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R&D SYSTEMS

Proteome Profiler™ Array

Mouse Phospho-RTK Array Kit

Catalog Number ARY014

For the parallel determination of the relative level of tyrosine phosphorylation of mouse 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 Mouse 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 39 different phosphorylated mouse RTKs without performing numerous individual immunoprecipitations and Western blots. Each capture antibody was carefully selected 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 RTK proteins 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 Mouse 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 coodinates 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 Mouse 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.

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
Mouse Phospho-RTK Array	893390	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)	
Lysis Buffer 17	895943	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	607681	1 template	

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

Note: *Additional wash buffer is available for purchase (R&D Systems®, # WA126).*

OTHER SUPPLIES REQUIRED

- Aprotinin (Tocris™, # 4139)
- Leupeptin hemisulfate (Tocris, # 1167)
- Pepstatin A (Tocris, # 1190)
- Pipettes and pipette tips
- Gloves
- Phosphate-Buffered Saline (PBS) (R&D Systems®, # RB01)
- 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
- Autoradiography cassette
- Film developer
- X-ray film
- Flat-tipped tweezers
- Flatbed scanner with transparency adapter capable of transmission mode
- Computer capable of running image analysis software and Microsoft® Excel

PRECAUTIONS

Chemi Reagents 1 and 2 contain Boric Acid which is suspected of damaging fertility or the unborn child.

Some components in this kit contain a preservative which may cause an allergic skin reaction. Avoid breathing mist.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Refer to the SDS on our website prior to use.

SAMPLE COLLECTION & STORAGE

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

Since the Mouse 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. The suggested starting range for cell lysates is 100-300 µg.*

Cell Lysates - Rinse cells with PBS and remove any remaining PBS before adding lysis buffer. Solubilize the cells at 1 x 10^7 cells/mL in Lysis Buffer 17 prepared with protease inhibitors. 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 concentration using a total protein assay is recommended. The maximum allowable lysate volume is 250 µL/array. Cell lysates should be used immediately or aliquoted and stored at ≤ -70 °C. Thawed lysates should be kept on ice prior to use.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Mouse Phospho-RTK Array - Four nitrocellulose membranes each containing 39 different anti-RTK antibodies 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.

Lysis Buffer 17 - Add 10 µg/mL Aprotinin, 10 µg/mL Leupeptin hemisulfate, and 10 µg/mL Pepstatin A to the volume of Lysis Buffer 17 required for cell lysate preparation. **Prepare fresh for each use.**

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 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 in 1.25 mL of Array Buffer 1. Adjust to a final volume of 1.5 mL with Lysis Buffer 17 as necessary. The maximum allowable cell lysate volume is 250 µL/array.
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 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 Anti-Phospho-Tyrosine-HRP and cover with the lid.
13. Incubate for 2 hours at room temperature on a rocking platform shaker.

ASSAY PROCEDURE *CONTINUED*

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

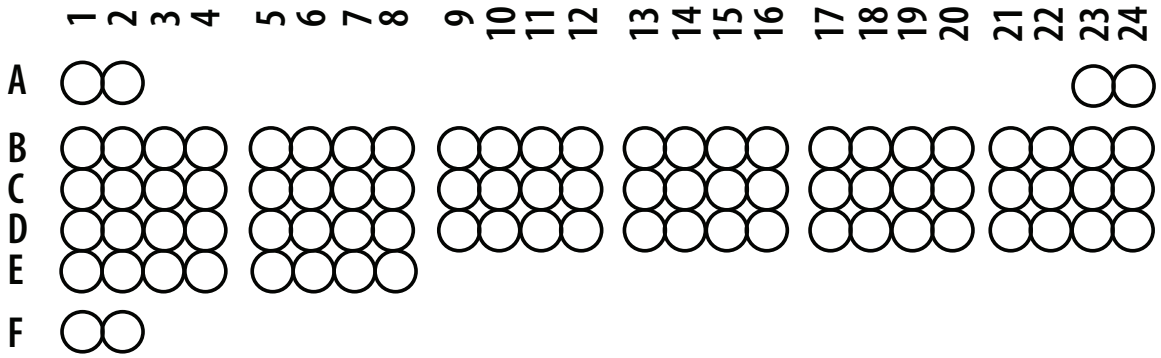
The positive signals seen on developed film can be quickly identified by placing the transparency overlay template on the array image and aligning it with the pairs of reference spots in three corners of each array. The stamped identification number on the array should be placed on the left hand side. 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 of specific RTKs between samples.

Mouse Phospho-RTK Array Coordinates



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

PROFILING MOUSE RTK TYROSINE PHOSPHORYLATION

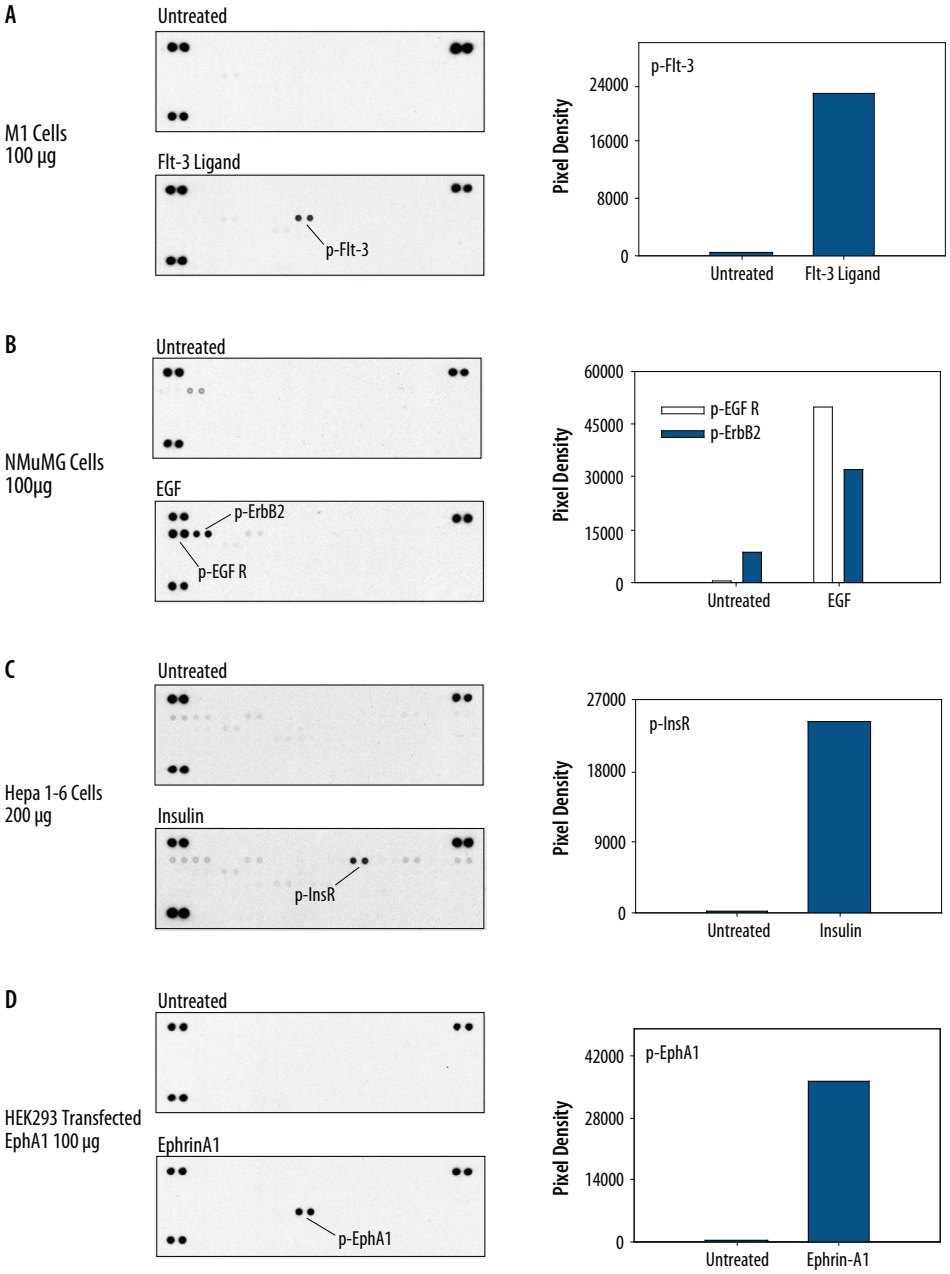


Figure 1: The Mouse Phospho-RTK Array detects multiple tyrosine phosphorylated receptors in untreated and ligand-treated cell lysates. The amount of lysate incubated with each array is indicated in the figure. Data shown are from 2 minute (A, B, and C) or 5 minute (D) exposure to X-ray film.

A. M1 mouse myeloid leukemia cells were either untreated or treated with 500 ng/mL Recombinant Mouse Flt-3 Ligand (R&D Systems®, # 427-FL) for 5 minutes.

B. NMuMG mouse mammary gland epithelial cells were either untreated or treated with 200 ng/mL Recombinant Mouse EGF (R&D Systems, # 2028-EG) for 5 minutes.

C. Hepa 1-6 mouse hepatoma cells were either untreated or treated with 1 µg/mL Recombinant Human Insulin (Sigma™, # I9278).

D. HEK293 human embryonic kidney cells transfected with mouse EphA1 were either untreated or treated with 3 µg/mL Mouse Ephrin-A1 (R&D Systems, # 602-A1) and 0.3 µg/mL Human IgG Fc Antibody (R&D Systems, # G-102-C) for 20 minutes.

SPECIFICITY

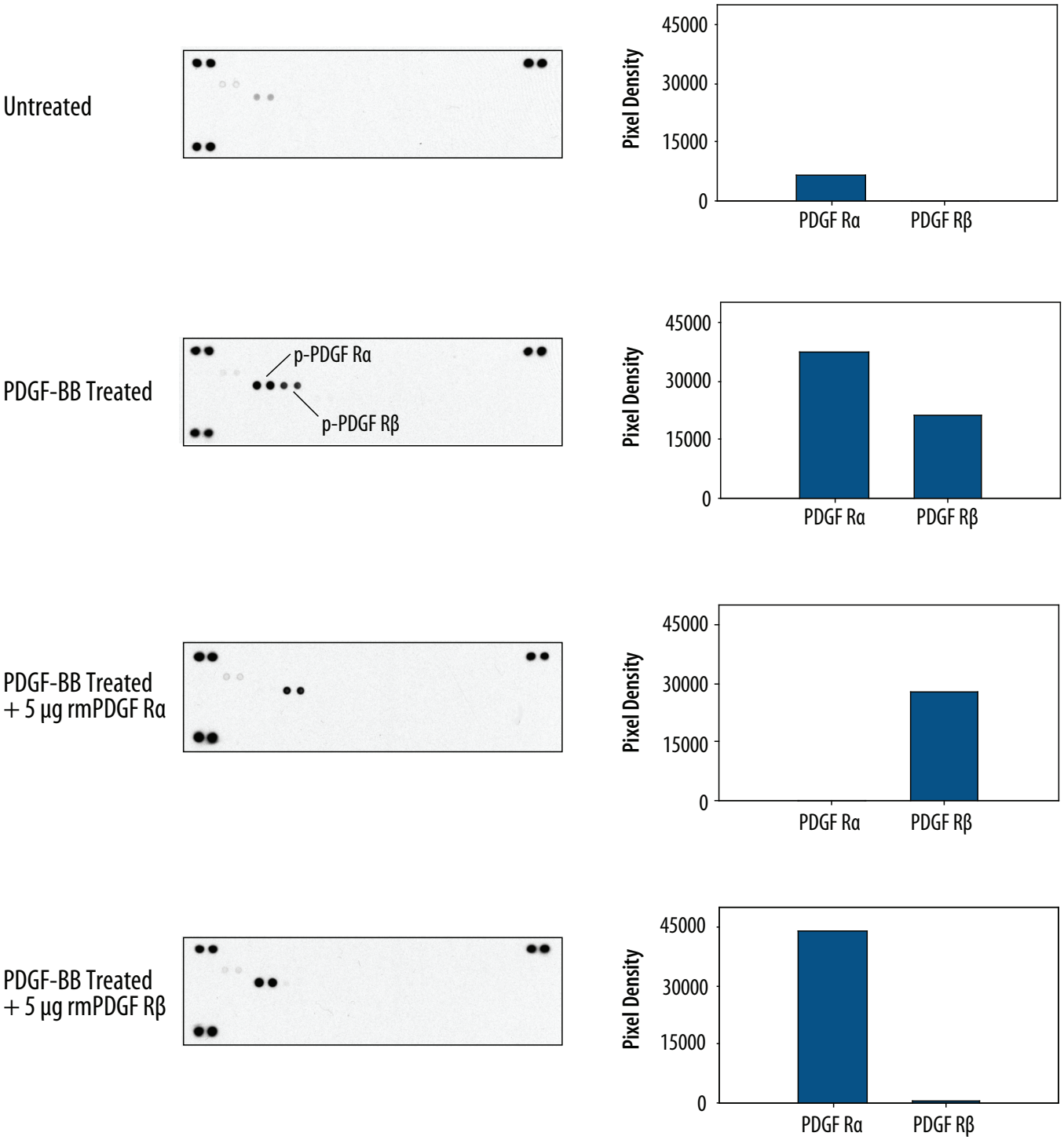


Figure 2: The Mouse Phospho-RTK Array is specific for PDGF Rα and PDGF Rβ as shown by receptor competition. NIH-3T3 mouse embryonic fibroblast cells were treated with 100 ng/mL of Recombinant Rat PDGF-BB (R&D Systems®, # 520-BB) for 5 minutes. To determine specificity, 5 µg of Recombinant Mouse PDGF Rα (R&D Systems, # 1062-PR) or 5 µg of Recombinant Mouse PDGF Rβ (R&D Systems, # 1042-PR) was added to 100 µg of lysate and analyzed using the Mouse Phospho-RTK Array. Data shown are from 2 minute exposures to X-ray film.

SENSITIVITY

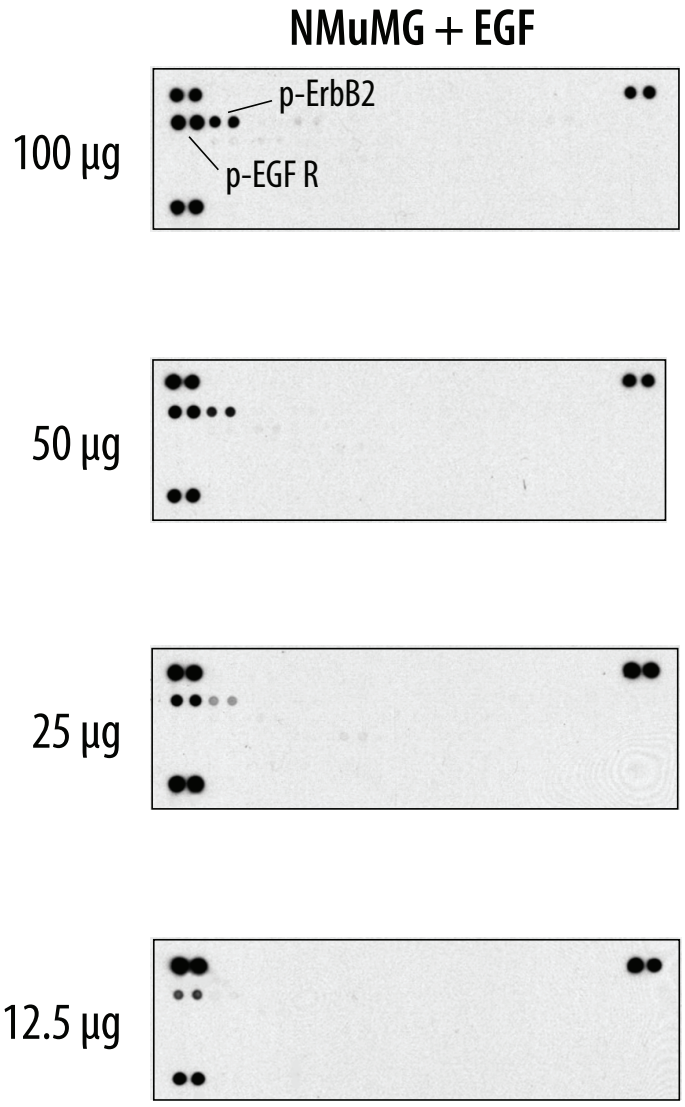


Figure 3: Signal intensities for tyrosine phosphorylated receptors may be modulated by the quantity of cell lysate incubated with the Mouse Phospho-RTK Array. The NMuMG mouse mammary gland epithelial cell line was treated with 200 ng/mL Recombinant Mouse EGF (R&D Systems, # 2028-EG) for 5 minutes to induce tyrosine phosphorylation of EGF R and ErbB2. Arrays were incubated with 12.5-100 µg of EGF treated NMuMG lysates. Data shown are from 2 minute exposures to X-ray film.

APPENDIX

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

Coordinate	Receptor Family	RTK/Control	Coodinate	Receptor Family	RTK/Control
A1, A2	Reference Spots	——	C17, C18	Tie	Tie-1
A23, A24	Reference Spots	——	C19, C20	Tie	Tie-2
B1, B2	EGF R	EGF R	C21, C22	NGF R	TrkA
B3, B4	EGF R	ErbB2	C23, C24	NGF R	TrkB
B5, B6	EGF R	ErbB3	D1, D2	NGF R	TrkC
B7, B8	EGF R	ErbB4	D3, D4	VEGF R	VEGF R1
B9, B10	FGF R	FGF R2 (IIIc)	D5, D6	VEGF R	VEGF R2
B11, B12	FGF R	FGF R3	D7, D8	VEGF R	VEGF R3
B13, B14	FGF R	FGF R4	D9, D10	MuSK	MuSK
B15, B16	Insulin R	Insulin R	D11, D12	Eph R	EphA1
B17, B18	Insulin R	IGF-I R	D13, D14	Eph R	EphA2
B19, B20	Axl	Axl	D15, D16	Eph R	EphA3
B21, B22	Axl	Dtk	D17, D18	Eph R	EphA6
B23, B24	Axl	Mer	D19, D20	Eph R	EphA7
C1, C2	HGF R	HGF R	D21, D22	Eph R	EphA8
C3, C4	HGF R	MSP R	D23, D24	Eph R	EphB1
C5, C6	PDGF R	PDGF Rα	E1, E2	Eph R	EphB2
C7, C8	PDGF R	PDGF Rβ	E3, E4	Eph R	EphB4
C9, C10	PDGF R	SCF R	E5, E6	Eph R	EphB6
C11, C12	PDGF R	Flt-3	E7, E8	Control (-)	PBS
C13, C14	PDGF R	M-CSF R	F1, F2	Reference Spots	——
C15, C16	RET	c-Ret			