Proteome Profiler™ Array

Human Phospho-Kinase Array Kit

Catalog Number ARY003

For the parallel determination of the relative levels of protein phosphorylation.

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

TABLE OF CONTENTS

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

Analyzing the phosphorylation profiles of kinases and their protein substrates is essential for understanding how cells recognize and respond to changes in their environment. The Human Phospho-Kinase Array is a rapid, sensitive, and economical tool to simultaneously detect the relative levels of phosphorylation of 46 kinase phosphorylation sites without performing numerous immunoprecipitations and Western blots. Each capture antibody was carefully selected using cell lysates prepared from cell lines known to express the target protein.

PRINCIPLE OF THE ASSAY

Capture and control antibodies have been spotted in duplicate on nitrocellulose membranes. Cell lysates are diluted and incubated overnight with the Human Phospho-Kinase Array. The array is washed to remove unbound proteins followed by incubation with a cocktail of biotinylated detection antibodies. Streptavidin-HRP and chemiluminescent detection reagents are applied and a signal is produced at each capture spot corresponding to the amount of phosphorylated protein bound. 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-Kinase 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-Kinase Array	893591	8 membranes (4 Part A, 4 Part B)	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)	
Array Buffer 3	895008	1 vial (21 mL)	
Lysis Buffer 6	895561	1 vial (21 mL)	
Wash Buffer Concentrate, 25X	895003	2 vials (21 mL/vial)	
Detection Antibody Cocktail A, Human Phospho-Kinase Array	893592	1 vial	
Detection Antibody Cocktail B, Human Phospho-Kinase Array	893593	1 vial	
Streptavidin-HRP	890803	1 vial	
Chemi Reagent 1	894287	1 vial (2.5 mL)	
Chemi Reagent 2	894288	1 vial (2.5 mL)	
8-Well Rectangular Multi-dish	607591	1 dish	Store at room temperature.
Transparency Overlay Template	607592	1 template	

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

OTHER SUPPLIES REQUIRED

- 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 Human Phospho-Kinase 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, making sure to remove any remaining PBS before adding lysis buffer. Solubilize 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. Quantitation of sample protein concentration using a total protein assay is recommended. The maximum allowable lysate volume is 167 µL per array part. A 5:1 ratio of Array Buffer 1 to Lysis Buffer 6 is required for optimal assay performance. 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-Kinase Array - Eight nitrocellulose membranes; Part A contains 28 antibodies printed in duplicate, and Part B contains 18 antibodies printed in duplicate. Part A and Part B should be used together for optimal analysis efficiency. **Handle the membranes only with gloved hands and flat-tipped tweezers.**

Detection Antibody Cocktail A (red cap) - One vial of lyophilized biotinylated antibodies for use with Part A membranes. Before use, reconstitute Detection Antibody Cocktail A in 100 µL of deionized or distilled water.

Detection Antibody Cocktail B (blue cap) - One vial of lyophilized biotinylated antibodies for use with Part B membranes. Before use, reconstitute Detection Antibody Cocktail B in 100 µL of deionized or distilled water.

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

Chemi Reagent Mix - Chemi Reagent 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 for each set of membranes (A and B).**

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. The Human Phospho-Kinase Array is divided into two parts (A and B) to maximize sensitivity and minimize cross-reactivity. For best results, incubate Part A and B in the same lysate preparation but in separate wells of the 8-Well Multi-dish.
3. Pipette 1.0 mL of Array Buffer 1 into each well of the 8-Well Multi-dish to be used. Array Buffer 1 serves as a block buffer.
4. Using flat-tip tweezers, remove each membrane to be used from between the protective sheets. Place one Part A membrane and one Part B membrane into adjacent wells of the 8-Well Multi-dish and place the lid on the 8-Well Multi-dish. The number on the membrane should be facing upward.

   Note: *Upon contact with Array Buffer 1, the blue dye from the spots will disappear, but the capture antibodies are retained in their specific locations.*
5. Incubate for one hour on a rocking platform shaker. Orient the tray so that each membrane rocks end to end in its well.
6. While the membranes are blocking, prepare samples by adding up to 334 µL of cell lysate to 1666 µL of Array Buffer 1. Adjust to a final volume of 2.0 mL with Lysis Buffer 6 as necessary.
7. Aspirate Array Buffer 1 from the 8-Well Multi-dish. Add 1.0 mL of the prepared samples to both the Part A and Part B membrane.
8. Place the lid on the 8-Well Multi-dish. 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 membrane and place into individual plastic containers with 20 mL of 1X Wash Buffer. The corresponding parts (A and B) of the membrane should be washed in the **same container** at this point. The recommended container size for washing is approximately 8 x 11 cm. Rinse the 8-Well Multi-dish with deionized or distilled water and dry thoroughly.
10. Wash each set (A and B) of membranes with 1X Wash Buffer for 10 minutes on a rocking platform shaker. Repeat two times for a total of three washes.
11. For each Part A membrane, dilute 20 µL of reconstituted Detection Antibody Cocktail A (red cap) to 1.0 mL with 1X Array Buffer 2/3. Pipette 1.0 mL per well of diluted Detection Antibody Cocktail A into the 8-Well Multi-dish.
12. Carefully remove each Part A membrane from its wash container. Allow excess Wash Buffer to drain from the membrane. Return the membrane to the 8-Well Multi-dish containing the diluted Detection Antibody Cocktail A.
13. For each Part B membrane, dilute 20 µL of reconstituted Detection Antibody Cocktail B (blue cap) to 1.0 mL with 1X Array Buffer 2/3. Pipette 1.0 mL per well of diluted Detection Antibody Cocktail B into the 8-Well Multi-dish.
14. Carefully remove each Part B membrane from its wash container. Allow excess Wash Buffer to drain from the membrane. Return the membrane to the 8-Well Multi-dish containing the diluted Detection Antibody Cocktail B, and cover it with the lid.

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

16. Carefully remove each membrane and place into individual plastic containers with 20 mL of 1X Wash Buffer. At this point, the corresponding parts (A and B) of the membrane should be washed in **separate containers** to minimize detection antibody cross-reactivity. Rinse the 8-Well Multi-dish with deionized or distilled water and dry thoroughly.

17. Wash each membrane with 1X Wash Buffer for 10 minutes on a rocking platform shaker. Repeat two times for a total of three washes.

18. Dilute the Streptavidin-HRP in 1X Array Buffer 2/3 using the dilution factor on the vial label. Pipette 1.0 mL into each well of the 8-Well Multi-dish.

19. Carefully remove each membrane from its wash container. Allow excess Wash Buffer to drain from the membrane. Return the membranes to the 8-Well Multi-dish containing the diluted Streptavidin-HRP, and cover with the lid. Incubate for 30 minutes at room temperature on a rocking platform shaker.

20. Carefully remove each membrane and place into plastic containers with 20 mL of 1X Wash Buffer. The corresponding parts (A and B) of the membrane should be washed in the **same container** at this point. Rinse the 8-Well Multi-dish with deionized or distilled water and dry thoroughly.

21. Wash each set (A and B) of membranes with 1X Wash Buffer for 10 minutes on a rocking platform shaker. Repeat two times for a total of three washes.

    Note: *Complete the remaining steps without interruption.*

22. 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. Place corresponding Part A and Part B membranes end-to-end.

23. Pipette 1 mL of the prepared Chemi Reagent Mix evenly onto each set of membranes.

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

24. 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.

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

26. 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.

27. 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.

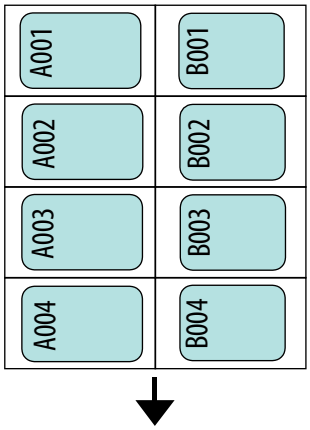
28. Place the membranes with the identification numbers facing up in an autoradiography film cassette.

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

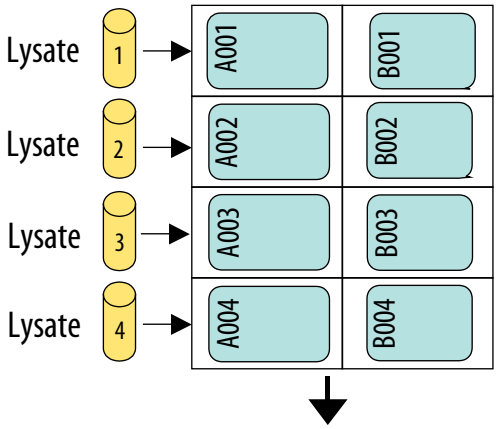
29. Expose membranes to X-ray film for 1-10 minutes. Multiple exposure times are recommended.

ARRAY PROCEDURE SUMMARY

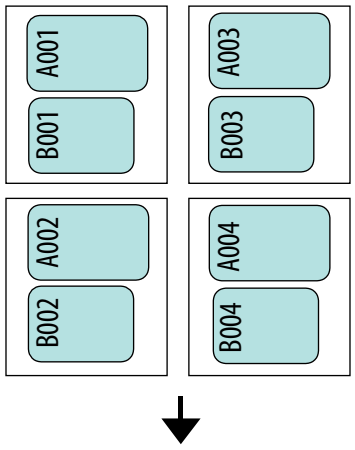
Step 1 (Blocking): Add 1.0 mL of Array Buffer 1 per well. Rock for 1 hour at room temperature.



Step 2 (Cell Lysates): Prepare 2.0 mL of diluted cell lysate. Remove Array Buffer 1. Add 1.0 mL of lysate to both Part A and Part B. Incubate overnight at 2-8 °C on a rocking platform shaker.



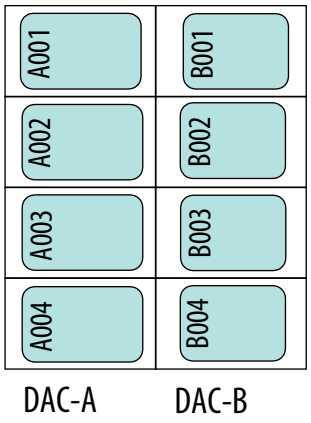
Step 3: (Wash 1) Wash in 1X Wash Buffer; 20 mL per dish. Wash a total of 3 times; 10 minutes on a rocking platform per wash. Wash corresponding parts (A and B) together.



Step 4 (**Detection Antibody Cocktail):** Pipette 1.0 mL of diluted Detection Antibody Cocktail A (DAC-A) into wells for Part A membranes.

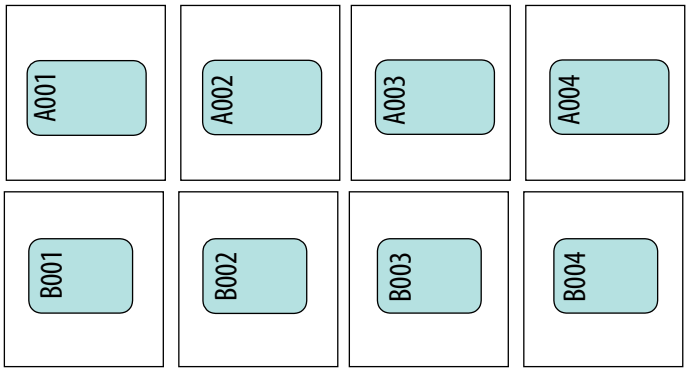
Pipette 1.0 mL of diluted Detection Antibody Cocktail B (DAC-B) into wells for Part B membranes.

Transfer the membranes to appropriate wells. Incubate for 2 hours at room temperature on a rocking platform.

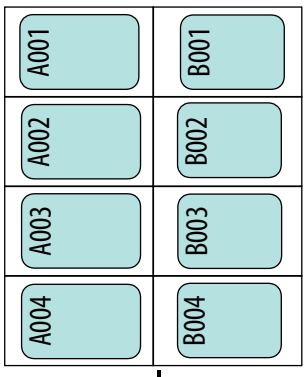


ARRAY PROCEDURE SUMMARY *CONTINUED*

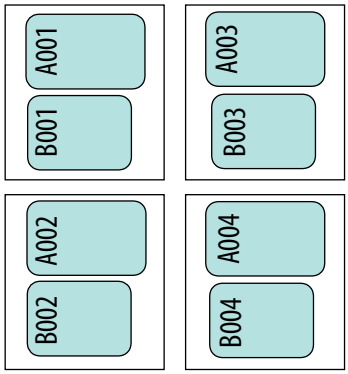
Step 5 (Wash 2): Wash in 1X Wash Buffer; 20 mL per dish. Wash a total of 3 times; 10 minutes on a rocking platform shaker per wash. **Wash all membranes separately.**



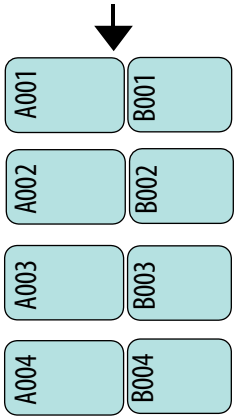
Step 6 (Streptavidin-HRP): Pipette 1.0 mL of diluted Streptavidin- HRP into each well. Transfer the membranes to the appropriate wells. Incubate for 30 minutes at room temperature on a rocking platform.



Step 7 (Wash 3): Wash in 1X Wash Buffer; 20 mL per dish. Wash a total of 3 times; 10 minutes per wash. Wash corresponding parts (A and B) together.



Step 8 (Signal Detection): Arrange the membranes on a sheet protector. Apply the Chemi Reagent Mix and expose to film.



DATA ANALYSIS

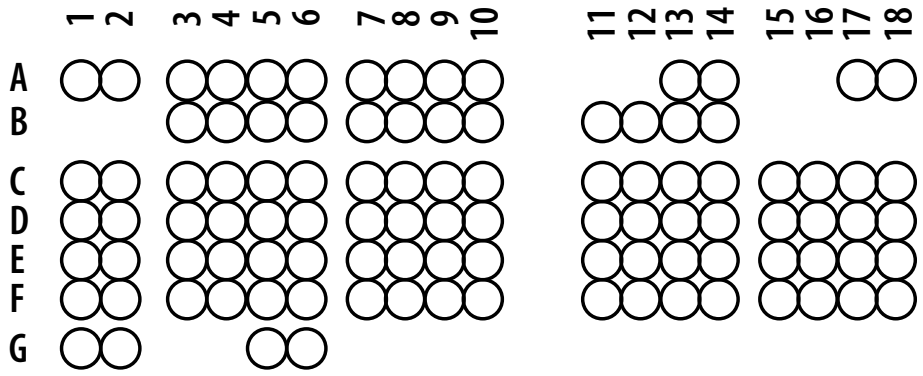
The positive signals seen on developed film can be quickly identified by placing the transparency overlay on the array image and aligning it with the pairs of reference spots in the corners of each membrane (two pairs on the left side of Part A and one pair on the right side of Part B). The stamped identification numbers on the membranes should be placed on the left hand side. The location of controls and capture antibodies is listed in the Appendix. It may be necessary to adjust the position of the transparency overlay template if the two parts of the membrane are not aligned.

Note: *Reference spots are included to align the transparency overlay template and to demonstrate that the array has been incubated with Streptavidin-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 phosphorylated kinase protein.
4. Subtract an averaged background signal from each spot. Use a signal from a clear area of the array or negative control spots as a background value.
5. Compare corresponding signals on different arrays to determine the relative change in phosphorylated kinase proteins between samples.

Human Phospho-Kinase Array Coordinates



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

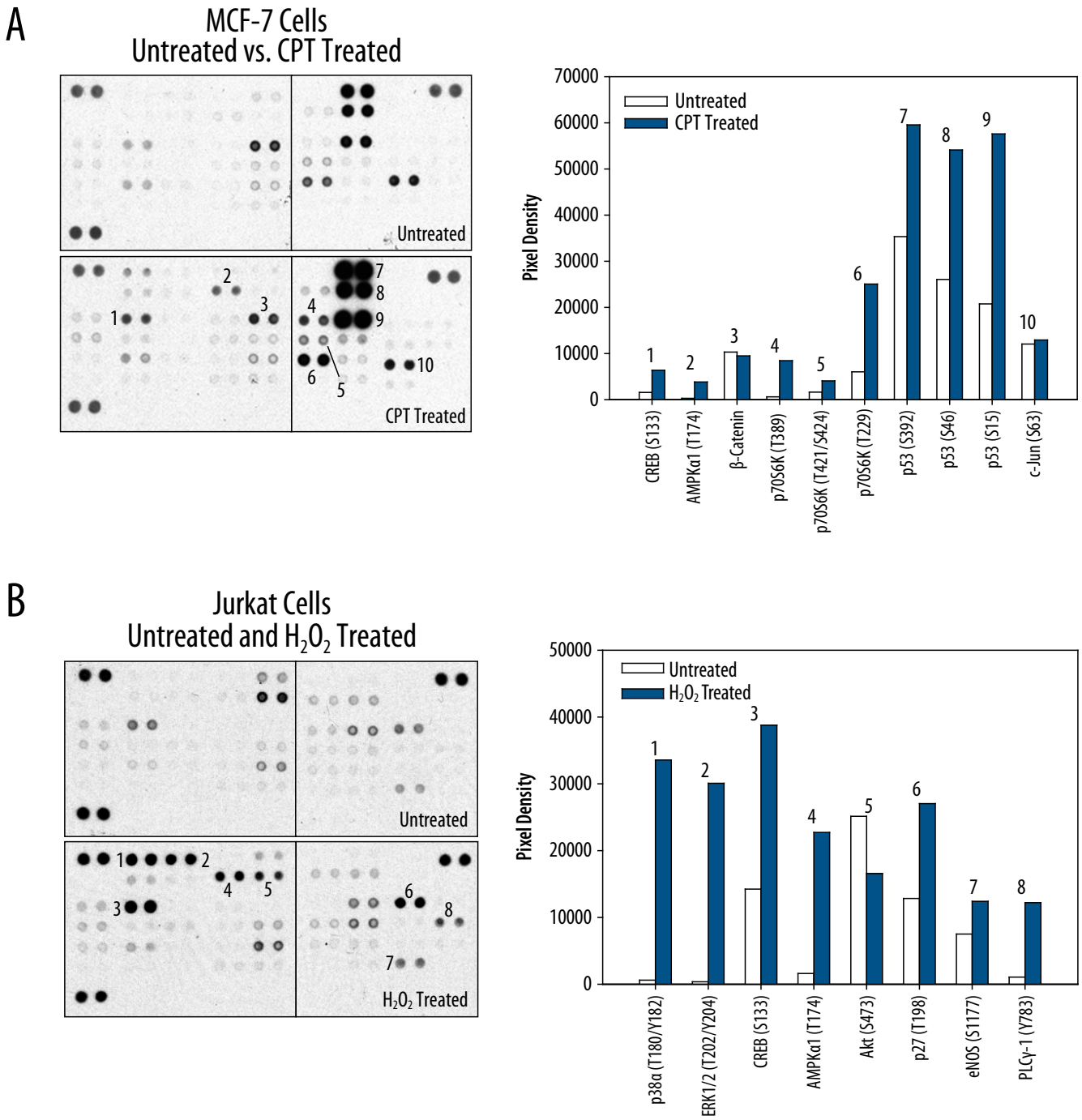


Figure 1A: The Human Phospho-Kinase Array detects phosphorylated proteins in untreated and treated cell lysates. Parts A and B of the arrays were each incubated with 300 µg of cell lysate.

A. MCF-7 human breast cancer cells were either left untreated or treated with 1 µM camptothecin (CPT) for 6 hours.

B. Jurkat human acute T cell leukemia cells were either left untreated or treated with 2 mM H_2O_2 for 2 minutes.

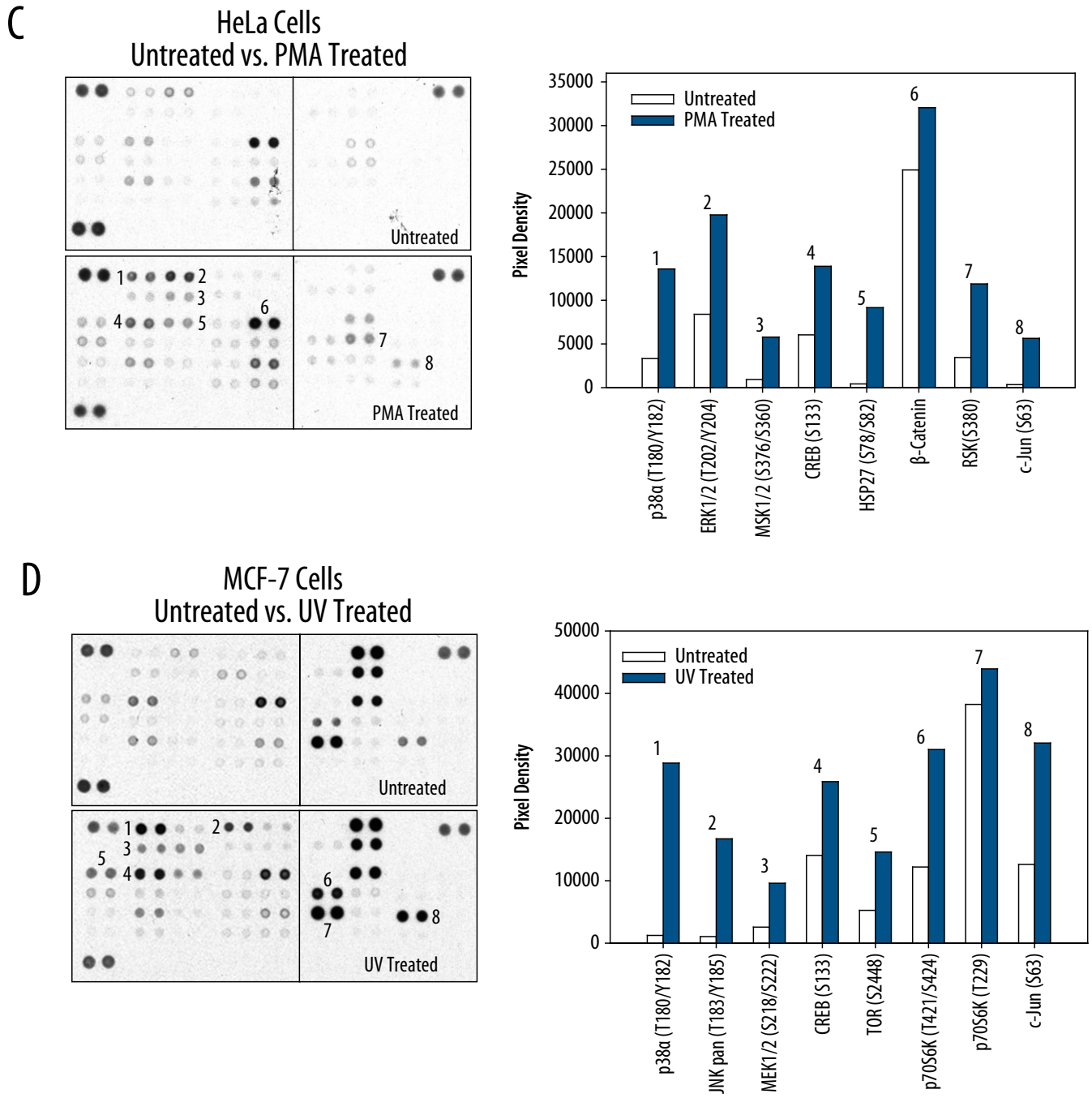


Figure 1B: The Human Phospho-Kinase Array detects phosphorylated proteins in untreated and treated cell lysates. Parts A and B of the arrays were each incubated with 300 µg of cell lysate.

C. HeLa human cervical epithelial carcinoma cells were either left untreated or treated with 200 nM PMA for 20 minutes.

D. MCF-7 human breast cancer cells were either left untreated or exposed to 50 J/m^2 of UV light followed by a 4 hour recovery period before lysis.

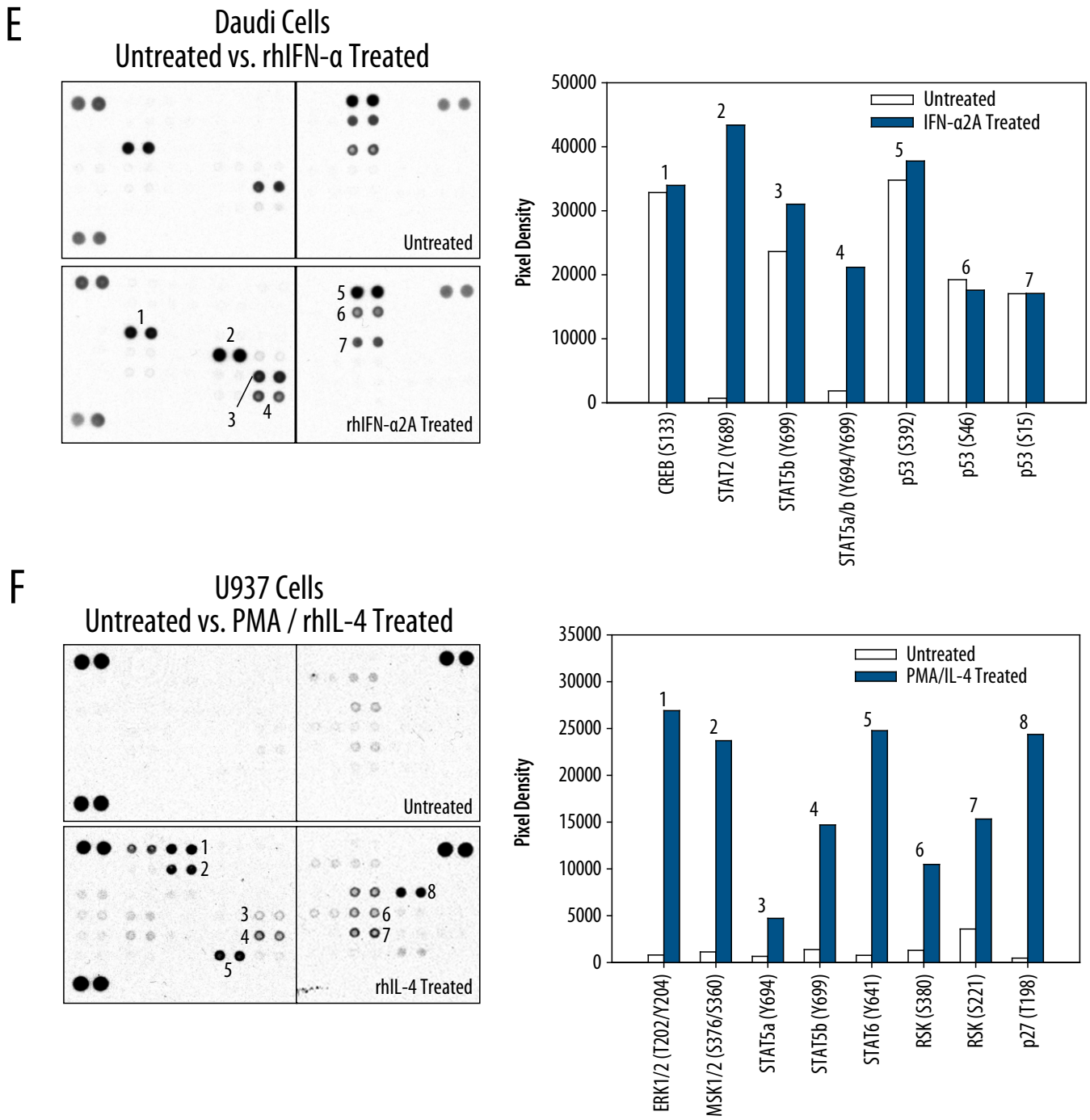


Figure 1C: The Human Phospho-Kinase Array detects phosphorylated proteins in untreated and treated cell lysates. Parts A and B of the arrays were each incubated with 500 µg of cell lysate.

E. Daudi human Burkitt's lymphoma cells were either left untreated or treated with 500 U/mL rhIFN-α2A (R&D Systems, Catalog # 11100-1) for 5 minutes.

F. U937 human histiocytic lymphoma cells were either left untreated or treated with 20 nM PMA for 24 hours followed by treatment with 100 ng/mL rhIL-4 (R&D Systems, Catalog # 204-IL) for 30 minutes.

APPENDIX

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

Membrane/Coordinate	Target/Control	Phosphorylation Site	Cell Source/Treatment
A-A1, A2	Reference Spot	——	——
A-A3, A4	p38α	T180/Y182	MCF-7/UV*
A-A5, A6	ERK1/2	T202/Y204, T185/Y187	HeLa/PMA*
A-A7, A8	JNK pan	T183/Y185, T221/Y223	MCF-7/UV*
A-A9, A10	GSK-3α/β	S21/S9	MCF-7/rhIGF-I
B-A13, A14	p53	S392	MCF-7/CPT*
B-A17, A18	Reference Spot	——	——
A-B3, B4	MEK1/2	S218/S222, S222/S226	MCF-7/UV*
A-B5, B6	MSK1/2	S376/S360	HeLa/PMA*
A-B7, B8	AMPKα1	T174	Jurkat/H_2O_2*
A-B9, B10	Akt	S473	MCF-7/rhIGF-I*
B-B11, B12	Akt	T308	K562/Pervanadate
B-B13, B14	p53	S46	MCF-7/CPT*
A-C1, C2	TOR	S2448	MCF-7/UV*
A-C3, C4	CREB	S133	Jurkat/H_2O_2*
A-C5, C6	HSP27	S78/S82	HeLa/PMA*
A-C7, C8	AMPKα2	T172	HepG2/Metformin*
A-C9, C10	β-Catenin	——	HeLa/PMA*
B-C11, C12	p70 S6 Kinase	T389	MCF-7/CPT*
B-C13, C14	p53	S15	MCF-7/CPT*
B-C15, C16	p27	T198	Jurkat/H_2O_2*
B-C17, C18	Paxillin	Y118	K562/Pervanadate
A-D1, D2	Src	Y419	COLO 205/SFM*
A-D3, D4	Lyn	Y397	Daudi/Pervanadate*
A-D5, D6	Lck	Y394	Jurkat/Pervanadate
A-D7, D8	STAT2	Y689	Daudi/rhIFN-α2A
A-D9, D10	STAT5a	Y694	U937/PMA + rhIL-4
B-D11, D12	p70 S6 Kinase	T421/S424	MCF-7/UV*
B-D13, D14	RSK1/2/3	S380/S386/S377	HeLa/PMA*
B-D15, D16	p27	T157	K562/Pervanadate
B-D17, D18	PLCγ-1	Y783	Jurkat/H_2O_2*

*A change in phosphorylation for this protein was detected in multiple cell sources.

Membrane/Coordinate	Target/Control	Phosphorylation Site	Cell Source/Treatment
A-E1, E2	Fyn	Y420	Daudi/Pervanadate*
A-E3, E4	Yes	Y426	K562/Pervanadate*
A-E5, E6	Fgr	Y412	K562/Pervanadate
A-E7, E8	STAT3	Y705	LNCap/rhIL-6
A-E9, E10	STAT5b	Y699	Daudi/rhIFN-α2A*
B-E11, E12	p70 S6 Kinase	T229	MCF-7/CPT*
B-E13, E14	RSK1/2	S221/S227	U937/PMA + rhIL-4
B-E15, E16	c-Jun	S63	HeLa/PMA*
B-E17, E18	Pyk2	Y402	K562/Pervanadate*
A-F1, F2	Hck	Y411	Daudi/Pervanadate
A-F3, F4	Chk-2	T68	HeLa/CPT*
A-F5, F6	FAK	Y397	HepG2/Pervanadate
A-F7, F8	STAT6	Y641	U937/PMA + rhIL-4
A-F9, F10	STAT5a/b	Y694/Y699	Daudi/rhIFN-α2A
B-F11, F12	STAT1	Y701	Daudi/rhIFN-α2A
B-F13, F14	STAT4	Y693	NC-37/Pervanadate
B-F15, F16	eNOS	S1177	Jurkat/H_2O_2*
B-F17, F18	PBS (Negative Control)	——	——
A-G1, G2	Reference Spot	——	——
A-G5, G6	PBS (Negative Control)	——	——

*A change in phosphorylation for this protein was detected in multiple cell sources.

NOTES