Proteome Profiler[™] Array

Human Phospho-Kinase Array Kit

Catalog Number ARY003B

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.

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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 43 kinase phosphorylation sites and 2 related total proteins 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.
- For a procedure demonstration video, please visit: <u>www.RnDSystems.com/ProteomeProfilerVideo</u>.

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 ProClin[®] which may cause an allergic skin reaction. Avoid breathing mist.

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

MATERIALS PROVIDED & STORAGE CONDITIONS

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

			STORAGE OF OPENED/			
PART	PART #	DESCRIPTION	RECONSTITUTED MATERIAL			
Human Phospho-Kinase Array	894552	8 nitrocellulose membranes (4 Part A, 4 Part B) each containing 43 different capture antibodies printed in duplicate.	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.*		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	21 mL of a buffered protein base with preservatives.				
Array Buffer 2 5X Concentrate	895478	21 mL of a concentrated buffered protein base with preservatives.				
Array Buffer 3	895008	21 mL of a buffered protein base with preservatives.				
Lysis Buffer 6	895561	21 mL of a denaturing buffered solution.				
Wash Buffer Concentrate	895003	2 vials (21 mL/vial) of a 25-fold concentrated solution of buffered surfactant with preservative. <i>May turn yellow over time</i> .	May be stored for up to 3 months at 2-8 °C.*			
Detection Antibody Cocktail A, Human Phospho-Kinase Array	894553	1 vial of biotinylated antibody cocktail; lyophilized.				
Detection Antibody Cocktail B, Human Phospho-Kinase Array	894554	1 vial of biotinylated antibody cocktail; lyophilized.				
Streptavidin-HRP	893019	200 μL of streptavidin conjugated to horseradish-peroxidase.				
Chemi Reagent 1	894287	2.5 mL of stabilized hydrogen peroxide with preservative.				
Chemi Reagent 2	894288	2.5 mL of stabilized luminol with preservative.				
8-Well Multi-dish	607591	Clear 8-well rectangular multi-dish.				
Transparency Overlay Template	607815	1 transparency overlay template for coordinate reference.	Store at room temperature.			

* 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 200-600 µg per array set (A and B).

Cell Lysates - Rinse cells with PBS, making sure to remove any remaining PBS before adding lysis buffer. Solubilize cells at 1 x 10⁷ 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 334 µL per array set (A and B). Lysates should be used immediately or aliquoted and stored at \leq -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 29 antibodies printed in duplicate, and Part B contains 16 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 μ L 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 - Add 2 mL of 5X Array Buffer 2 Concentrate to 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. Add 40 mL of 25X Wash Buffer Concentrate to 960 mL of deionized or distilled water to prepare 1000 mL of 1X Wash Buffer. *Wash Buffer may turn yellow over time*.

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 Part 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 diluting the desired quantity of cell lysate (up to 334μ L) to a final volume of 2 mL with Array Buffer 1.
- 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, add 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, add 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 transmissionmode 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.

PROFILING KINASE PHOSPHORYLATION IN SAMPLES

The Human Phospho-Kinase Array detects phosphorylated proteins in cell lysates.

Parts A and B of each array were incubated with 200 µg of cell lysate. Data shown are from a 5 minute exposure to film.



Figures A and B:

- **A.** MCF-7 human breast cancer cells were either left untreated or exposed to 50 J/m² of UV light followed by a 4 hour recovery before lysis.
- **B.** A431 human epithelial carcinoma cells were either left untreated or treated with 200 ng/mL recombinant human (rh) EGF (R&D Systems, Catalog # 236-EG) for 5 minutes.

PROFILING KINASE PHOSPHORYLATION IN SAMPLES CONTINUED



Figures C and D:

- **C.** MCF-7 human breast cancer cells were either left untreated or treated with 100 ng/mL of rhIGF-I (R&D Systems, Catalog # 291-G1) for 1 hour.
- **D.** CCD-1070Sk human foreskin fibroblast cells were either left untreated or treated with 100 ng/mL rhPDGF-BB (R&D Systems, Catalog # 220-BB) for 5 minutes.

PROFILING KINASE PHOSPHORYLATION IN SAMPLES CONTINUED





Figures E and F:

- **E.** HeLa human cervical epithelial carcinoma cells were either left untreated or treated with 200 nM PMA for 20 minutes.
- **F.** MCF-7 human breast cancer cells were either left untreated or treated with 1 μ M Camptothecin (CPT) for 4 hours.

PROFILING KINASE PHOSPHORYLATION IN SAMPLES CONTINUED



Figure G: Jurkat human acute T cell leukemia cells were either left untreated or treated with $2 \text{ mM H}_2\text{O}_2$ for 2 minutes.

APPENDIX

Refer to the table below for the Human Phospho-Kinase Array coordinates. DuoSet IC catalog numbers are provided for follow-up analysis of array data when desired.

Membrane/ Coordinate	Target/Control	Phosphorylation Site	DuoSet® IC (Total) ELISA Development System	DuoSet [®] IC (Phospho) ELISA Development System
A-A1, A2	Reference Spot			
A-A3, A4	p38a	T180/Y182	DYC8691B	DYC869B
A-A5, A6	ERK1/2	T202/Y204, T185/ Y187		DYC1018B
А-А7, А8	JNK 1/2/3	T183/Y185, T221/ Y223	DYC1205	DYC1387
A-A9, A10	GSK-3α/β	S21/S9	DYC2157	DYC2630
B-A13, A14	p53	S392	DYC1043	DYC2996
B-A17, A18	Reference Spot			
A-B3, B4	EGF R	Y1086	DYC1854	
A-B5, B6	MSK1/2	S376/S360		
A-B7, B8	ΑΜΡΚα1	T183	DYC3197	DYC3528
A-B9, B10	Akt 1/2/3	S473		DYC887B
B-B11, B12	Akt 1/2/3	T308		
B-B13, B14	p53	S46	DYC1043	DYC1489
A-C1, C2	TOR	S2448		DYC1665
A-C3, C4	CREB	S133		DYC2510
A-C5, C6	HSP27	S78/S82	DYC1580	DYC2314
A-C7, C8	ΑΜΡΚα2	T172		
A-C9, C10	β-Catenin		DYC1329	
B-C11, C12	p70 S6 Kinase	T389	DYC8962	DYC896
B-C13, C14	p53	S15	DYC1043	DYC1839
B-C15, C16	c-Jun	S63		
A-D1, D2	Src	Y419		DYC2685
A-D3, D4	Lyn	Y397		DYC3936
A-D5, D6	Lck	Y394		
A-D7, D8	STAT2	Y689		
A-D9, D10	STAT5a	Y694		
B-D11, D12	p70 S6 Kinase	T421/S424	DYC8962	DYC8965
B-D13, D14	RSK1/2/3	S380/S386/S377		DYC889B
B-D15, D16	eNOS	S1177		

APPENDIX CONTINUED

Membrane/	Target/Control	Phosphorylation	DuoSet [®] IC (Total) ELISA	DuoSet [®] IC (Phospho) ELISA
				Development System
A-E1, EZ	ryn	1420		
A-E3, E4	Yes	Y426		DYC3929
A-E5, E6	Fgr	Y412		
A-E7, E8	STAT6	Y641		
A-E9, E10	STAT5b	Y699		
B-E11, E12	STAT3	Y705		DYC4607B
B-E13, E14	p27	T198	DYC2256	
B-E15, E16	PLC-γ1	Y783		
A-F1, F2	Hck	Y411		
A-F3, F4	Chk-2	T68		DYC1626
A-F5, F6	FAK	Y397	DYC4467	DYC4528
A-F7, F8	PDGF Rβ	Y751	DYC385	DYC3096
A-F9, F10	STAT5a/b	Y694/Y699		
B-F11, F12	STAT3	S727		
B-F13, F14	WNK1	T60		DYC4720
B-F15, F16	РҮК2	Y402		
A-G1, G2	Reference Spot			
A-G3, G4	PRAS40	T246		DYC6890
A-G9, G10	PBS (Negative Control)			
B-G11, G12	HSP60		DYC1800	
B-G17, G18	PBS (Negative Control)			

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