RayBio® Phosphorylation Antibody Array I

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

User Manual

(Revised Jul 25, 2007)

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

RayBiotech, Inc.

We Provide You With Excellent Protein Array System And Service

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RayBiotech, Inc.

RayBio® Phosphorylation Antibody Array I Protocol

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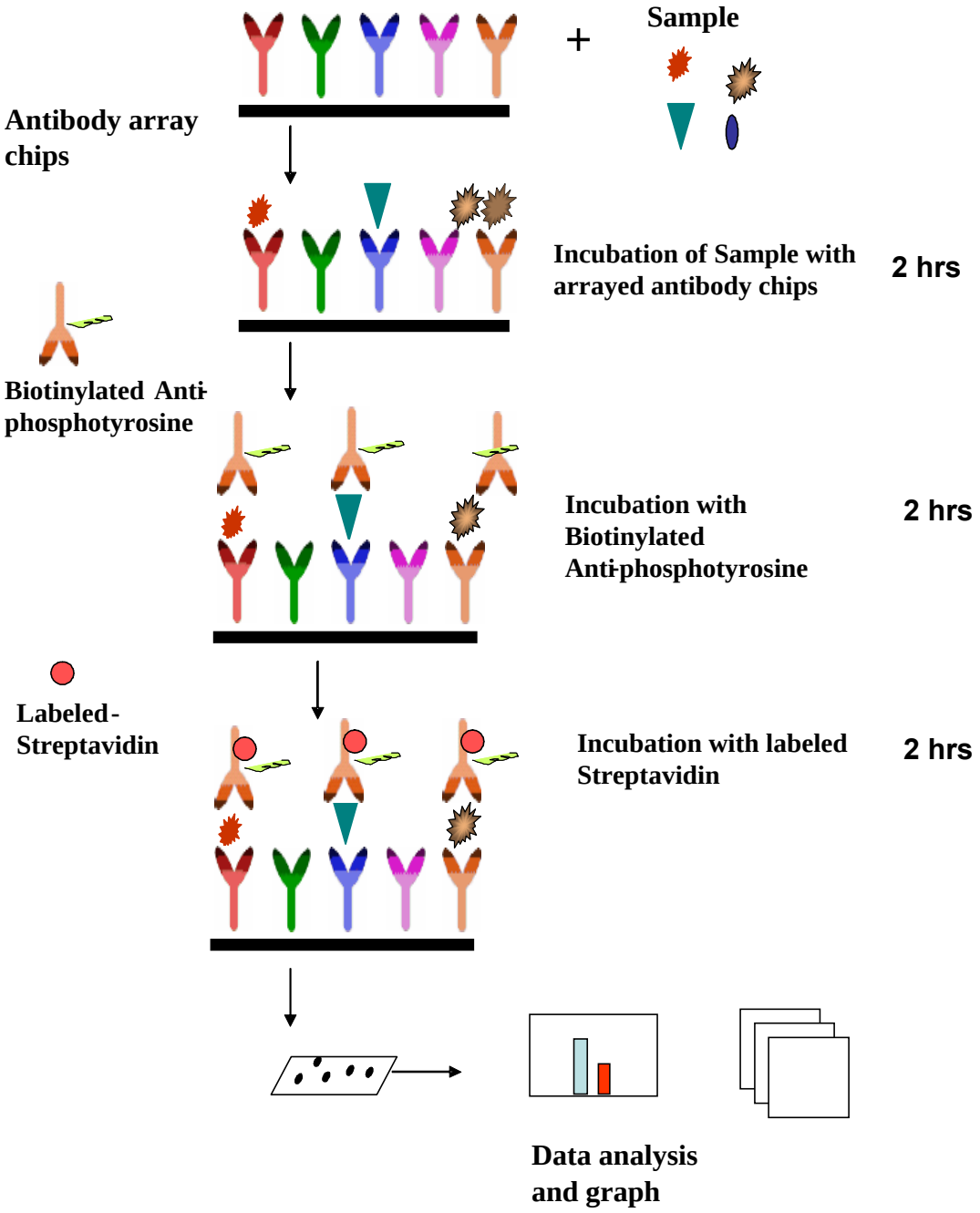
I. Introduction

Protein phosphorylation plays an unusually prominent role in cell signaling, development and growth. The RayBio® Phosphorylation Antibody Array G-series I 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® Phosphorylation Antibody Array I, which is specifically designed for simultaneously identifying the relative levels of phosphorylation of 71 different human receptor tyrosine kinases (RTKs) in cell lysates. 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.

By using RayBio® Phosphorylation antibody array I, treated or untreated cell lysate is added into antibody array membranes. The antibody array membranes are washed and biotinylated anti-phosphotyrosine antibody is used to detect phosphorylated tyrosines on activated receptors. 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 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 HRP-Conjugated Streptavidin should be stored at 4 °C to avoid repeated freeze-thaw cycles. Array I membrane, Protease Inhibitor Cocktail and Phosphatase Inhibitor Cocktail should be kept at –20 ° to -80°C. Use within three months after initial use.

- RayBio® Phosphorylation Antibody Array I 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)
- Phosphatase Inhibitor Cocktail (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)
- Biotin-Conjugated Anti-phosphotyrosine (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 membranes, 2.5 ml for 8 membranes)
- Eight-Well Tray (1 each)
- Plastic sheets

III. Additional Materials Required

- Small plastic boxes or containers
- Orbital 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: Briefly spin down the Phosphatase Inhibitor Cocktail tube before use. Add 180 µl of 1X Lysis Buffer into the vial to prepare 25X Phosphatase Inhibitor Cocktail 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 Protease Inhibitor Cocktail Concentrate and 80 µl Phosphatase Inhibitor Cocktail Concentrate 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. Biotinylated Anti-phosphotyrosine: 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 2.5 ml of Blocking Buffer to prepare 1X Biotinylated Anti-phosphotyrosine.

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 Streptavin 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. 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 Phosphorylation Antibody Array I, 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 ("-" mark is on the antibody printed side).

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

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

3. Decant Blocking Buffer from each container. Add 1.2 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 1.2 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 fold 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 1.2 ml of diluted biotin-conjugated antibodies to each membrane. Incubate at room temperature 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 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.

Because the spots are very small, you may need to magnify signals after scanning your films If the signals are too weak or can't be seen in your films.

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

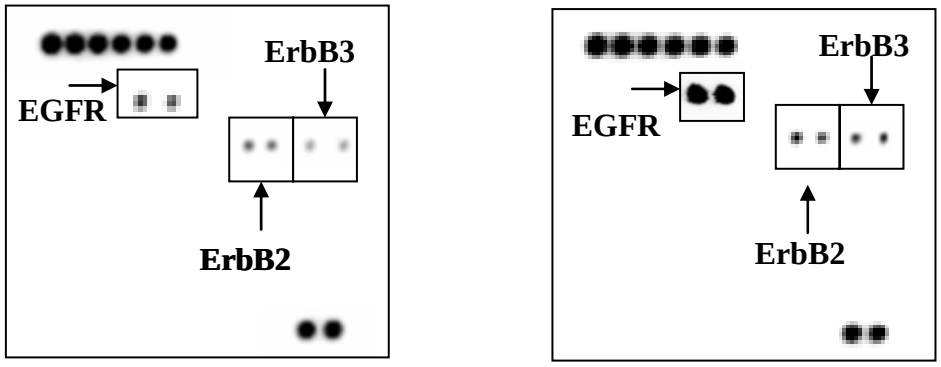
VII. Interpretation of Results:

The following figure shows RayBio® Phosphorylation Antibody Array I membranes probed with different cell lines. The signals were detected by using a chemiluminescene imaging device. Membranes also can be exposed to Kodak X-Omat™ film at room temperature. A biotinylated protein provides positive signals, which can be used to identify the orientation and to normalize the results from different wells being compared.

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 signals can be quantified by densitometry. Positive control can be used to normalize the results from different membranes being compared.

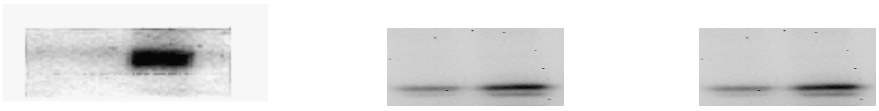
Antibody affinity to its target varies significantly between antibodies. The intensity detected on the array with each antibody depends on this affinity; therefore, signal intensity comparison can be performed only within the same antibody/antigen system and not between different antibodies.



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 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®Phosphorylation Antibody Array I, treated or untreated cell lysate was added into antibody array membrane. The antibody array membranes were washed and biotinylated anti-phosphotyrosine antibody was used to detect phosphorylated tyrosines on activated receptors. After incubation with HRP-Conjugated Streptavidin, the signals were visualized by chemiluminescence.



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 taget protein receptors. Bands were visualized with Streptavidin-HRP followed by chemiluminescent detection substrate.

RayBio® Phosphorylation Antibody Array I Map

	A	B	C	D	E	F	G	H	I	J	K	L
1	POS 1	POS 1	POS 2	POS 2	POS3	POS3	ABL1	ABL1	ACK1	ACK1	ALK	ALK
2	NEG	NEG	NEG	NEG	Axl	Axl	Blk	Blk	BMX	BMX	Btk	Btk
3	Csk	Csk	Dtk	Dtk	EGFR	EGFR	EphA1	EphA1	EphA2	EphA2	EphA3	EphA3
4	EphA4	EphA4	EphA5	EphA5	EphA6	EphA6	EphA7	EphA7	EphA8	EphA8	EphB1	EphB1
5	EphB2	EphB2	EphB3	EphB3	EphB4	EphB4	EphB6	EphB6	ErbB2	ErbB2	ErbB3	ErbB3
6	ErbB4	ErbB4	FAK	FAK	FER	FER	FGFR1	FGFR1	FGFR2	FGFR2	FGFR2 (α isoform)	FGFR2 (α isoform)
7	Fgr	Fgr	FRK	FRK	Fyn	Fyn	Hck	Hck	HGFR	HGFR	IGF-I R	IGF-I R
8	Insulin R	Insulin R	Itk	Itk	JAK1	JAK1	JAK2	JAK2	JAK3	JAK3	LCK	LCK
9	LTK	LTK	Lyn	Lyn	MATK	MATK	M-CSFR	M-CSFR	MUSK	MUSK	NGFR	NGFR
10	PDGFR-α	PDGFR-α	PDGFR-β	PDGFR-β	PYK2	PYK2	RET	RET	ROR1	ROR1	ROR2	ROR2
11	ROS	ROS	RYK	RYK	SCFR	SCFR	SRMS	SRMS	SYK	SYK	Tec	Tec
12	Tie-1	Tie-1	Tie-2	Tie-2	TNK1	TNK1	TRKB	TRKB	TXK	TXK	NEG	NEG
13	Tyk2	Tyk2	TYRO10	TYRO10	VEGFR2	VEGFR2	VEGFR3	VEGFR3	ZAP70	ZAP70	POS2	POS2

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 concentrated.	3. Use more diluted sample.

IX. Reference List

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