# RayBio® G-Series Human RTK Phosphorylation Antibody Array 1

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

## User Manual

## (Revised Oct. 14th, 2020)

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

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RayBiotech Life, Inc.

# RayBio® G-Series Human RTK Phosphorylation Antibody Array 1 Protocol

## TABLE OF CONTENTS

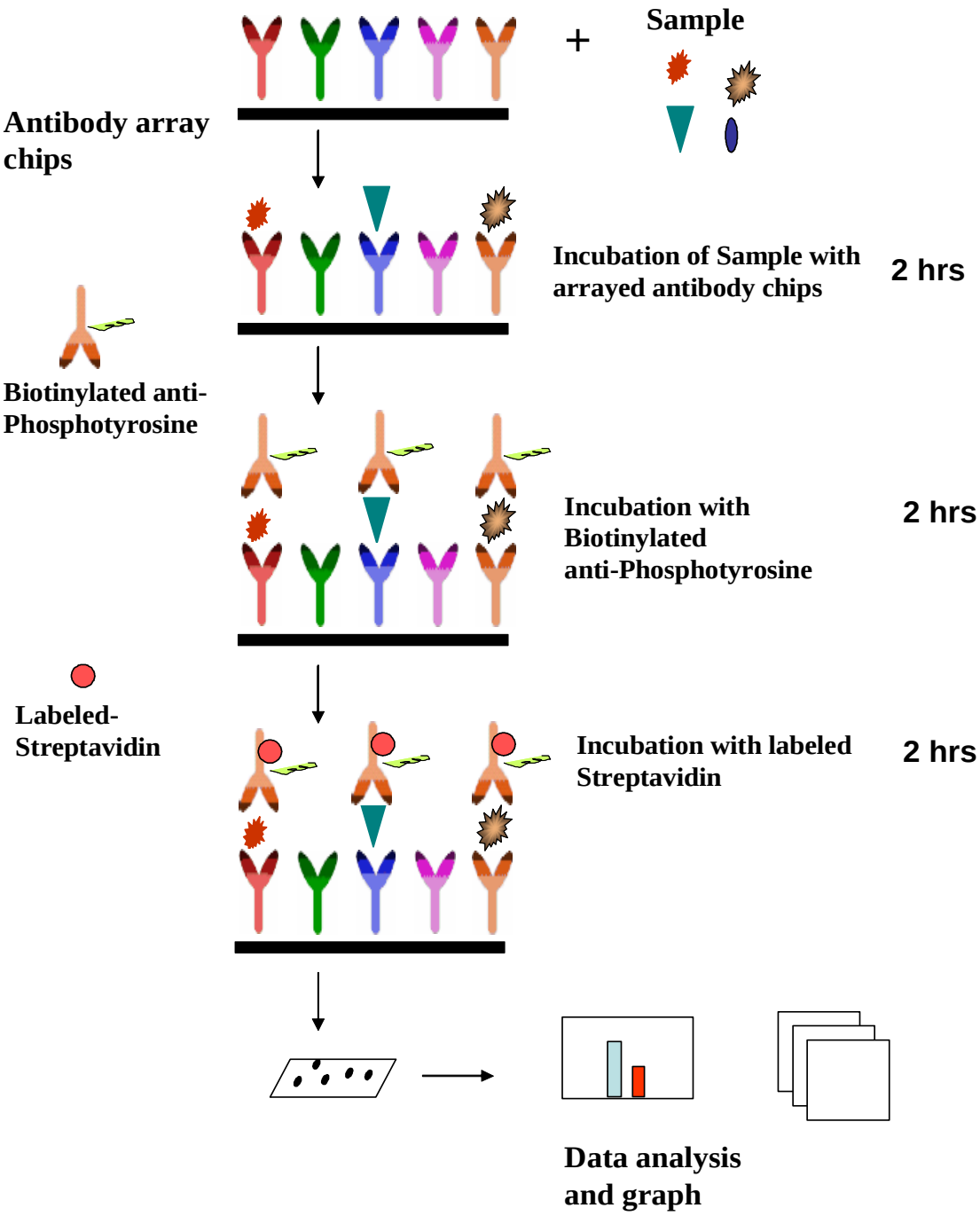
# I. Introduction

Protein phosphorylation plays an unusually prominent role in cell signaling, development and growth. The RayBio® G-Series Human RTK Phosphorylation Antibody Array 1 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 developing a series of phosphorylation antibody arrays. Our first product in this series is the RayBio® Human RTK Phosphorylation Antibody Array 1, which is specifically designed for simultaneous identification of 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 performing an analysis of immunoprecipitation and/or Western Blot.

To use the RayBio® G-Series Human RTK Phosphorylation Antibody Array 1, treated or untreated cell lysate is added into antibody array glass slide wells. The antibody array slide wells are washed, and biotinylated anti-phosphotyrosine antibodies are then used to detect the phosphorylated tyrosines on activated receptors. 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

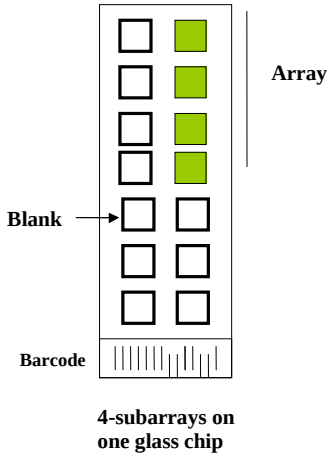
Upon receipt, the kit should be stored at -20 °C to -80 °C. Please use within 6 months from the date of shipment. After initial use, the 2X Cell Lysis Buffer, Blocking Buffer, 20X Wash Buffer I, 20X Wash Buffer II, Biotin-Conjugated Anti-phosphotyrosine and Fluorescent dye-Conjugated Streptavidin (Cy3 equivalent) should be stored at 4 °C to avoid repeated freeze-thaw cycles. The Array I Glass Slide, Protease Inhibitor Cocktail and Phosphatase Inhibitor Cocktail Set II should be kept at -20 °C to -80 °C. Use within 3 months after initial use.

- RayBio® G-Series Human RTK Phosphorylation Antibody Array 1 Glass Slide with Frame (each slide contains 4 Subarrays, with 1 slide included for 4-subarray slides, and 2 slides included for 8-subarray slides)
- 2X Cell Lysis Buffer (10 ml)
- Protease Inhibitor Cocktail (1/2 tubes, 1 tube included for the 4-subarrary slides, and 2 for the 8-subarray slides)
- Phosphatase Inhibitor Cocktail Set II (1/2 tubes, 1 tube included for the 4-subarray slides, and 2 for the 8-subarray slides)
- Blocking Buffer (8 ml)
- 20X Wash Buffer I (30 ml)
- 20X Wash Buffer II (30 ml)
- Biotin-Conjugated Anti-phosphotyrosine (1/2 tubes, 1 tube included for the 4-subarrary slides, and 2 for the 8-subarray slides)
- Fluorescent dye-Conjugated Streptavidin (Cy3 equivalent) (1/2 tubes, 1 tube included for the 4-subarrary slides, and 2 for the 8-subarray slides)
- 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 Slide



## IV. Reagent Preparation

1. **Protease Inhibitor Cocktail:** Briefly spin down the Protease Inhibitor Cocktail vial before use. Add 60 µl 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 µl 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 µl of the Protease Inhibitor Cocktail Concentrate and 80 µl 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-Phosphotyrosine:** Briefly spin down the Detection Antibody vial before use. Add 65 µl of Blocking Buffer to the vial to prepare a Biotinylated Anti-phosphotyrosine Concentrate. Pipette up and down to mix gently (the Concentrate can be stored at 4 $^0$C for 5 days). Add 30 µl of Detection Antibody Concentrate to a tube with 570 µl of Blocking Buffer to prepare a 1X Biotinylated Anti-phosphotyrosine solution. Mix gently.

6. **Fluorescent dye-Conjugated Streptavidin (Cy3 equivalent):** Briefly spin down the Fluorescent dye-Conjugated Streptavidin vial before use. Add 50 µl of Blocking Buffer to the vial to prepare a Streptavidin

Concentrate. Pipette up and down to mix gently. Add 10 µl of Streptavidin Concentrate to a tube with 1 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 $2x10^7$ 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 1, use cell lysates at a concentration of 50–1000 µg/ml (as a starting point, we recommend using 400 µg/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. If signals are too weak, cell lysate can be pretreated by immunoprecipitation before incubation with the array slides. Immunoprecipitation can be done using an anti-phosphotyrosine antibody and Protein A.*

## 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 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 2 (sample incubation), step 6 (Biotin-conjugated Anti-phosphotyrosine 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 12 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 100 µl 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.

   *Note: Only add reagents to wells printed with antibodies.*

2. Decant the Blocking Buffer from each well (make sure to remove all of the buffer). Add 100 µl 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 100 µl of cell lysate at a concentration of 50–1000 ug/ml (as a starting point, we recommend using 400 µg/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.*

*Note: Cell lysate can be pretreated by immunoprecipitation before incubation with the array slides if signals are too weak. Immunoprecipitation can be done using an anti-phosphotyrosine antibody and Protein A. The elution samples from Protein A can then be diluted with Blocking Buffer and incubated with the array slides.*

3. Decant the samples from each well, and wash 3 times, 5 min per wash, with 100 µl 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 100 µl of the 1X Biotin-conjugated Anti-phosphotyrosine 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 100 µl of the 1X Fluorescent dye-Conjugated Streptavidin solution to each subarray. Cover the incubation chamber with the Adhesive film. Cover the plate

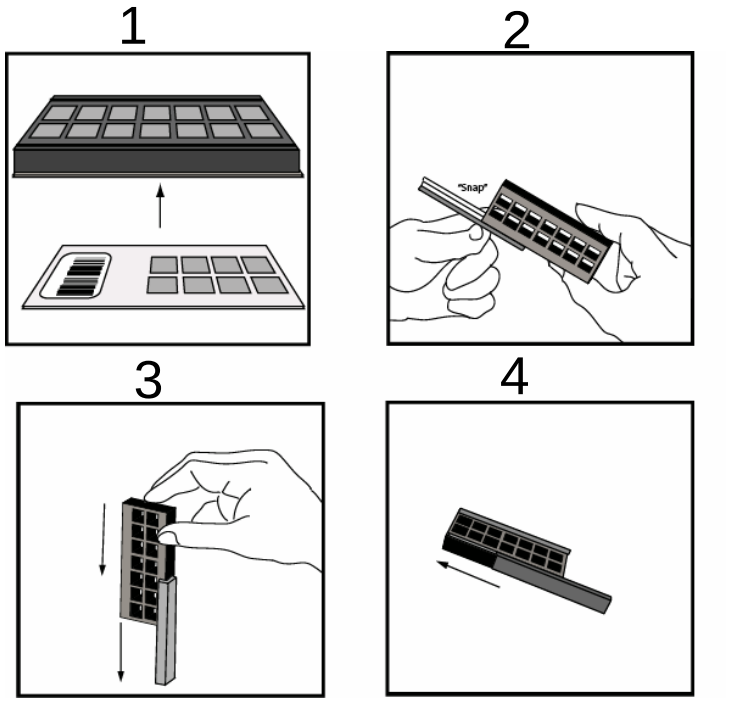
with aluminum foil to avoid exposure to light or incubate in a dark room.

*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 $N_2$ 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 RTK Phosphorylation Antibody Array 1 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 tyrosine may not be recognized by biotinylated anti-phosphotyrosine because of steric hindrance of the recognition site.

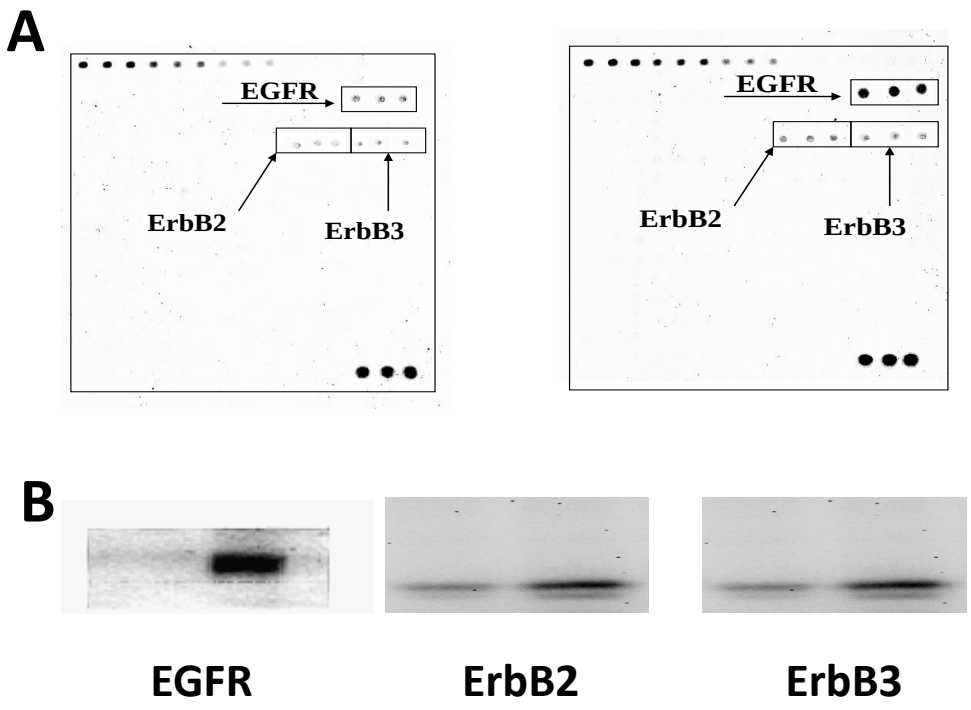



Fig 1. **(A)** Cells from a human epidermoid carcinoma cell line, A431, at 80-90% confluence were serum starved overnight, then exposed to 100 ng/ml EGF for 10 minutes at 37 °C. Control cells were serum starved without the subsequent stimulation with EGF. Cell lysates were prepared following the "Sample Preparation" portion IV of our protocol. To use the RayBio® G-Series Human RTK Phosphorylation Antibody Array 1, treated or untreated cell lysate was added to wells on the antibody array glass slides. The antibody array slides 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 scanning using the cy3 channel. **(B)** Immunoprecipitation was 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 a chemiluminescent detection substrate.

# RayBio® G-Series Human RTK Phosphorylation Antibody Array 1 Array Map

| | 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 | 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 |

## IX. Reference List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