during incubation, and cover eight-well tray with lid to avoid drying.
- Avoid foaming during incubation steps.
- Perform all incubation and wash steps under gentle rotation.

- Several incubation steps such as step 4 (sample incubation), or step 8 (biotin-Ab incubation) or step 10 (HRP-streptavidin incubation) may be done at 4 °C for overnight.

VI. Protocol

A. Blocking and Incubation

1. Place each membrane into the provided 8-well tray (top left corner marked with "**-**"). You can mark the membranes in a corner with a number using a pen to identify the different membranes prior to starting experiment.

   Note: *The printed side should be facing upward.*

2. Add 1 ml Blocking Buffer and incubate at room temperature with gentle shaking for 1 hour to block membranes.

3. Decant Blocking Buffer from each container. Add 1.0 ml of sample into each array membrane, and cover with the lid. Incubate at room temperature for 2 hours. Dilute sample using Blocking Buffer.

   Note: 1). We recommended using 1.0 ml of 50-1000 µg/ml concentration of cell lysates (as starting point, we recommended to use a concentration of 200 µg/ml of cell lysate. ***Dilute the cell lysates at least 5 folds with Blocking Buffer.***

   Note: 2). The amount of sample used depends on the abundance of protein. More of the sample can be used if signals are

too weak. If signals are too strong, the sample can be diluted further.

Note: 3). Incubation may be done at room temperature for 2 hours. Over night at 4°C

4. Decant the samples from each container, and wash 3 times with 2 ml of 1X Wash Buffer I at room temperature with shaking. 3 min per wash.

5. Carefully remove each array membrane and place all of membranes into a plastic container with a minimum of 20 ml of 1X Wash Buffer I. Rinse the 8-Well Multi-dish with deionized or distilled water and dry thoroughly. Wash array membranes with 1X Wash Buffer with shaking. Repeat 2 times for a total of 3 washes. 5 min per wash.

6. Wash 3 times with a minimum of 20 ml of 1X Wash Buffer II at room temperature with shaking. 5 min per wash.

7. Carefully remove each array membrane from the container, return it to the 8-well tray.

8. Add 1 ml of diluted Cocktail of Biotin-Conjugated Anti-EGFR to each membrane. Incubate at room temperature with gentle shaking for 2 hours.

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

9. Wash as directed in steps 5, 6 and 7.

10. Add 1.5 ml of 1X HRP-conjugated streptavidin to each membrane.

    Note: *Mix tube containing 1X HRP-Conjugated Streptavidin well before use since precipitation may form during storage.*

11. Incubate at room temperature for 2 hours.

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

12. Wash as directed in steps 5 and 6.

B. Detection

*** Do not let the membrane dry out during detection. The detection process must be completed within 40 minutes without stopping.**

1. Proceed with detection reaction.

   Add 250 µl of Detection Buffer C and 250 µl of Detection Buffer D for one membrane; mix both solutions; Drain off excess wash buffer by holding the membrane vertically with forceps. Place membrane protein side up ("-" mark is on the protein side top left corner) on a clean plastic sheet (provided in the kit). Pipette the mixed Detection Buffer on to the membrane and incubate at room temperature with gentle shaking for 2 minutes. Ensure that the detection mixture is completely and evenly covering the membrane without any air bubbles.

2. Drain off excess detection reagent by holding the membrane vertically with forceps and touching the edge against a tissue.

Gently place the membrane, protein side up, on a piece of plastic sheet ("-" mark is on the protein side top left corner). Cover the array with another piece of plastic sheet. Gently smooth out any air bubbles. Avoid using pressure on the membrane.

3. Detect signal directly from membrane using chemiluminescene imaging system or expose to x-ray film (we recommend to use Kodak X-Omat™ AR film) detect signal using film developer. Expose the membranes for 40 Seconds. Then re-expose the film according to the intensity of signals. If the signals are too strong (background too high), reduce exposure time (eg, 5–30 seconds). If the signals are too weak, increase exposure time (eg, 5–20 min or overnight). Or re-incubate membranes overnight with 1X HRP-conjugated streptavidin, and repeat detection on the second day.

4. Save membranes at –20 °C to –80 °C for future reference.

VII. Interpretation of Results:

The following figure shows RayBio Human EGFR Phosphorylation Antibody Array I membranes probed with different cell lines. The signals were detected by using a chemiluminescence imaging device. Alternatively, membranes may also be exposed to Kodak X-Omat film at room temperature. A biotinylated protein provides positive signals (indicated "Pos" on the array map, page 15) which can be used to orient the membrane and to normalize the results from different arrays being compared. The signals of pan EGFR, ErbB2, ErbB3, and ErbB4 can also be used to normalize the

results of their corresponding phospho-proteins if the pan proteins are detectable.

One important parameter is the background signal. To obtain the best results, we suggest that several exposures be attempted. We also strongly recommend using a negative control in which the sample is replaced with an appropriate mock buffer according to the array protocol, particularly during your first experiment.

By comparing the signal intensities, relative expression levels of target proteins can be made. The intensities of the signals can be quantified by densitometry. Positive controls may be used to normalize the results from different membranes. If the pan (total) EGFR, ErbB2, ErbB3, or ErbB4 signals are detectable, then they may also be used to normalize the signals of their corresponding phospho-proteins. If desired, the phospho-protein signals may be normalized to both the positive controls and the pan EGFR signals.

Normalization of Signals:

One array should defined as the "reference" to which the signal intensities of the other arrays should be compared. It is up to the researcher to define which array should be the reference. The normalization of the array signals to the positive controls may be calculated as follows:

Pos(1) = average signal intensity of positive controls on the reference array
Pos(2) = average signal intensity of positive controls on Array 2
X(2) = signal intensity for a particular spot on Array 2
X(N2)= the normalized value for that particular spot on Array 2

X(N2) = X(2) * Pos(1)/Pos(2)

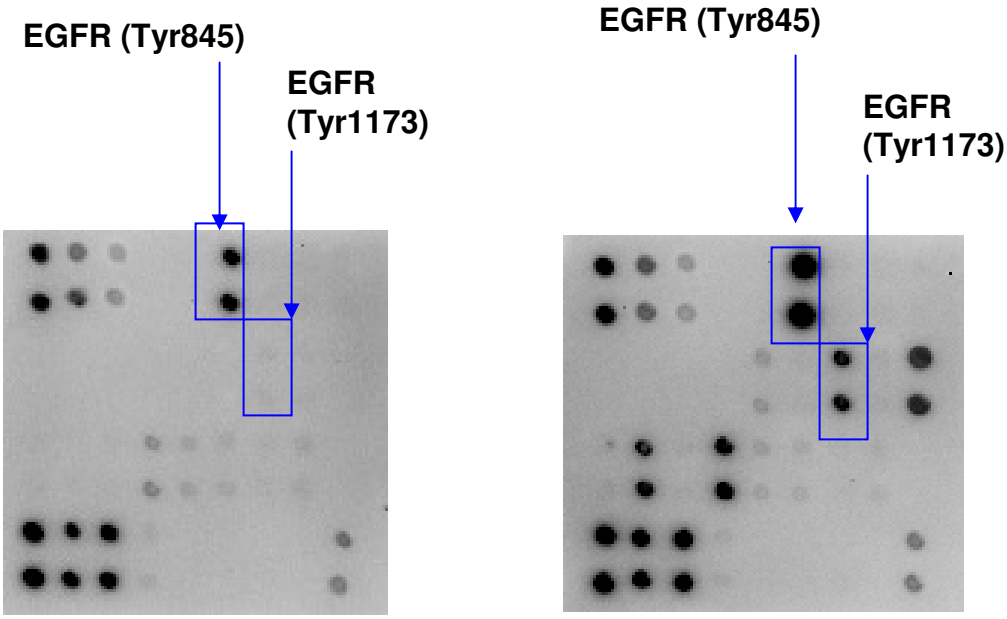
This calculation may be repeated for the remaining arrays 3, 4, 5, etc. for a particular experiment.

After positive control normalization, normalization of phospho-EGFR signals to the pan EGFR signals may be calculated according to the following example:

EGFR(1) = average signal intensity of pan EGFR on the reference array
EGFR(2) = average signal intensity of pan EGFR in array 2
Y845(2) = average signal intensity of EGFR (Tyr845) in array 2
Y845(N2) = normalized signal of EGFR (Tyr845) in array 2

Y845(N2) = Y845(2) * EGFR(1) / EGFR(2)

Antibody affinity to its target varies significantly between antibodies. The 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.



Untreated A431 cells
(Cell lysate: 200 µg/ml)

EGF treated A431 cells
(Cell lysate: 200 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 20 minutes at 37 °C. Control cells were serum starved without the subsequent stimulation with EGF. Cell lysates were prepared following the "Preparation of Sample" portion of our protocol V. To use the RayBio®Phosphorylation Antibody Array 1, treated or untreated cell lysate was added into antibody array membrane. The antibody array membranes were washed and cocktail of biotinylated anti-EGFR was used to detect phosphorylated proteins on activated receptors. After incubation with HRP-Conjugated Streptavidin, the signals were visualized by chemiluminescence.

RayBio® Human EGFR Phosphorylation Antibody Array 1 Map

	A	B	C	D	E	F	G	H	I
1	P1	P2	P3	Blank	Neg	EGFR (Tyr845)	EGFR (Tyr992)	EGFR (Tyr1045)	EGFR (Tyr1068)
2	P1	P2	P3	Blank	Neg	EGFR (Tyr845)	EGFR (Tyr992)	EGFR (Tyr1045)	EGFR (Tyr1068)
3	Blank	Blank	Blank	Blank	EGFR (Tyr1086)	EGFR(Tyr1148)	EGFR (Tyr1173)	EGFR (Ser1046/1047)	EGFR (Ser1070)
4	Blank	Blank	Blank	Blank	EGFR (Tyr1086)	EGFR(Tyr1148)	EGFR (Tyr1173)	EGFR (Ser1046/1047)	EGFR (Ser1070)
5	ErbB2 (Tyr877)	ErbB2 (Tyr1112)	ErbB2 (Tyr 1221/1222)	ErbB2 (Tyr1248)	ErbB2 (Thr686)	ErbB2 (Ser1113)	ErbB3 (Tyr1289)	ErbB4 (Tyr1284)	Blank
6	ErbB2 (Tyr877)	ErbB2 (Tyr1112)	ErbB2 (Tyr 1221/1222)	ErbB2 (Tyr1248)	ErbB2 (Thr686)	ErbB2 (Ser1113)	ErbB3 (Tyr1289)	ErbB4 (Tyr1284)	Blank
7	EGFR	ErbB2	ErbB3	ErbB4	Blank	Blank	Neg	Blank	P4
8	EGFR	ErbB2	ErbB3	ErbB4	Blank	Blank	Neg	Blank	P4

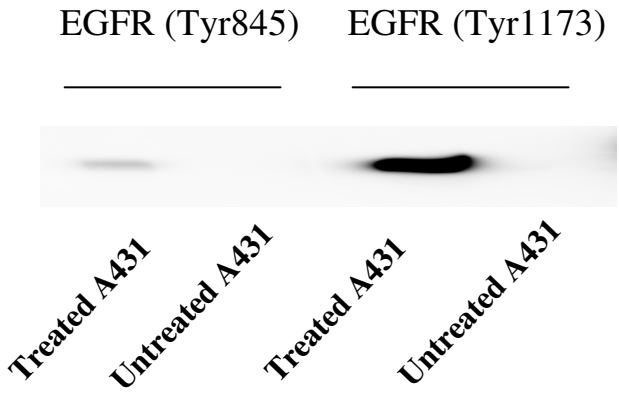


Fig. 2. Western blot analysis of extracts from 100 ng/ml hEGF treated A431 cells or untreated A431 cells. Phospho-EGFR (Tyr845) or Phospho-EGFR (Tyr1173) antibodies was used in this assay.

VIII. Troubleshooting Guide

Problem	Cause	Recommendation
Weak signal or no signal	1. Taking too much time for Detection.	1. The whole detection process must be completed in 30 min.
	2. Film developer does not work properly.	2. Fix film developer.
	3. Did not mix HRP-streptavidin well before use.	3. Mix tube containing HRP-Conjugate Streptavidin well before use since precipitates may form during storage.
	4. Sample is too dilute.	4. Increase sample concentration
	5. Other.	1.Reduce blocking concentration by diluting in 1X Wash Buffer II.
		2. Slightly increase HRP concentrations.
		3. Slightly increase biotinylate-antibody concentrations.
		4. Expose film for overnight to detect weak signal.
Uneven signal	1. Bubbles formed during incubation.	1. Remove bubbles during incubation.
	2. Membranes were not completely covered by solution.	2. Completely cover membranes with solution.
High background	1. Exposure to x-ray file is too long.	1. Decrease exposure time.
	2. Membranes were allowed to dry out during experiment.	2. Completely cover membranes with solution during experiment.
	3. Sample is too	3. Use more diluted sample.

concentrated.

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

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Recombinant protein production

Assay development

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

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

Note:

Note:

This product is for research use only.