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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MANUFACTURED AND DISTRIBUTED BY:
USA & Canada | R&D Systems, Inc.
614 McKinley Place NE, Minneapolis, MN 55413, USA
TEL: (800) 343-7475  (612) 379-2956  FAX: (612) 656-4400
E-MAIL: info@RnDSystems.com

DISTRIBUTED BY:
UK & Europe | R&D Systems Europe, Ltd.
19 Barton Lane, Abingdon Science Park, Abingdon OX14 3NB, UK
TEL: +44 (0)1235 529449  FAX: +44 (0)1235 533420
E-MAIL: info@RnDSystems.co.uk

China | R&D Systems China Co., Ltd.
24A1 Hua Min Empire Plaza, 726 West Yan An Road, Shanghai PRC 200050
TEL: +86 (21) 52380373  FAX: +86 (21) 52371001
E-MAIL: info@RnDSystemsChina.com.cn
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 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 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.

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.

<table>
<thead>
<tr>
<th>PART</th>
<th>PART #</th>
<th>AMOUNT PROVIDED</th>
<th>STORAGE OF OPENED/RECONSTITUTED MATERIAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse Phospho-RTK Array</td>
<td>893390</td>
<td>4 membranes</td>
<td>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.*</td>
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<tr>
<td>Array Buffer 1</td>
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<td>Array Buffer 2 Concentrate, 5X</td>
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<td>Lysis Buffer 17</td>
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<td>1 vial (21 mL)</td>
<td></td>
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<tr>
<td>Wash Buffer Concentrate, 25X</td>
<td>895003</td>
<td>2 vials (21 mL/vial)</td>
<td></td>
</tr>
<tr>
<td>Chemi Reagent 1</td>
<td>894287</td>
<td>1 vial (2.5 mL)</td>
<td></td>
</tr>
<tr>
<td>Chemi Reagent 2</td>
<td>894288</td>
<td>1 vial (2.5 mL)</td>
<td></td>
</tr>
<tr>
<td>Anti-Phospho-Tyrosine-HRP Detection Antibody</td>
<td>841403</td>
<td>1 vial (50 μL)</td>
<td>May be stored for up to 3 months at 2-8 °C.* DO NOT FREEZE.</td>
</tr>
<tr>
<td>4-Well Rectangular Multi-dish</td>
<td>607544</td>
<td>1 dish</td>
<td>Store at room temperature.</td>
</tr>
<tr>
<td>Transparency Overlay Template</td>
<td>607681</td>
<td>1 template</td>
<td></td>
</tr>
</tbody>
</table>

* 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)
- Pipettes and pipette tips
- Gloves
- Phosphate-Buffered Saline (PBS)
- Deionized or distilled water
- Flat-tipped tweezers
- Rocking platform shaker
- Microcentrifuge
- Plastic containers 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
- Absorbent lab wipes (KimWipes® or equivalent)
- Paper towels
- Autoradiography cassette
- Film developer
- X-ray film (Kodak® BioMax™ Light-1, Catalog # 1788207) or equivalent
- Flatbed scanner with transparency adapter capable of transmission mode
- Computer capable of running image analysis software and Microsoft® Excel
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, and 10 μg/mL Pepstatin 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 2.0 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 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 2.0 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 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 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.
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, Catalog # 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, Catalog # 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, Catalog # 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, Catalog # 602-A1) and 0.3 μg/mL goat anti-human IgG Fc (R&D Systems, Catalog # G-102-C) for 20 minutes.
SPECIFICITY

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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, Catalog # 520-BB) for 5 minutes. To determine specificity, 5 μg of recombinant mouse PDGF Rα (R&D Systems, Catalog # 1062-PR) or 5 μg of recombinant mouse PDGF Rβ (R&D Systems, Catalog # 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.
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, Catalog # 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 as shown above. Data shown are from 2 minute exposures to X-ray film.
# Appendix

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

<table>
<thead>
<tr>
<th>Coordinate</th>
<th>Receptor Family</th>
<th>RTK/Control</th>
<th>Coordinate</th>
<th>Receptor Family</th>
<th>RTK/Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1, A2</td>
<td>Reference Spots</td>
<td>——</td>
<td>C17, C18</td>
<td>Tie</td>
<td>Tie-1</td>
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<td>B1, B2</td>
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<td>M-CSF R</td>
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