

# RayBio<sup>®</sup> G-Series Human Protein Phosphorylation Antibody Array 2

For Simultaneously Detecting the Relative Level of Phosphorylation of Human Protein

**User Manual**

**(Revised Mar. 20<sup>th</sup>, 2024)**

**Cat#: AAH-PANP-G2-4 (4 Sample Kit)**

**Cat#: AAH-PANP-G2-8 (8 Sample Kit)**



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RayBiotech Life, Inc.

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**RayBio® G-Series Human Protein Phosphorylation Antibody Array 2  
Protocol**

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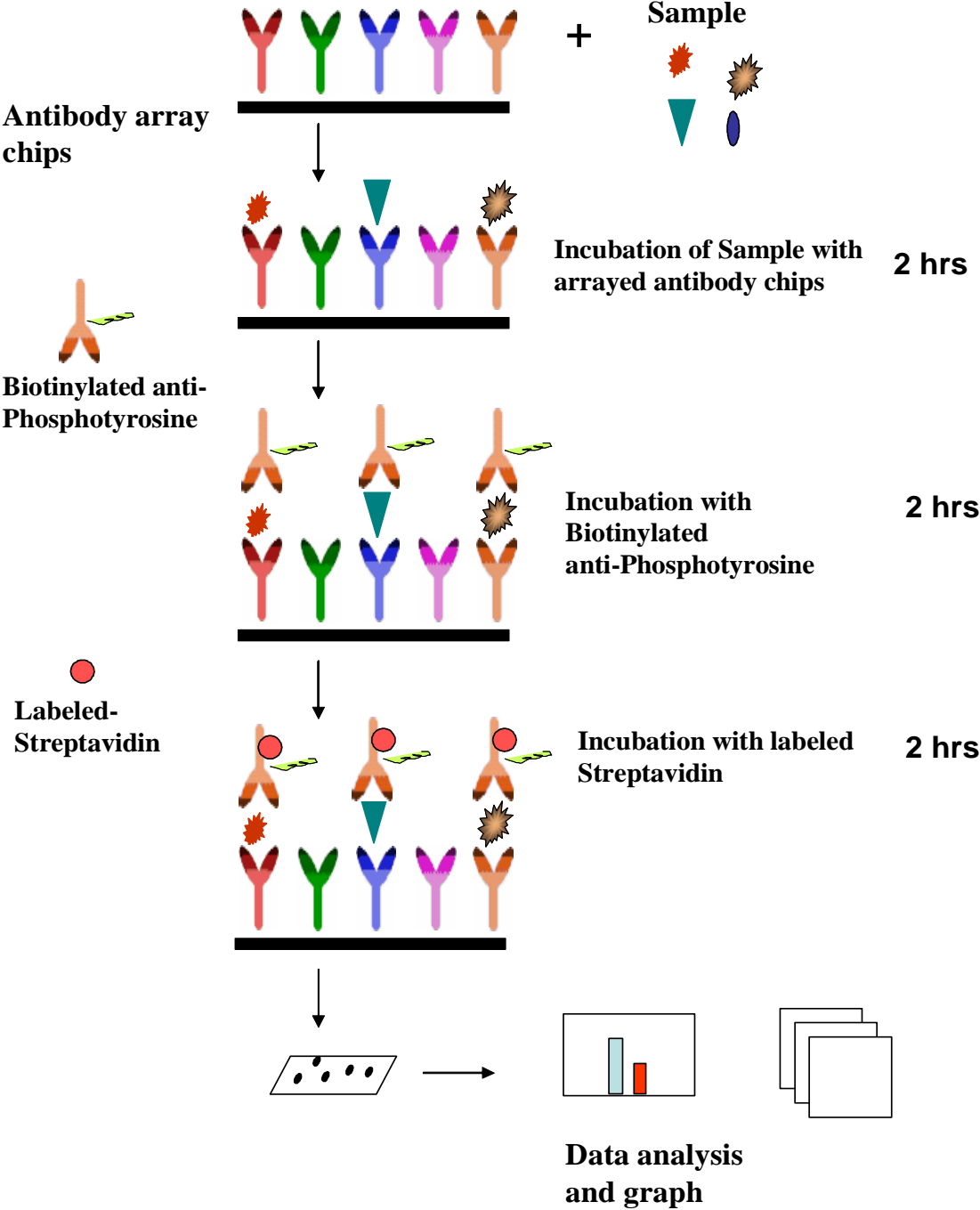
## **I. Introduction**

Protein phosphorylation plays an unusually prominent role in cell signaling, development and growth. The RayBio® G-Series Human Protein Phosphorylation Antibody Array 2 is a very rapid, convenient, and sensitive assay that can simultaneously detect multiple protein phosphorylations and be used to monitor the activation or function of important biological pathways.

RayBiotech is committed to develop a series of phosphorylation antibody arrays. RayBio® Human Protein Phosphorylation Antibody Array 2 is specifically designed for simultaneous identification of the relative levels of phosphorylation of 493 different Human Proteins 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 performing an analysis of immunoprecipitation and/or Western Blot.

To use the RayBio® G-Series Human Protein Phosphorylation Antibody Array 2, treated or untreated cell lysate is added into antibody array glass slide wells. The antibody array slide wells are washed, and biotinylated anti-phospho Tyr/Thr/Ser antibodies are then used to detect the phosphorylated Tyrosine/Threonine/Serine on target proteins. After incubation with a fluorescent dye-conjugated streptavidin (Cy3 equivalent), the slides can then be imaged using a laser scanner, such as the Axon GenePix, using the Cy3 channel.

# Here's how it works



## II. Materials Provided

Store kit at  $\leq -20^{\circ}\text{C}$  immediately upon arrival. Kit must use within the 6 months expiration date.

ITEM	COMPONENT	AAH-PANP-G2-4	AAH-PANP-G2-8	STORAGE TEMPERATURE AFTER THAWING**
1	RayBio® Glass Slide*	1	2	$\leq -20^{\circ}\text{C}$
2	Blocking Buffer	1 bottle (8ml/ea)	2 bottles (8ml/ea)	
3	Biotinylated Anti-Phospho Tyr/Thr/Ser Antibodies	1 vial	2 vials	2-8 °C
4	Cy3 equivalent-Conjugated Streptavidin	1 vial	2 vials	2-8 °C
5	20X Wash Buffer I Concentrate	1 bottle (30ml)		2-8 °C
6	20X Wash Buffer II Concentrate	1 bottle (30ml)		
7	Wash Buffer III	1 bottle (20ml)		
8	2X Cell Lysis Buffer Concentrate	1 bottle (10ml)		2-8 °C
9	Protease Inhibitor Cocktail	1 vial		$\leq -20^{\circ}\text{C}$
10	100x Phosphatase Inhibitor Cocktail I	1 vial		
11	Phosphatase Inhibitor Cocktail II	1 vial		
Other Kit Components: Adhesive film				

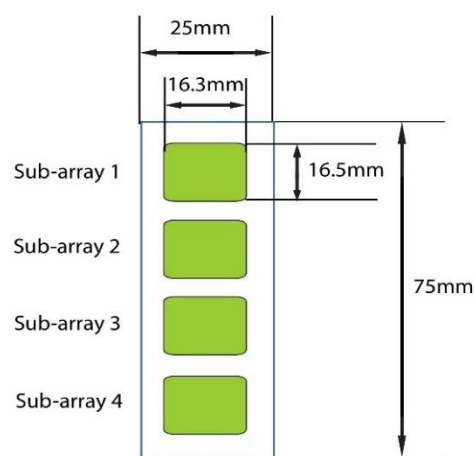
\*Each slide contains 4 identical subarrays

\*\*For up to 3 months (unless stated otherwise) or until expiration date

### 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 Slide



4 printed sub-arrays per glass chip

## IV. Reagent Preparation

1. **Protease Inhibitor Cocktail:** Briefly spin down the Protease Inhibitor Cocktail vial before use. Add 60  $\mu$ l of 1X Cell Lysis Buffer to 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 vial before use. Add 180  $\mu$ l of 1X Cell Lysis Buffer to the vial to prepare a 25X Phosphatase Inhibitor Cocktail Set II Concentrate. **Dissolve the powder thoroughly by gentle mixing.**
3. **2X Cell Lysis Buffer:** The 2X Cell Lysis Buffer should be diluted 2-fold with deionized or distilled water to prepare a 1X Cell Lysis Buffer solution. Then, add 20  $\mu$ l of the Protease Inhibitor Cocktail Concentrate, 20  $\mu$ l Phosphatase Inhibitor Cocktail Set I and 80  $\mu$ l of the Phosphatase Inhibitor Cocktail Set II Concentrate into 1.9 ml of the 1X Cell Lysis Buffer to prepare a 1X Cell Lysis Buffer with Protease and Phosphatase Inhibitor Cocktail solution. Mix well before use.
4. **20X Wash Buffer I or II:** If the 20X Wash Buffer Concentrate contains visible crystals, warm to room temperature and mix gently until dissolved. Dilute 25 ml of the 20X Wash Buffer Concentrate into deionized or distilled water to yield 500 ml of 1X Wash Buffer.
5. **Biotinylated anti-Phospho Tyr/Thr/Ser Antibodies:** Briefly spin down the Detection Antibody vial before use. Add 90  $\mu$ l of Blocking Buffer to the vial to prepare a Biotinylated Anti-phospho Tyr/Thr/Ser Concentrate. Pipette up and down to mix gently (the Concentrate can be stored at 4  $^{\circ}$ C for 5 days). Add 90  $\mu$ l of Detection Antibody Concentrate to a tube with 1710  $\mu$ l of Blocking Buffer to prepare a 1X Biotinylated Anti-phospho Tyr/Thr/Ser solution. Mix gently.
6. **Fluorescent dye-Conjugated Streptavidin (Cy3 equivalent):** Briefly spin down the Fluorescent dye-Conjugated Streptavidin vial before use.

Add 180  $\mu$ l of Blocking Buffer to the vial to prepare a Streptavidin Concentrate. Pipette up and down to mix gently. Transfer all Streptavidin Concentrate to a tube with 1.7 ml of Blocking Buffer to prepare a 1X Fluorescent dye-Conjugated Streptavidin solution. Mix gently.

## **V. Overview and General Considerations**

### **A. Preparation of Samples**

Cells can be prepared using the following convention.

For attached cells, remove the supernatant from the cell culture, and wash the cells twice with cold 1X PBS (for cells in suspension, pellet the cells by spinning down at 1500 rpm for 10 min). Make sure to remove any remaining PBS. Then, solubilize the cells at  $2 \times 10^7$  cells/ml in the 1X Cell Lysis Buffer with Protease and Phosphatase Inhibitor Cocktail solution. Pipette up and down to resuspend the cells, and rock the lysates gently at 2–8 °C for 30 min. Transfer the lysates 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 G-series 2, use cell lysates at a concentration of 50–1000  $\mu$ g/ml (as a starting point, we recommend using 400  $\mu$ g/ml of cell lysate diluted at least 5-fold with the Blocking Buffer).

Lysates should be used immediately or aliquoted and stored at –80 °C. Thawed lysates should be kept on ice prior to use.

*If you experience high background, you may further dilute your sample.*

### **B. Handling glass slides**



- The microarray slides are very sensitive. Do not touch the array surface with tips, forceps or hands. Hold the slides by the edges only.
- Handle all buffers and slides with latex free gloves.
- Avoid breaking the glass slide.
- Maintain a clean environment.

## **C. Incubation**

- Completely cover the array area with sample or buffer during incubation, and cover the incubation chamber with the 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 the adhesive film during incubation, particularly when the incubation is more than 2 hours.
- Avoid cross-contamination from overflowing solution to neighboring wells.
- Several incubation steps such as step 2 (sample incubation), step 6 (Biotin-conjugated Anti-phospho Tyr/Thr/Ser incubation) or step 9 (Fluorescent dye-Conjugated Streptavidin incubation) may be done at 4 °C overnight. Please make sure to cover the incubation chamber tightly to prevent evaporation.
- Avoid exposing the array slide to light from step 9 in page 10 on.

## VI. Protocol

### A. Dry the Glass Slide

Open the box containing the Glass Slide with Frame and take it out. Then let it air dry for 1 hour in a clean environment before use.

*Note: Protect the slide from dust or other contaminants.*

### B. Blocking and Incubation

1. Add 400  $\mu$ l of 1X Blocking Buffer to each well and incubate at room temperature with gentle shaking for 30 min to block the slides. Make sure no bubbles are in the wells.
2. Decant the Blocking Buffer from each well (make sure to remove all of the buffer). Add 400  $\mu$ l of each sample into appropriate wells. Incubate the arrays with sample at room temperature with gentle shaking for 2 hours or at 4 °C overnight.

*Note: We recommend using 400  $\mu$ l of cell lysate at a concentration of 50–1000  $\mu$ g/ml (as a starting point, we recommend using 400  $\mu$ g/ml cell lysate). **Dilute the lysate at least 5-fold with the Blocking Buffer. Make sure there are no bubbles 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. The optimal sample dilution must be determined empirically by the researcher.*

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

3. Decant the samples from each well, and wash 3 times, 5 min per wash, with 800  $\mu$ l of 1X Wash Buffer I at room temperature with gentle shaking.

*Note: Avoid the solution overflowing into neighboring wells.*

4. Put the Glass Slide 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 Slide with Frame into a box with Wash Buffer II (cover the whole glass slide and frame with Wash Buffer II), and wash 2 times, 5 min per wash, at room temperature with gentle shaking.
6. Remove all of Wash Buffer II from each well. Add 400  $\mu$ l of the 1X Biotin-conjugated Anti-phospho Tyr/Thr/Ser solution to each corresponding well. Incubate at room temperature with gentle shaking for 2 hours.
7. Decant the antibody solution and wash as directed in step 4 three times (wash 3 times, 20 min per wash).

8. Wash as directed in step 5.

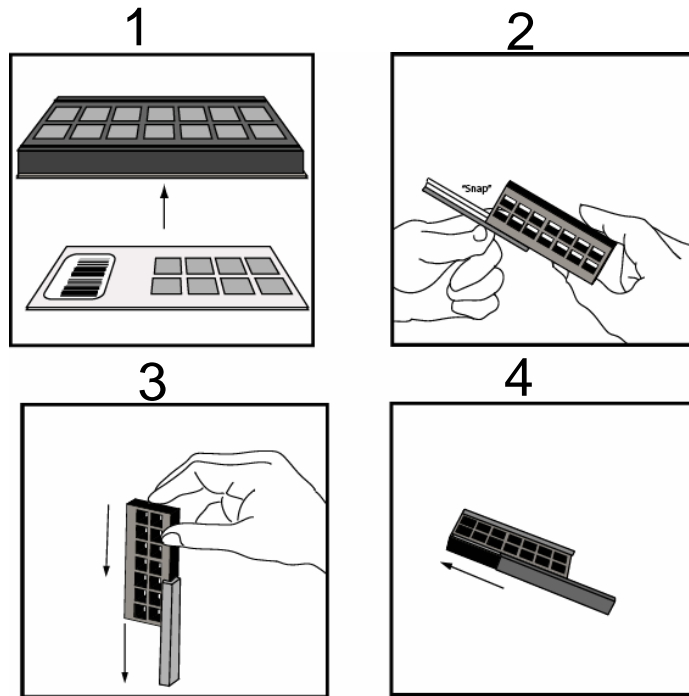
9. Remove all of Wash Buffer II from each well. Add 400  $\mu$ l of the 1X Fluorescent dye-Conjugated Streptavidin solution to each subarray. Cover the incubation chamber with the Adhesive film. Cover the plate with aluminum foil to avoid exposure to light or incubate in a dark room.

*Note: Avoid exposing the array slide to light from this step forward.*

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

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

11. Decant the Fluorescent dye-Conjugated Streptavidin solution and disassemble the Glass Slide and Frame by removing the incubation frame and chamber from the slide as illustrated below.



*Note: You may assemble and disassemble the glass slide into an incubation chamber and glass slide using the following steps.*

- 1. To assemble, apply the incubation chamber to the slide with the printed side facing upward as illustrated in (1) above.*
- 2. Gently snap one edge of a snap-on side as shown in (2).*
- 3. Adjust the position of the snap-on by gently pressing the edge of the snap-on side against a lab bench and pushing down as shown in (3).*
- 4. Repeat steps 2 – 3 with a second snap-on as shown in (4).*

12. Gently put the glass slide into a 50 ml centrifuge tube or a plastic box with 40 ml of 1X Wash Buffer I as illustrated below. Gently roll or shake the tube for 5 min. Remove the Wash Buffer I. Repeat 2 more times for a total of 3 washes.



13. Wash the glass slide with 40 ml of Wash Buffer II for 5 min. Repeat one more time for a total of 2 washes.
14. Finally, wash the glass slide with 40 ml of deionized or distilled water.

### **C. Fluorescence Detection**

1. To dry the glass slide, do one of the following:
  - a. Put the glass slide into a 50 ml centrifuge tube and centrifuge at 1,000 rpm for 3 min
  - or*
  - b. Apply a compressed N<sub>2</sub> stream, or let glass slide air dry completely under clean air conditions (protected from light)

Make sure the slides are absolutely dry before scanning.

2. Image the slides using a laser scanner, such as the Axon GenePix, using the Cy3 channel.

*Note: We recommend scanning the slides immediately after completing the experiment. Slides can also be stored at -20 °C in the dark for*

*several days. If you do not have a laser scanner, we can scan and extract the data for free for you.*

*Note: Put the glass slide into a tube with 40 ml of 30% Wash Buffer III in isopropanol (add 15 ml of Wash Buffer III to a tube with 35 ml of isopropanol and 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 a dark room). Dry the slide completely and re-scan the slide.*

## **VII. Interpretation of Results**

The following figure shows the RayBio® G-Series Human Protein Phosphorylation Antibody Array 2 probed with different cell lysates. The images were captured using a laser scanner. A biotinylated protein produces positive control signals, which can be used to identify the orientation of the slide and to normalize the results for comparison of different wells.

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







## VIII. Troubleshooting Guide

<b>Problem</b>	<b>Cause</b>	<b>Recommendation</b>
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	Reduce sample 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

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