Proteome Profiler™ Array

Human Phospho-MAPK Array Kit

Catalog Number ARY002

For the parallel determination of the relative levels of phosphorylation of Mitogen-Activated Protein Kinases (MAPKs) and other serine/threonine kinases.

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

Analyzing the phosphorylation status of all three major families of mitogen-activated protein kinases (MAPKs), the extracellular signal-regulated kinases (ERK1/2), c-Jun N-terminal kinases (JNK1 - 3), and different p38 isoforms (α/β/δ/γ), is essential in understanding the roles these signaling molecules play in mechanisms underlying cell function and disease. Other intracellular kinases, such as Akt, GSK-3, and p70 S6 Kinase, are additional important regulators of signal transduction, mediating development and cell proliferation. The Human Phospho-MAPK Array is a rapid, sensitive, and economical tool used to simultaneously detect the relative levels of phosphorylation of nine MAPKs and nine other serine/threonine kinases without performing numerous immunoprecipitations and/or Western blots. Each capture antibody was carefully selected using lysate samples prepared from treated cell lines known to express the target proteins.

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-MAPK Array. After binding both phosphorylated and unphosphorylated kinases, unbound material is washed away. A cocktail of phospho-site specific biotinylated antibodies is then used to detect phosphorylated kinases via Streptavidin-HRP and chemiluminescence. Refer to the Appendix on page 11 for the recognized phosphorylation sites.

TECHNICAL HINTS AND LIMITATIONS

- 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.
- Any variation in buffers, operator, pipetting technique, washing technique, and incubation time or temperature can alter the performance of the kit.
- R&D Systems validates the Human Phospho-MAPK Array membranes for one use only.
- Always use gloved hands and flat-tipped tweezers to handle the array.
- Pick up the array from the edge on the side with the identification number.
- The amount of lysate used can be varied to create an array assay with a different sensitivity.
- A thorough and consistent wash technique is essential for proper assay performance. Wash Buffer should be removed completely from the array before proceeding to the next step.
- Do not allow the array to dry out. This will cause high background.
- Avoid microbial contamination of reagents and buffers.

MATERIALS PROVIDED

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

Component	Part #	Storage Conditions	Amount Provided
Phospho-MAPK Array	893052	2 - 8° C	4 membranes
Array Buffer 1	895477	2 - 8° C	1 vial (21 mL)
Array Buffer 2 Concentrate, 5X	895478	2 - 8° C	1 vial (21 mL)
Array Buffer 3	895008	2 - 8° C	1 vial (21 mL)
Lysis Buffer 6	895561	2 - 8° C	1 vial (21 mL)
Wash Buffer Concentrate, 25X	895003	2 - 8° C	2 vials (21 mL each)
Anti-Phospho-MAPK Detection Antibody Cocktail	893051	2 - 8° C	1 vial
Streptavidin-HRP	890803	2 - 8° C	1 vial
4-Well Rectangular Multi-dish	607544	Room Temperature	1 dish
Transparency Overlay Template	607562	Room Temperature	1 template

OTHER MATERIALS REQUIRED

- Pipettes and pipette tips
- Gloves
- Phosphate-Buffered Saline (PBS)
- Deionized or distilled water
- Rocking platform shaker
- Microcentrifuge
- Flat-tipped tweezers
- A plastic container with the capacity to hold 50 mL (for washing the arrays)
- Plastic transparent sheet protector
- Plastic wrap
- 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
- Chemiluminescent detection reagent (Pierce, Catalog # 32106 or Amersham, Catalog # RPN2132)

SAMPLE PREPARATION

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 6. 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. It is recommended that sample protein concentrations be determined using a total protein assay. For incubation with the Human Phospho-MAPK Array, use a quantity of lysate similar to that used for immunoprecipitation or Western blot (100 - 300 µg). The maximum allowable lysate volume is 250 µL/array. Lysates should be used immediately or aliquoted and stored at ≤ -70° C. A 5:1 ratio of Array Buffer 1 to Lysis Buffer 6 is required for optimal assay performance. Thawed lysates should be kept on ice prior to use.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Human Phospho-MAPK Array - Four nitrocellulose membranes each containing 21 different anti-kinase antibodies and 7 different controls printed in duplicate. **Handle arrays only with gloved hands and flat-tipped tweezers.** After opening, reseal unused membranes in the foil pouch with desiccant and store at 2 - 8° C for up to 3 months.*

Anti-Phospho-MAPK Detection Antibody Cocktail Concentrate - One vial of lyophilized phospho-site specific biotinylated antibodies. Before use, reconstitute the Detection Antibody Cocktail in 100 µL of deionized or distilled water. Store at 2 - 8° C for up to 3 months after reconstitution.*

Lysis Buffer 6 - Ready for use. Store at 2 - 8° C for up to 3 months after initial use.*

Array Buffer 1 - Ready for use. Store at 2 - 8° C for up to 3 months after initial use.*

1X Array Buffer 2/3 - Dilute 2 mL of 5X Array Buffer 2 Concentrate into 8 mL of Array Buffer 3. Prepare fresh for each use.

1X Wash Buffer - Dilute 40 mL of 25X Wash Buffer Concentrate into 960 mL of deionized or distilled water. Store at 2 - 8° C for up to 3 months after initial use.*

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

ARRAY PROTOCOL

Bring all reagents to room temperature before use. Keep samples on ice.

1. Prepare all reagents and samples as directed in the previous sections.

2. For blocking each array, add 2.0 mL of Array Buffer 1 into each well of the 4-Well Multi-dish which will be used.

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: *The blue dye will disappear from the spots. The capture antibodies are retained in their specific locations.*

5. Incubate for 1 hour on a rocking platform shaker. Orient the tray so that each array rocks end to end in its well.

6. In a separate tube, add the desired quantity of lysate to 1.25 mL of Array Buffer 1. Adjust to a final volume of 1.5 mL with Lysis Buffer 6 as necessary. The maximum allowable lysate volume is 250 μL/array.

7. Remove Array Buffer 1 from the 4-Well Multi-dish.

8. Add the diluted lysates and place the lid on the 4-Well Multi-dish.

9. Incubate overnight at 2 - 8° C on a rocking platform shaker.

   Note: *Although sensitivity may be compromised, a 2 hour incubation may be done in place of the overnight incubation.*

10. 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.

11. Wash each array with 1X Wash Buffer by soaking for 10 minutes on a rocking platform shaker. Repeat two times for a total of three washes.

12. For each array, dilute 15 μL of Detection Antibody Cocktail Concentrate to 1.5 mL with 1X Array Buffer 2/3. Pipette 1.5 mL per well of diluted Detection Antibody Cocktail into the 4-Well Multi-dish.

13. Carefully remove each array from its wash container. Allow excess Wash Buffer to drain from the array. Return the array to the 4-Well Multi-dish containing the diluted Antibody Cocktail Concentrate, and cover with the lid.

14. Incubate for 2 hours at room temperature on a rocking platform shaker.

15. Wash each array as described in steps 10 and 11.

16. Dilute the Streptavidin-HRP 1:2000 in 1X Array Buffer 2/3. Pipette 1.5 mL into each well of the 4-Well Multi-dish.

17. Carefully remove each array from its wash container. Allow excess wash buffer to drain from the array. Return the array to the 4-Well Multi-dish containing the diluted Streptavidin-HRP, and cover it with the lid.

18. Incubate for 30 minutes at room temperature on a rocking platform shaker.

19. Wash each array as described in steps 10 and 11.

20. Carefully remove each array from the wash container. Allow excess Wash Buffer to drain from the array. Place the array on a plastic sheet protector. Expose each array to chemiluminescent detection reagent.

21. Cover with plastic wrap and expose to X-ray film for 1 - 10 minutes.

DATA ANALYSIS

The positive signals seen on developed film can be quickly identified by placing the transparency overlay on the array image and aligning it with the three pairs of positive control spots in the corners of each array. The stamped identification number on the array should be placed on the left hand side. The location of controls and kinase capture antibodies is listed in the Appendix on page 11.

Phospho-MAPK Array data on developed X-ray film can be quantitated by scanning the film on a transmission-mode scanner and analyzing the array image file using image analysis software.

1. Create a template to analyze pixel density in each spot of the array.

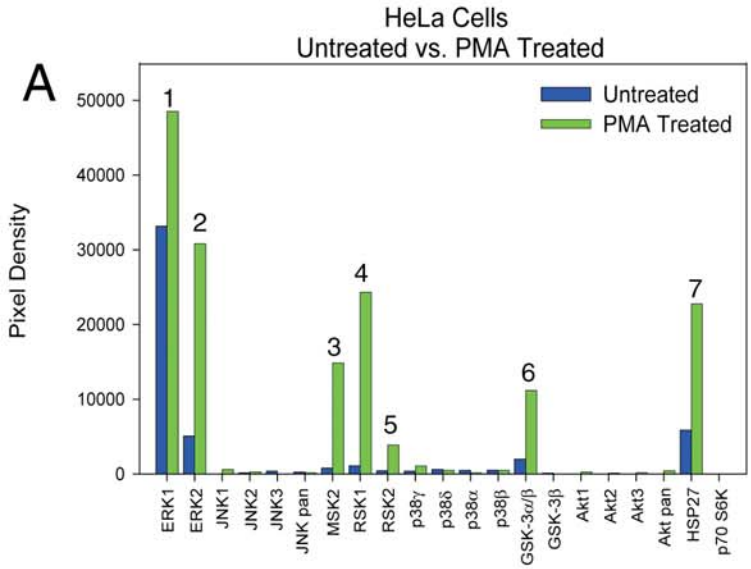
2. Subtract an averaged background signal from each kinase spot. Use a signal from a clear area of the array or one of the six negative control spots as a background value.

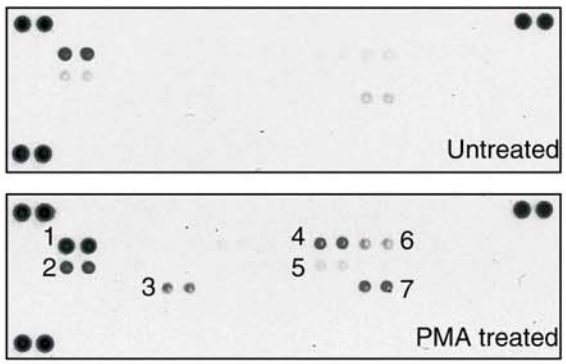
3. Export signal values to a spreadsheet file for manipulation in a program such as Microsoft Excel.

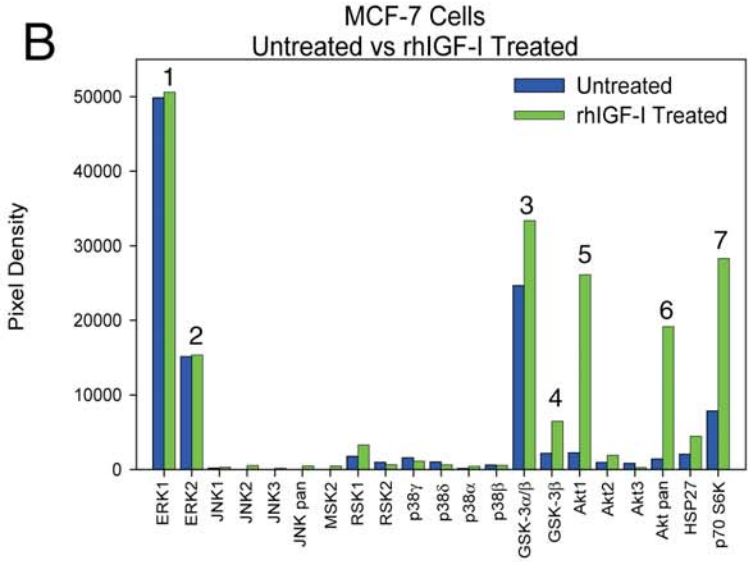
4. Determine the average signal (pixel density) of the pair of duplicate spots representing each kinase.

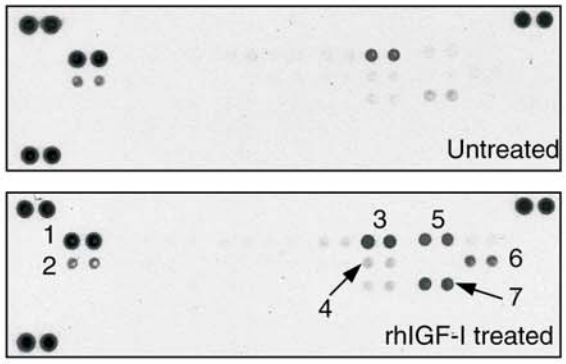
5. Compare corresponding signals on different arrays to determine the relative change in phosphorylation state of specific kinases between samples.

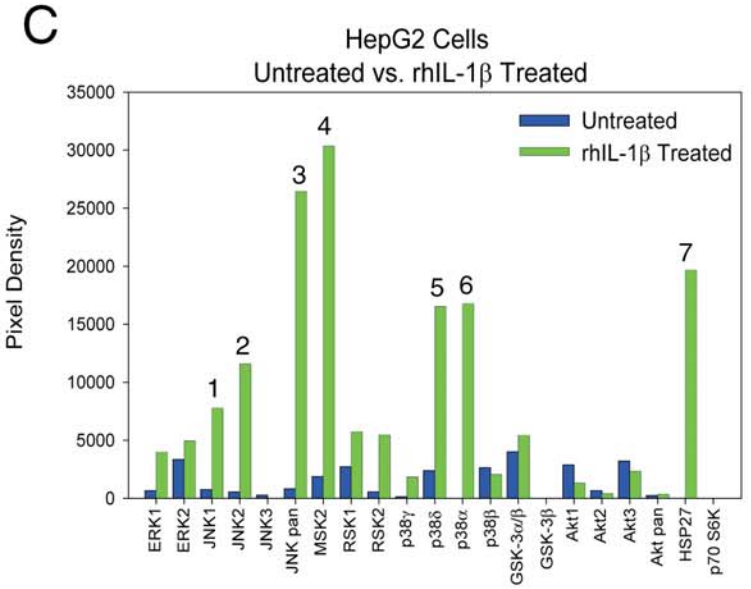
PROFILING KINASE PHOSPHORYLATION











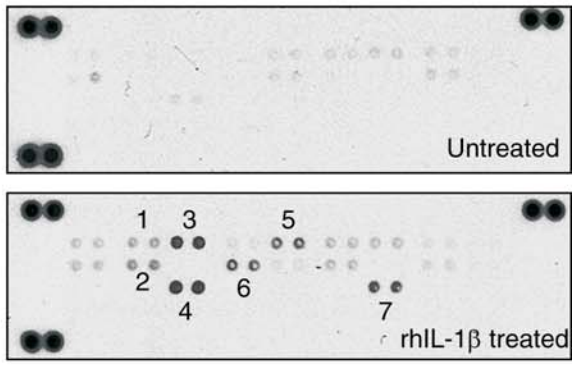


Figure 1: The Human Phospho-MAPK Array detects multiple phosphorylated kinases in untreated and treated cell lysates. (A) HeLa cells were either untreated or treated with 200 nM PMA for 20 minutes. (B) MCF-7 cells were either untreated or treated with 100 ng/mL of rhIGF-I (R&D Systems, Catalog # 291-G1) for 1 hour. (C) HepG2 cells were either untreated or treated with 25 ng/mL of rhIL-1β (R&D Systems, Catalog # 201-LB) for 30 minutes. 200 μg of lysate was used on each array. Array signals from scanned X-ray film images were analyzed using image analysis software. Phosphorylated kinases detected for each treatment are listed below.

Phosphorylated Kinases*		
HeLa Cells Treated with PMA	MCF-7 Cells Treated with rhIGF-I	HepG2 Cells Treated with rhIL-1β
ERK1	ERK1	JNK1
ERK2	ERK2	JNK2
MSK2	GSK-3α/β	JNK pan
RSK1	GSK-3β	p38δ
RSK2	Akt1	p38α
GSK-3α/β	Akt pan	MSK2
HSP27	p70 S6 Kinase	HSP27

*Refer to the Appendix on page 11 for the recognized phosphorylation sites.

SPECIFICITY - COMPETITION

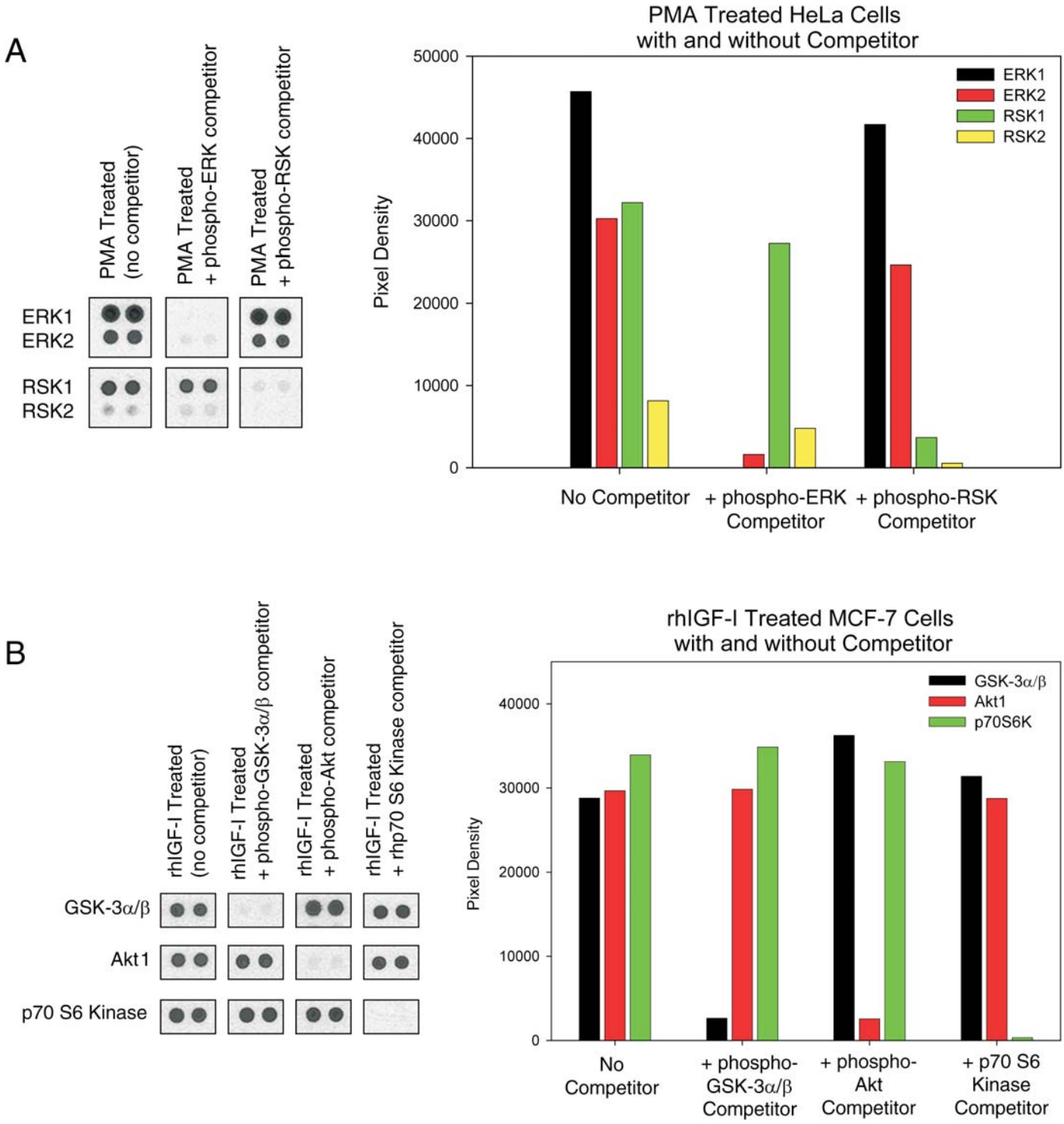


Figure 2: The specificity of the Human Phospho-MAPK Array is shown by competition. (A) HeLa cells were treated with 200 nM PMA for 20 minutes. 200 μg of lysate was used on each array. The arrays were incubated with the Detection Antibody Cocktail either with or without 0.2 μg/mL of competing phospho-peptide for ERK1 and ERK2 or RSK1 and RSK2. MCF-7 cells (B) were treated with 100 ng/mL of rhIGF-I (R&D Systems, Catalog # 291-G1) for 1 hour. 200 μg of lysate was used on each array. Lysates for the p70 S6 Kinase competition were incubated with 5 μg of recombinant human p70 S6 Kinase protein. The arrays were incubated with the Detection Antibody Cocktail either with or without 0.2 μg/mL of competing phospho-peptide for GSK-3α/β or Akt.

SPECIFICITY - PATHWAY INHIBITION

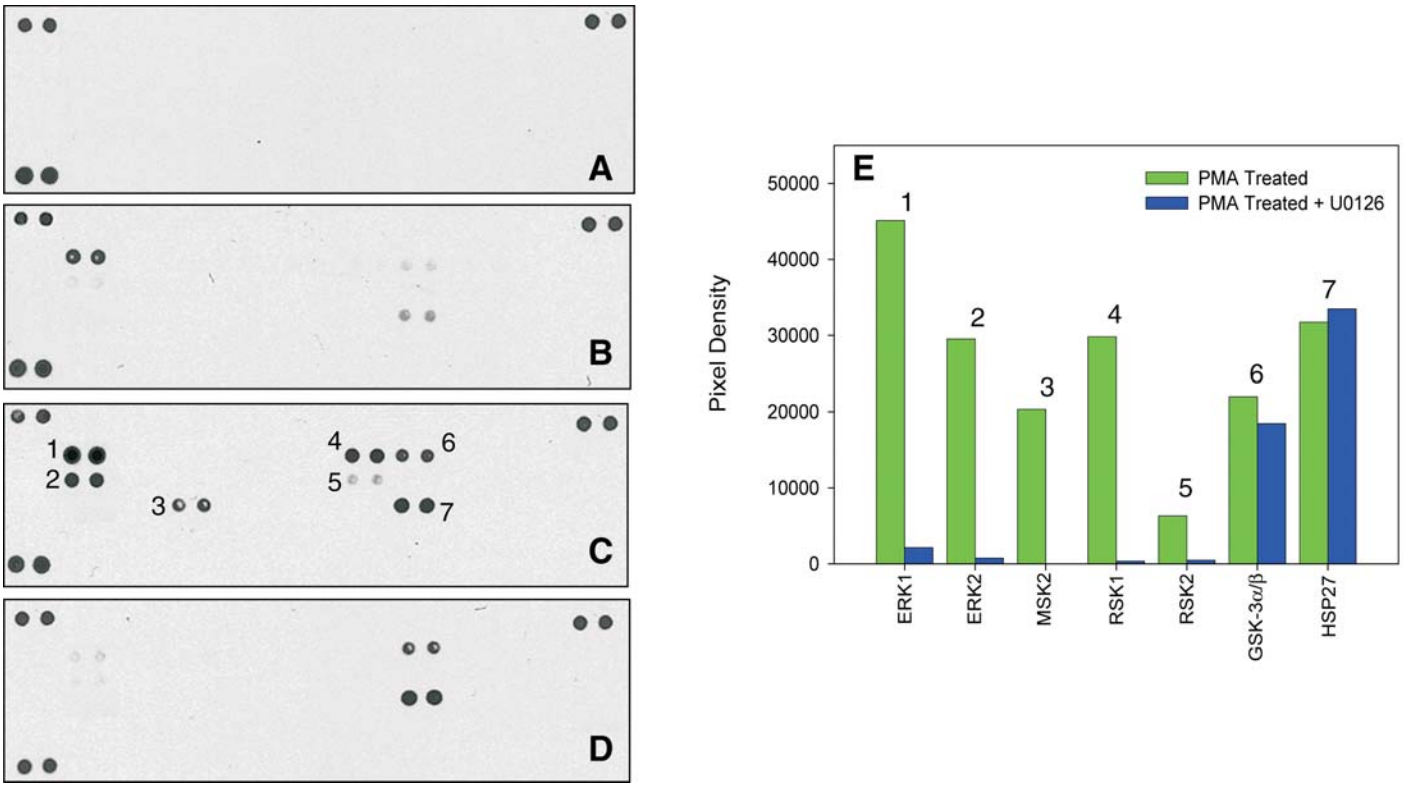


Figure 3: The Human Phospho-MAPK Array shows the effect of the MEK1/2 inhibitor U0126 on the ERK pathway. HeLa cells were incubated with no additions or with 200 nM PMA for 20 minutes either with or without U0126. R&D Systems' Proteome Profiler™ Human Phospho-MAPK Arrays (Catalog # ARY002) were incubated with (A) no lysate, (B) 175 μg of lysate from untreated cells, (C) 175 μg of lysate from PMA treated cells, or (D) 175 μg of lysate from cells treated with PMA in the presence of U0126. Graphical representation (E) of the data of the MEK1/2 inhibitor in panels C and D is shown.

APPENDIX

Refer to the table below for the Phospho-MAPK Array coordinates.

Coordinate	Target/Control	Alternate Nomenclature	Phosphorylation Site Detected
A1, A2	Positive Control	Control (+)	—
A21, A22	Positive Control	Control (+)	—
B3, B4	ERK1	MAPK3, p44 MAPK	T202/Y204
B5, B6	JNK1	MAPK8, SAPK1γ	T183/Y185
B7, B8	JNK pan	—	T183/Y185, T221/Y223
B9, B10	p38γ	MAPK12, SAPK3, ERK6	T183/Y185
B11, B12	p38δ	MAPK13, SAPK4	T180/Y182
B13, B14	RSK1	MAPKAPK1α, RPS6KA1	S380
B15, B16	GSK-3α/β	GSK3A/GSK3B	S21/S9
B17, B18	Akt1	PKBα, RACα	S473
B19, B20	Akt2	PKBβ, RACβ	S474
C3, C4	ERK2	MAPK1, p42 MAPK	T185/Y187
C5, C6	JNK2	MAPK9, SAPK1α	T183/Y185
C9, C10	p38α	MAPK14, SAPK2A, CSBP1	T180/Y182
C11, C12	p38β	MAPK11, SAPK2B, p38-2	T180/Y182
C13, C14	RSK2	ISPK-1, RPS6KA3	S386
C15, C16	GSK-3β	GSK3B	S9
C17, C18	Akt3	PKBγ, RACγ	S472
C19, C20	Akt pan	—	S473, S474, S472
D5, D6	JNK3	MAPK10, SAPK1β	T221/Y223
D7, D8	MSK2	RSKβ, RPS6KA4	S360
D15, D16	HSP27	HSPB1, SRP27	S78/S82
D17, D18	p70 S6 Kinase	S6K1, p70α, RPS6KB1	T421/S424
E3, E4	Rabbit IgG	Control (-)	—
E5, E6	Mouse IgG_1	Control (-)	—
E7, E8	Mouse IgG_{2A}	Control (-)	—
E9, E10	Mouse IgG_{2B}	Control (-)	—
E11, E12	Goat IgG	Control (-)	—
E13, E14	PBS	Control (-)	—
F1, F2	Positive Control	Control (+)	—