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R&D SYSTEMS

Proteome Profiler™ Array

Human Phospho-Immunoreceptor Array Kit

Catalog Number ARY004B

For the parallel determination of the relative levels of tyrosine phosphorylation of human immunoreceptors.

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 tyrosine phosphorylation of immunoreceptors and adaptor signaling molecules on ITAMs (immunoreceptor tyrosine-based activation motifs) and ITIMs (immunoreceptor tyrosine-based inhibitory motifs) plays an important role in the control of cellular activation in the immune system. The Human Phospho-Immunoreceptor Array is a rapid, sensitive, and economical tool used to detect changes in phosphorylation between samples. Protein array technology allows the screening of fifty-nine different immunoreceptors without numerous individual immunoprecipitations and Western blots. The non-denaturing conditions used in the methods recommended for this array maintain the non-covalent association of activating receptors with phosphorylated ITAM-containing transmembrane signaling adaptors. The efficacy of each capture antibody was confirmed using lysate samples prepared from treated cell lines known to express the immunoreceptors. Recombinant immunoreceptors were used to validate capture antibodies by competition with lysates prepared from activated cell lines.

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-Immunoreceptor Array. After binding the extracellular domain of both phosphorylated and unphosphorylated immunoreceptors, 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.

This kit can be converted to obtain a total immunoreceptor profile by using lysates prepared from cell-surface biotinylated cells and Streptavidin-HRP (R&D Systems®, # DY998) for detection. A biotinylated positive control for streptavidin-HRP detection is spotted on three corners of the array next to the phospho-tyrosine positive control.

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-Immunoreceptor 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.
- The use of lysis buffers other than Lysis Buffer 17 may change the phospho-immunoreceptor profile. Some immunoreceptors may have decreased solubility in different lysis buffers due to their association with lipid rafts upon their activation. Lysis buffers may also affect the association of receptors with ITAM-containing adaptor proteins.
- For a procedure demonstration video, please visit: www.RnDSystems.com/ProteomeProfilerVideo.

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.

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-Immunoreceptor Array	894684	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	607464	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

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-Immunoreceptor Array detects relative phosphorylation levels of individual analytes, it is important to include appropriate control samples.

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 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. Cellular 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.
Note: *Sample amount may be empirically adjusted to attain optimal sensitivity with minimal background. The suggested starting ranges for cell lysates is 200-600 µg per array (A and B).*

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Human Phospho-Immunoreceptor Array - Four nitrocellulose membranes each containing 59 anti-receptor antibodies printed in duplicate. **Handle the membranes only with gloved hands and flat-tipped tweezers.**

Anti-Phospho-Tyrosine-HRP - 50 µL of mouse anti-phospho-tyrosine antibody conjugated to HRP. Immediately before each 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 required for cell lysate preparation. **Prepare fresh for each use.**

Note: *The use of lysis buffers other than Lysis Buffer 17 may change the phospho-immunoreceptor profile. Some immunoreceptors may have decreased solubility in different lysis buffers due to their association with lipid rafts upon their activation.*

1X Array Buffer 2 - Dilute 2 mL of 5X Array Buffer 2 Concentrate into 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 completey dissolved. Add 40 mL of 25X Wash Buffer Concentrate to 960 mL of deionized or distilled water. *Wash Buffer Concentrate 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 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 to be used. Array Buffer 1 serves as a block buffer.
3. Using flat-tip tweezers, remove each membrane to be used from between the protective sheets and place in a well of the 4-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.*
4. Incubate for one hour on a rocking platform shaker. Orient the tray so that each membrane rocks end to end in its well.
5. While the arrays are blocking, prepare samples by diluting the desired quantity of cell lysate to a final volume of 1.5 mL with Array Buffer 1.
6. Remove Array Buffer 1 from the 4-Well Multi-dish. Add the prepared samples and place the lid on the 4-Well Multi-dish.
7. 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.*
8. 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.
9. Wash each array with 1X Wash Buffer for 10 minutes on a rocking platform shaker. Repeat two times for a total of three washes.
10. 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.
11. Carefully remove each array from its wash container. Allow excess Wash Buffer to drain from the membrane. Return the membrane to the 4-Well Multi-dish and cover the wells with the lid.
12. Incubate for 2 hours at room temperature on a rocking platform shaker.
13. Wash each array as described in steps 8 and 9.

    Note: *Complete the remaining steps without interruption.*

ASSAY PROCEDURE *CONTINUED*

14. 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.

15. 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.*

16. 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.

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

18. 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.

19. 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.

20. 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.*

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

DATA ANALYSIS

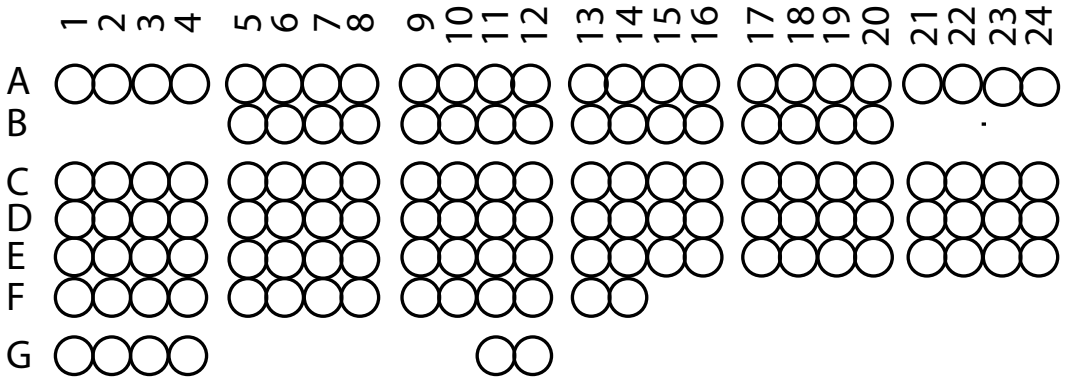
The positive signals seen on developed film can be quickly identified by placing the transparency overlay template on the array image and aligning it with the pairs of reference spots in three corners of each array. The stamped identification number on the array should be placed on the left hand side.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

Human Phospho-Immunoreceptor Array Coordinates



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

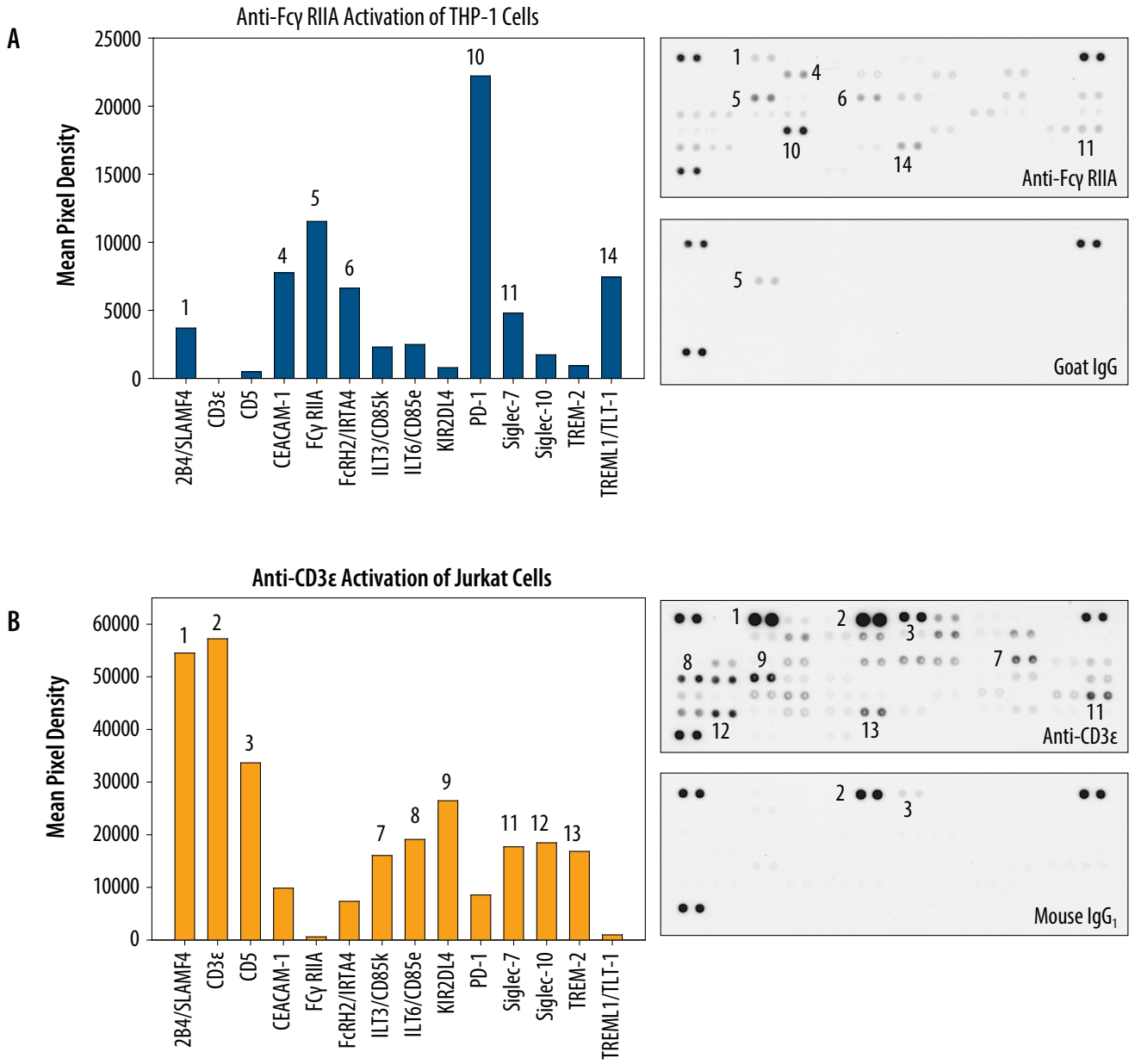


Figure 1: The Human Phospho-Immunoreceptor Array detects multiple tyrosine phosphorylated immunoreceptors in lysates prepared from cells activated by antibody-mediated cross-linking of cell-surface immunoreceptors. (A) THP-1 human acute monocytic leukemia cells were incubated with Human Fc gamma RII/CD32 Antibody (R&D Systems®, # AF1875) or Normal Goat IgG (R&D Systems, # AB-108-C) followed by incubation with a Donkey Anti-Goat IgG Antibody (R&D Systems, # AF109) for 5 minutes [Maresco, D.L. *et al.* (1999) J. Immunol. **162**:6458]. **(B)** Jurkat human acute T cell leukemia cells were incubated with a mouse monoclonal anti-CD3ε antibody (R&D Systems, # MAB100) or Mouse IgG_1 Isotype Control (R&D Systems, # MAB002) followed by incubation with a Mouse IgG Antibody (R&D Systems, # AF007) for 5 minutes. 100 µg of lysate was run on each array.

APPENDIX

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

Coordinate	Receptor
A1, A2	Reference Spots
A3, A4	Bt-Control*
A5, A6	2B4/SLAMF4
A7, A8	BLAME/SLAMF8
A9, A10	BTLA
A11, A12	CD3ε
A13, A14	CD5
A15, A16	CD6
A17, A18	CD28
A19, A20	CD84/SLAMF5
A21, A22	Bt-Control*
A23, A24	Reference Spots
B5, B6	CD229/SLAMF3
B7, B8	CEACAM-1
B9, B10	CLEC-1
B11, B12	CLEC-2
B13, B14	CRACC/SLAMF7
B15, B16	CTLA-4/CD152
B17, B18	DCIR/CLEC4A
B19, B20	Dectin-1/CLEC7A
C1, C2	DNAM-1
C3, C4	Fcε RII/CD23
C5, C6	Fcγ RIIA
C7, C8	Fcγ RIIIA/B
C9, C10	FcRH1/IRTA5
C11, C12	FcRH2/IRTA4
C13, C14	FcRH4/IRTA1
C15, C16	FcRH5/IRTA2
C17, C18	ILT2/CD85j
C19, C20	ILT3/CD85k
C21, C22	ILT4/CD85d
C23, C24	ILT5/CD85a

*Biotinylated Control

Coordinate	Receptor
D1, D2	ILT6/CD85e
D3, D4	Integrin β3/CD61
D5, D6	KIR2DL4
D7, D8	LAIR-1
D9, D10	LAIR-2
D11, D12	LMIR1/CD300A
D13, D14	LMIR2/CD300C
D15, D16	LMIR3/CD300LF
D17, D18	LMIR6/CD300LE
D19, D20	MDL-1/CLEC5A
D21, D22	NKp30/NCR3
D23, D24	NKp44/NCR2
E1, E2	NKp46/NCR1
E3, E4	NKp80/KLRF1
E5, E6	NTB-A/SLAMF6
E7, E8	PD-1
E9, E10	PECAM/CD31
E11, E12	SHIP-1
E13, E14	SHP-1
E15, E16	SHP-2
E17, E18	Siglec-2/CD22
E19, E20	Siglec-3/CD33
E21, E22	Siglec-5
E23, E24	Siglec-7
F1, F2	Siglec-9
F3, F4	Siglec-10
F5, F6	SIRP-β1
F7, F8	SLAM/CD150
F9, F10	TREM-1
F11, F12	TREM-2
F13, F14	TREML1/TLT-1
G1, G2	Reference Spots
G3, G4	Bt-Control*
G11, G12	PBS

NOTES