Proteome Profiler™ Array

Human Phospho-RTK Array Kit

Catalog Number ARY001

For the parallel determination of the relative level of tyrosine phosphorylation of human receptor tyrosine kinases (RTKs).

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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INTRODUCTION

The Human Phospho-Receptor Tyrosine Kinase (Phospho-RTK) Array is a rapid, sensitive, and economical tool used to detect changes in phosphorylation between samples. The development of protein array technology allows the screening of 42 different phosphorylated human RTKs without performing numerous immunoprecipitations and Western blots. Each capture antibody was carefully selected by using lysate samples prepared from ligand-treated cell lines known to express the target receptor or cell lines transfected with a cDNA encoding a particular RTK. Recombinant tyrosine phosphorylated RTKs were used to choose capture antibodies when ligand-treated lysates were not available.

PRINCIPLE OF THE ASSAY

Capture and control antibodies have been spotted in duplicate on nitrocellulose membranes. Cell lysates are diluted and incubated with the Human Phospho-RTK Array. After binding the extracellular domain of both phosphorylated and unphosphorylated RTKs, unbound material is washed away. A pan anti-phospho-tyrosine antibody conjugated to horseradish peroxidase (HRP) is then used to detect phosphorylated tyrosines on activated receptors by chemiluminescence. Refer to the Appendix for a list and coordinates of analytes and controls.

TECHNICAL HINTS

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- This kit should not be used beyond the expiration date on the kit label.
- **Do not mix or substitute reagents with those from other lots or sources. Substitution of some high intensity chemiluminescent reagents for Chemi Reagents 1 and 2 may cause either increased background or diminished signal depending on the reagent.**
- Any variation in sample handling, buffers, operator, pipetting technique, washing technique, and incubation time or temperature can alter the performance of the kit.
- The Human Phospho-RTK Array membranes are validated for single use only.
- Always use gloved hands and flat-tipped tweezers to handle the membranes.
- Pick up the membranes from the edge on the side with the identification number avoiding the area with the printed antibodies.
- A thorough and consistent wash technique is essential for proper assay performance. Individual arrays should be washed in separate containers to minimize background. Wash Buffer should be removed completely from the membrane before proceeding to the next step.
- Do not allow the membrane to dry out. This will cause high background.
- Avoid microbial contamination of reagents and buffers.

PRECAUTION

Chemi Reagents 1 and 2 contain Boric Acid which is suspected of damaging fertility or the unborn child. Do not handle until all safety precautions in the MSDS have been read and understood. Wear protective gloves, clothing, eye, and face protection when using these reagents.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

PART	PART #	AMOUNT PROVIDED	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Human Phospho-RTK Array	892900	4 membranes	Return unused membranes to the foil pouch containing the desiccant pack. Reseal along entire edge of the zip-seal. May be stored for up to 3 months at 2-8 °C.*
Array Buffer 1	895477	1 vial (21 mL)	May be stored for up to 3 months at 2-8 °C.*
Array Buffer 2 Concentrate, 5X	895478	1 vial (21 mL)	
Wash Buffer Concentrate, 25X	895003	2 vials (21 mL/vial)	
Chemi Reagent 1	894287	1 vial (2.5 mL)	
Chemi Reagent 2	894288	1 vial (2.5 mL)	
Anti-Phospho-Tyrosine-HRP Detection Antibody	841403	1 vial (50 µL)	May be stored for up to 3 months at 2-8 °C.* **DO NOT FREEZE**
4-Well Rectangular Multi-dish	607544	1 dish	Store at room temperature.
Transparency Overlay Template	607545	1 template	

* Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- Aprotinin (Sigma, Catalog # A6279)
- Leupeptin (Tocris, Catalog # 1167)
- Pepstatin (Tocris, Catalog # 1190)
- Igepal® CA-630 (Sigma, Catalog # I3021)
- Pipettes and pipette tips
- Gloves
- Deionized or distilled water
- Rocking platform shaker
- Microcentrifuge
- A plastic container with the capacity to hold 50 mL (for washing the arrays)
- Plastic transparent sheet protector (trimmed to 10 cm x 12 cm and open on three sides)
- Plastic wrap
- Paper towels
- Absorbent lab wipes (KimWipes® or equivalent)
- Autoradiography cassette
- Film developer
- X-ray film (Kodak® BioMax™ Light-1, Catalog # 1788207) or equivalent
- Flat-tipped tweezers
- Flatbed scanner with transparency adapter capable of transmission mode
- Computer capable of running image analysis software and Microsoft® Excel

If using cell lysate samples, the following buffers are also required:

- Phosphate-Buffered Saline (PBS)
- Lysis buffer (1% Igepal CA-630, 20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 10% glycerol, 2 mM EDTA, 10 µg/mL Aprotinin, 10 µg/mL Leupeptin, and 10 µg/mL Pepstatin)

SAMPLE COLLECTION & STORAGE

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Since the Human Phospho-RTK Array detects relative phosphorylation levels of individual analytes, it is important to include appropriate control samples.

Note: *Sample amount may be empirically adjusted to attain optimal sensitivity with minimal background. Suggested starting range for cell lysates is 100-300 µg.*

Cell Lysates - Rinse cells with PBS, making sure to remove any remaining PBS before adding lysis buffer. Solubilize the cells at 1 x 10^7 cells/mL in lysis buffer. Pipette up and down to resuspend and rock the lysates gently at 2-8 °C for 30 minutes. Microcentrifuge at 14,000 x g for 5 minutes, and transfer the supernate into a clean test tube. Quantitation of sample protein concentrations using a total protein assay is recommended. Lysates should be used immediately or aliquoted and stored at ≤ -70 °C. Avoid repeated freeze-thaw cycles. Thawed lysates should be kept on ice prior to use.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Human Phospho-RTK Array - Four nitrocellulose membranes each containing 42 different anti-RTK antibodies and 6 controls printed in duplicate. **Handle arrays only with gloved hands and flat-tipped tweezers.**

Anti-Phospho-Tyrosine-HRP Detection Antibody - 50 µL of mouse anti-phospho-tyrosine antibody conjugated to HRP. Immediately before use, dilute the Detection Antibody to the working concentration specified on the vial label using 1X Array Buffer 2. Prepare only as much Detection Antibody as needed to run each experiment.

1X Array Buffer 2 - Add 2 mL of Array Buffer 2 Concentrate to 8 mL of deionized or distilled water. **Prepare fresh for each use.**

1X Wash Buffer - If crystals have formed in the concentrate, warm the bottles to room temperature and mix gently until the crystals have completely dissolved. Dilute 40 mL of 25X Wash Buffer Concentrate into 960 mL of deionized or distilled water.

Chemi Reagent Mix - Chemi Reagents 1 and 2 should be mixed in equal volumes within 15 minutes of use. **Protect from light. 1 mL of the resultant mixture is required per membrane.**

ARRAY PROCEDURE

Bring all reagents to room temperature before use. Keep samples on ice. To avoid contamination, wear gloves while performing the procedures.

1. Prepare all reagents and samples as directed in the previous sections.
2. Pipette 2.0 mL of Array Buffer 1 into each well of the 4-Well Multi-dish that will be used. Array Buffer 1 is used as a block buffer.
3. Using flat-tip tweezers, remove each array to be used from between the protective sheets.
4. Place one array into each well of the 4-Well Multi-dish. The array number should be facing upward.

   Note: *Upon contact with Array Buffer 1 the blue dye will disappear from the spots. The capture antibodies are retained in their specific locations.*
5. Incubate for 1 hour at room temperature on a rocking platform shaker. Orient the tray so that each array rocks from end to end in its well.
6. While the arrays are blocking, prepare samples by diluting the desired quantity of cell lysate to a final volume of 1.5 mL with Array Buffer 1.
7. Aspirate Array Buffer 1 from the 4-Well Multi-dish. Add the prepared samples and place the lid on the 4-Well Multi-dish.
8. Incubate overnight at 2-8 °C on a rocking platform shaker.

   Note: *A shorter incubation time may be used if optimal sensitivity is not required.*
9. Carefully remove each array and place into individual plastic containers with 20 mL of 1X Wash Buffer. Rinse the 4-Well Multi-dish with deionized or distilled water and dry thoroughly.
10. Wash each array with 1X Wash Buffer for 10 minutes on a rocking platform shaker. Repeat two times for a total of three washes.
11. Dilute the Anti-Phospho-Tyrosine-HRP Detection Antibody in 1X Array Buffer 2 using the dilution factor on the vial label. Pipette 2.0 mL into each well of the 4-Well Multi-dish.
12. Carefully remove each array from its wash container. Allow excess buffer to drain from the array. Return the array to the 4-Well Multi-dish containing the diluted Anti-Phospho-Tyrosine-HRP and cover with the lid.
13. Incubate for 2 hours at room temperature on a rocking platform shaker.

14. Wash each array as described in steps 9 and 10.

    Note: *Complete the remaining steps without interruption.*

15. Carefully remove each membrane from its wash container. Allow excess Wash Buffer to drain from the membrane by blotting the lower edge onto paper towels. Place each membrane on the bottom sheet of the plastic sheet protector with the identification number facing up.

16. Pipette 1 mL of the prepared Chemi Reagent Mix evenly onto each membrane.

    Note: *Using less than 1 mL of Chemi Reagent Mix per membrane may result in incomplete membrane coverage.*

17. Carefully cover with the top sheet of the plastic sheet protector. Gently smooth out any air bubbles and ensure Chemi Reagent Mix is spread evenly to all corners of each membrane. Incubate for 1 minute.

18. Position paper towels on the top and sides of the plastic sheet protector containing the membranes and carefully squeeze out excess Chemi Reagent Mix.

19. Remove the top plastic sheet protector and carefully lay an absorbent lab wipe on top of the membranes to blot off any remaining Chemi Reagent Mix.

20. Leaving membranes on the bottom plastic sheet protector, cover the membranes with plastic wrap taking care to gently smooth out any air bubbles. Wrap the excess plastic wrap around the back of the sheet protector so that the membranes and sheet protector are completely wrapped.

21. Place the membranes with the identification numbers facing up in an autoradiography film cassette.

    Note: *Use an autoradiography cassette that is not used with radioactive isotope detection.*

22. Expose membranes to X-ray film for 1-10 minutes. Multiple exposure times are recommended.

DATA ANALYSIS

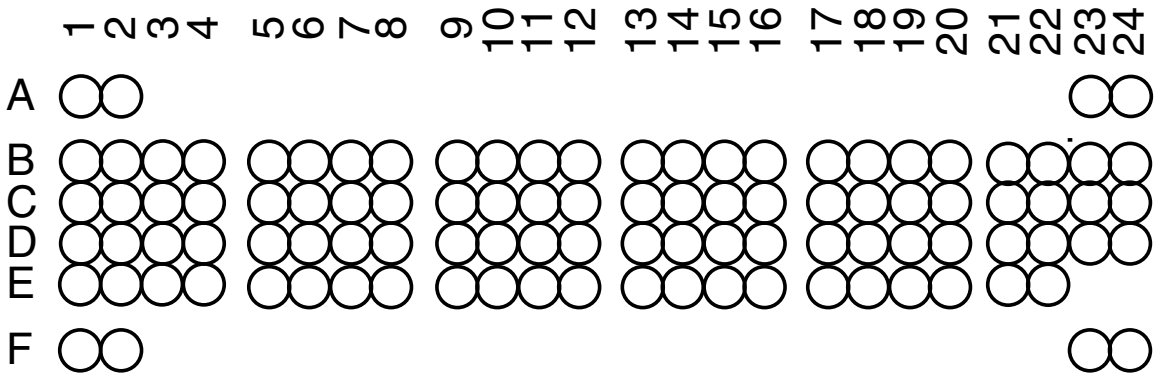
The positive signals seen on developed film can be quickly identified by placing the transparency overlay template on the array image and aligning it with the pairs of reference spots in the four corners of each array. The stamped identification number on the array should be placed on the left hand side. The location of controls and capture antibodies is listed in the Appendix.

Note: *Reference spots are included to align the transparency overlay template and to demonstrate that the array has been incubated with Anti-Phospho-Tyrosine-HRP during the assay procedure.*

Pixel densities on developed X-ray film can be collected and analyzed using a transmission-mode scanner and image analysis software.

1. Create a template to analyze pixel density in each spot of the array.
2. Export signal values to a spreadsheet file for manipulation in a program such as Microsoft Excel.
3. Determine the average signal (pixel density) of the pair of duplicate spots representing each RTK.
4. Subtract an averaged background signal from each RTK. Use a signal from a clear area of the array or the PBS negative control spots as a background value.
5. Compare corresponding signals on different arrays to determine the relative change in tyrosine phosphorylation if specific RTKs between samples.

Human Phospho-RTK Array Coordinates



This image is not to scale. It is for coordinate reference only.
Please use the transparency overlay for analyte identification.

SENSITIVITY

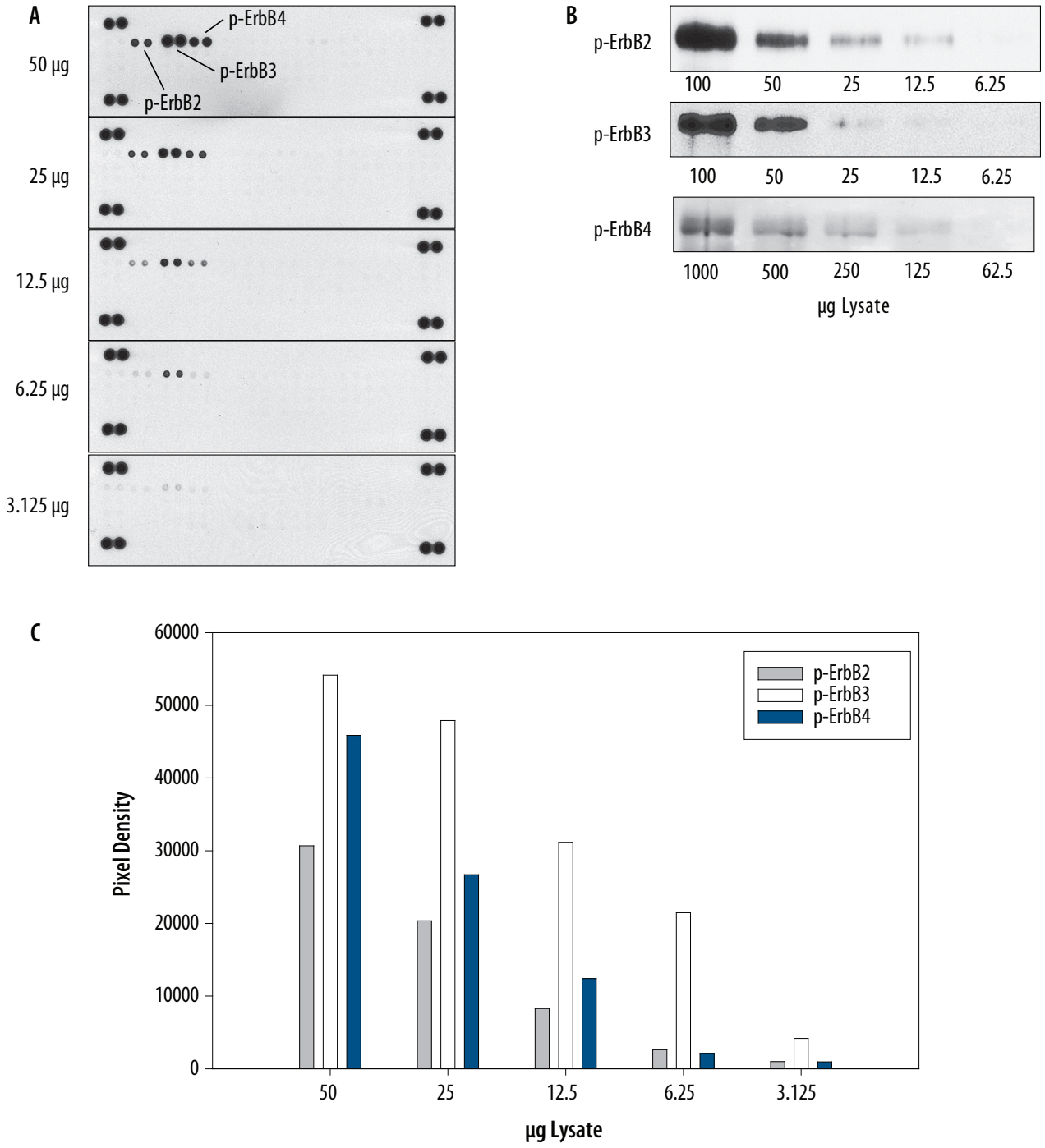


Figure 1: The Human Phospho-RTK Array detects tyrosine-phosphorylated receptors over a range of lysate concentrations. MDA-MB-453 human breast cancer cells were treated with 100 ng/mL recombinant NRG1-β1/HRG1-β1 (R&D Systems, Catalog # 396-HB) for 5 minutes to induce tyrosine phosphorylation of the ErbB receptors. Serial dilutions of lysates were analyzed by **(A)** the Human Phospho-RTK Array and **(B)** IP-Western blot. Immunoprecipitations were done using anti-ErbB monoclonal antibodies and anti-mouse IgG agarose. Immunoblots were incubated with a biotinylated anti-phospho-tyrosine monoclonal antibody (R&D Systems, Catalog # BAM1676) to detect phosphorylated ErbB receptors. Bands were visualized with Streptavidin-HRP (R&D Systems, Catalog # DY998) followed by chemiluminescent detection. **(C)** Array signals from scanned X-ray film images were analyzed using image analysis software.

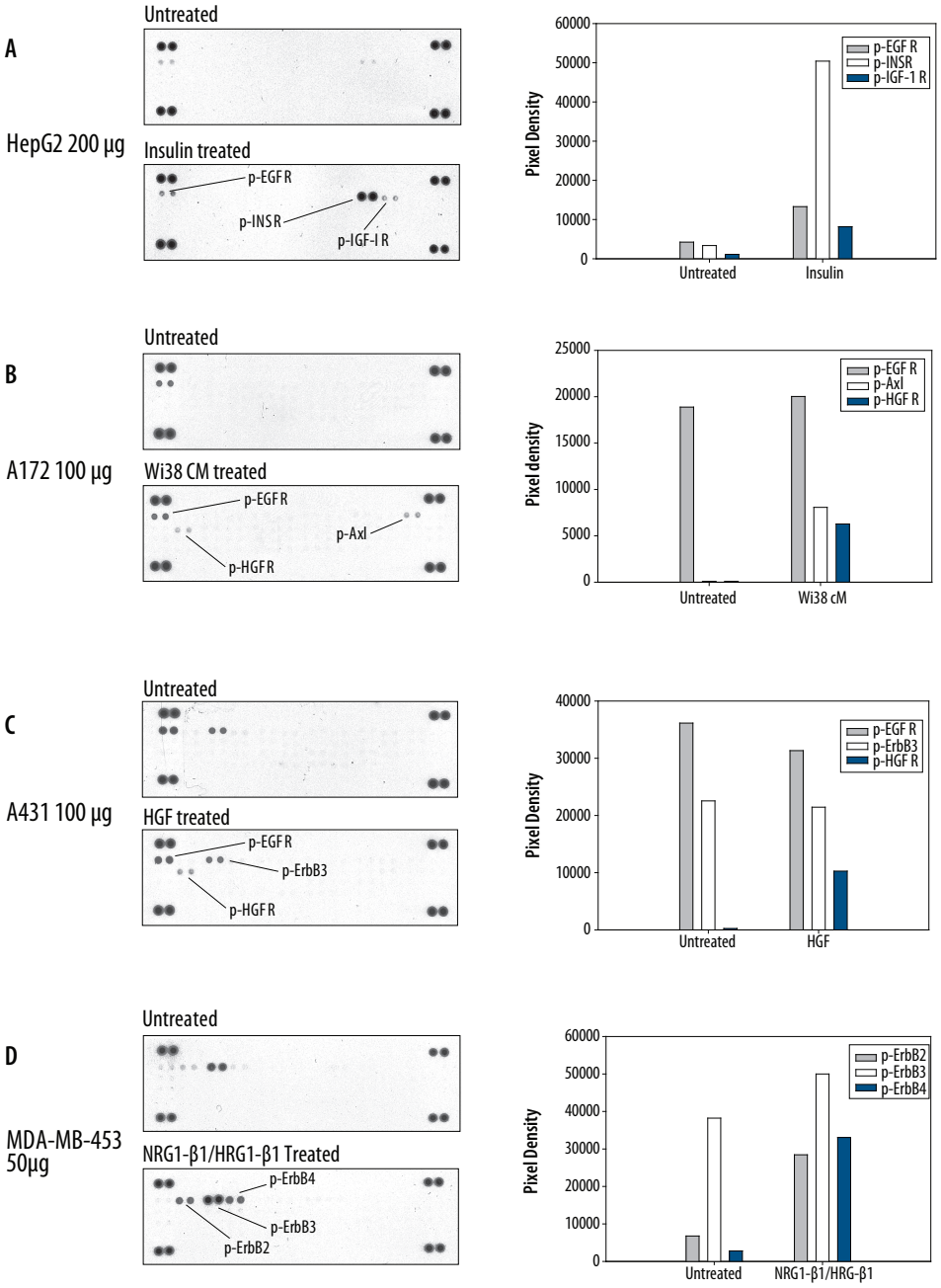


Figure 2: The Human Phospho-RTK Array detects multiple tyrosine phosphorylated receptors in untreated and ligand-treated cell lysates.

A. HepG2 human hepatocellular carcinoma cells were untreated or treated with 1 µg/mL of insulin for 5 minutes.

B. A172 human glioblastoma cells were untreated or treated with conditioned media (CM) from Wi38 human lung cells for 15 minutes.

C. A431 human epithelial carcinoma cells were untreated or treated with 100 ng/mL of recombinant human HGF (R&D Systems, Catalog # 294-HGN) for 5 minutes.

D. MDA-MB-453 human breast cancer cells were untreated or treated with 100 ng/mL of recombinant human NRG1-β1/HRG1-β1 (R&D Systems, Catalog # 396-HB) for 5 minutes.

The quantity of lysate incubated with each array is indicated in the figure.

SPECIFICITY

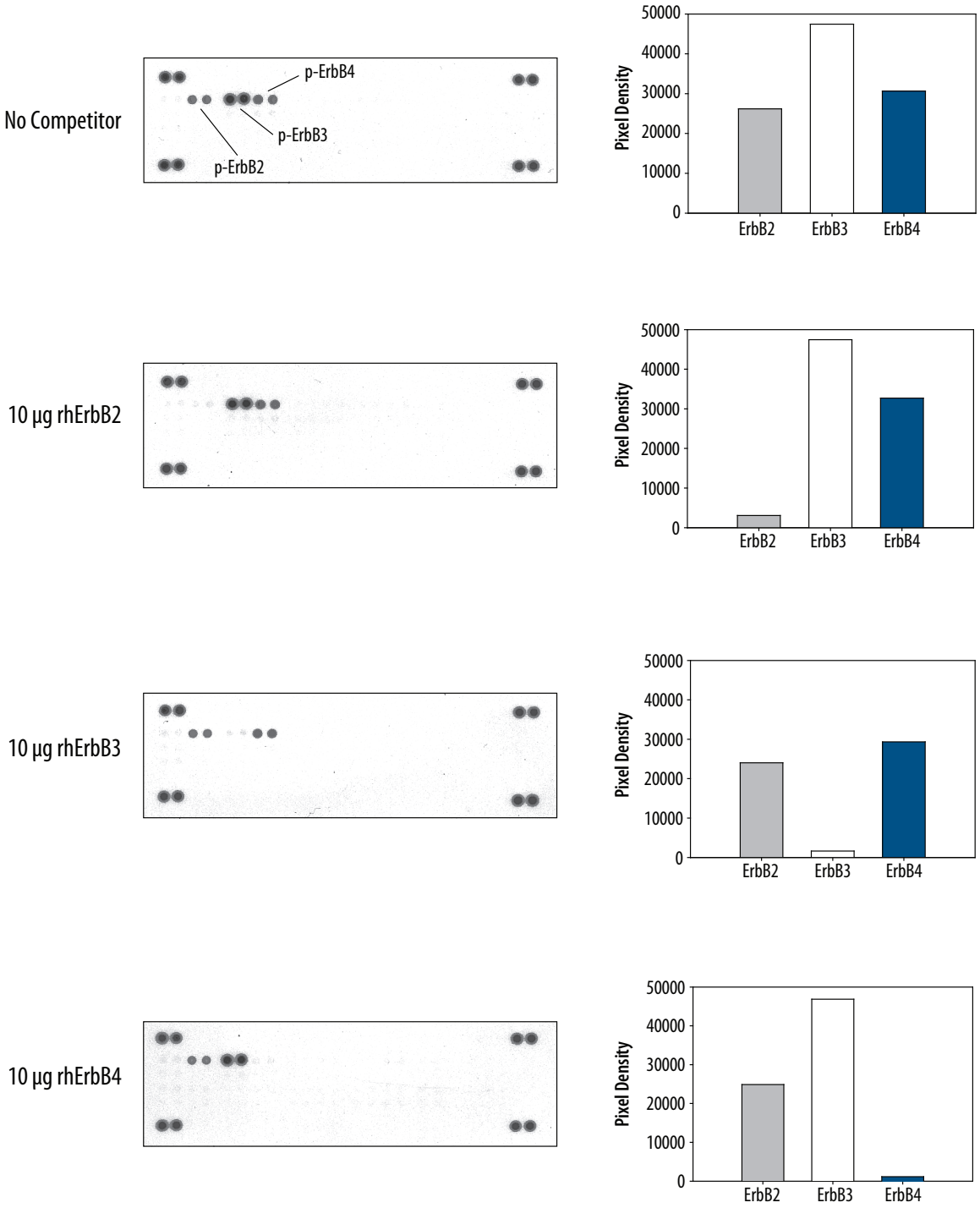


Figure 3: The Human Phospho-RTK Array is specific for ErbB2, ErbB3, and ErbB4 as shown by receptor competition. MDA-MB-453 human breast cancer cells were treated with 100 ng/mL of recombinant human (rh) NRG1-β1/HRG1-β1 for 5 minutes. The indicated amounts of rhErbB2 (R&D Systems, Catalog # 1129-ER), rhErbB3 (R&D Systems, Catalog # 348-RB) and rhErbB4 (R&D Systems, Catalog # 1131-ER) extracellular domains were added to 50 µg of lysate and analyzed using the Human Phospho-RTK Array. Competition of a particular ErbB receptor was observed only with the corresponding recombinant soluble receptor.

APPENDIX

Refer to the table below for the Human Phospho-RTK Array coordinates.

Coordinate	Receptor Family	RTK/Control	Coodinate	Receptor Family	RTK/Control
A1, A2	Reference Spots	——	D1, D2	Tie	Tie-2
A23, A24	Reference Spots	——	D3, D4	NGF R	TrkA
B1, B2	EGF R	EGF R	D5, D6	NGF R	TrkB
B3, B4	EGF R	ErbB2	D7, D8	NGF R	TrkC
B5, B6	EGF R	ErbB3	D9, D10	VEGF R	VEGF R1
B7, B8	EGF R	ErbB4	D11, D12	VEGF R	VEGF R2
B9, B10	FGF R	FGF R1	D13, D14	VEGF R	VEGF R3
B11, B12	FGF R	FGF R2α	D15, D16	MuSK	MuSK
B13, B14	FGF R	FGF R3	D17, D18	Eph R	EphA1
B15, B16	FGF R	FGF R4	D19, D20	Eph R	EphA2
B17, B18	Insulin R	Insulin R	D21, D22	Eph R	EphA3
B19, B20	Insulin R	IGF-I R	D23, D24	Eph R	EphA4
B21, B22	Axl	Axl	E1, E2	Eph R	EphA6
B23, B24	Axl	Dtk	E3, E4	Eph R	EphA7
C1, C2	Axl	Mer	E5, E6	Eph R	EphB1
C3, C4	HGF R	HGF R	E7, E8	Eph R	EphB2
C5, C6	HGF R	MSP R	E9, E10	Eph R	EphB4
C7, C8	PDGF R	PDGF Rα	E11, E12	Eph R	EphB6
C9, C10	PDGF R	PDGF Rβ	E13, E14	Control (-)	Mouse IgG_1
C11, C12	PDGF R	SCF R	E15, E16	Control (-)	Mouse IgG_{2A}
C13, C14	PDGF R	Flt-3	E17, E18	Control (-)	Mouse IgG_{2B}
C15, C16	PDGF R	M-CSF R	E19, E20	Control (-)	Goat IgG
C17, C18	RET	c-Ret	E21, E22	Control (-)	PBS
C19, C20	ROR	ROR1	F1, F2	Reference Spots	——
C21, C22	ROR	ROR2	F23, F24	Reference Spots	——
C23, C24	Tie	Tie-1			