

Proteome Profiler™ Array

Human Phospho-Immuneceptor Array Kit

Catalog Number ARY004

For the parallel determination of the relative levels of tyrosine phosphorylation of immuneceptors.

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 simultaneously detect the relative levels of phosphorylation of fifty-nine different immunoreceptors without numerous immunoprecipitations and/or 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 membranes. Capture antibodies will bind both phosphorylated and unphosphorylated immunoreceptors. After unbound material is washed away, a pan anti-phospho-tyrosine antibody conjugated to horseradish peroxidase (HRP) is used for chemiluminescent detection of phosphorylated tyrosines on activated receptors.

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, Catalog # 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. The protocol for this application of the Human Phospho-Immunoreceptor Array can be obtained at www.RnDSystems.com/go/BiotinIRAProtocol.

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-Immunoreceptor 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.
- The use of lysis buffers containing different detergents 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.

MATERIALS PROVIDED

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

Component	Part #	Storage Conditions	Amount Provided
Phospho-Immunoreceptor Array	893156	2 - 8° C	4 membranes
Array Buffer 1	895477	2 - 8° C	1 vial (21 mL)
Array Buffer 2, 5X Concentrate	895478	2 - 8° C	1 vial (21 mL)
Lysis Buffer 15	895567	2 - 8° C	1 vial (21 mL)
Wash Buffer Concentrate, 25X	895003	2 - 8° C	2 vials (21 mL each)
Anti-Phospho-Tyrosine-HRP Detection Antibody	841403	2 - 8° C Do Not Freeze	1 vial (20 µL)
4-Well Rectangular Multi-dish	607544	Room Temperature	1 dish
Transparency Overlay Template	607573	Room Temperature	1 template

OTHER MATERIALS REQUIRED

- Pipettes and pipette tips
- Gloves
- Aprotinin (Sigma # A6279)
- Leupeptin (Sigma # L8511)
- Phosphate-Buffered Saline (PBS)
- Deionized or distilled water
- Rocking platform shaker
- Microcentrifuge
- Plastic transparent sheet protector
- Plastic wrap
- Autoradiography cassette
- Film developer
- X-ray film (Kodak[®] BioMax ML, Catalog # 1788207) or equivalent
- Flat-tipped tweezers
- A plastic container with the capacity to hold 50 mL (for washing the arrays)
- Flatbed scanner with transparency adapter capable of transmission mode
- Computer capable of running image analysis software and Microsoft[®] Excel
- Chemiluminescent detection substrate

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SAMPLE PREPARATION

Cell Lysates - Rinse cells with PBS, making sure to remove any remaining PBS before adding lysis buffer. Solubilize the cells at 1×10^7 cells/mL in Lysis Buffer 15. 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-Immunoreceptor Array, use a quantity of lysate similar to that used for immunoprecipitation or Western blot (50 - 500 µg). Use the lysates immediately or aliquot and store samples at $\leq -70^\circ$ 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-Immunoreceptor Array - Four nitrocellulose membranes each containing 59 different anti-receptor antibodies and 11 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-Tyrosine-HRP - 20 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. Store undiluted anti-phospho-tyrosine-HRP at 2 - 8° C for up to 3 months after initial use.* **DO NOT FREEZE.**

Lysis Buffer 15 - NP-40 Lysis buffer. Immediately before use, add 10 µg/mL Aprotinin and 10 µg/mL Leupeptin. Store unused Lysis Buffer 15 at 2 - 8° C for up to 3 months after initial use.*

Note: *The use of lysis buffers containing different detergents 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.*

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

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. Store unused 5X Array Buffer 2 at 2 - 8° C for up to 3 months after initial use.*

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

*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 to 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. Dilute the lysates to 1.5 mL with Array Buffer 1.
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 (or 2 hours at room temperature) on a rocking platform shaker. Orient the tray so that each array rocks end to end in its well.
10. Carefully remove each array and place into individual plastic containers with a minimum of 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. Add 1.5 mL of the freshly diluted Anti-Phospho-Tyrosine-HRP to each well of the 4-Well Multi-dish.
13. Carefully remove each array from its wash container, return it to the 4-Well Multi-dish, and cover with the lid.
14. Incubate for 2 hours at room temperature on a rocking platform shaker. Orient the tray so that each array rocks end to end in its well.
15. Wash each array as described in steps 10 and 11.
16. Remove each array from the wash container and place it on a plastic sheet protector. Expose each array to chemiluminescent substrate according to the manufacturer's instructions.
17. 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 immunoreceptor capture antibodies is listed in the Appendix on page 10.

Phospho-Immunoreceptor 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 immunoreceptors spot. Use a signal from a clear area of the array or one of the 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 immunoreceptor.
5. Compare corresponding signals on different arrays to determine the relative change in the phosphorylation state of specific immunoreceptors between samples.

PROFILING IMMUNORECEPTOR PHOSPHORYLATION

Figure 1.

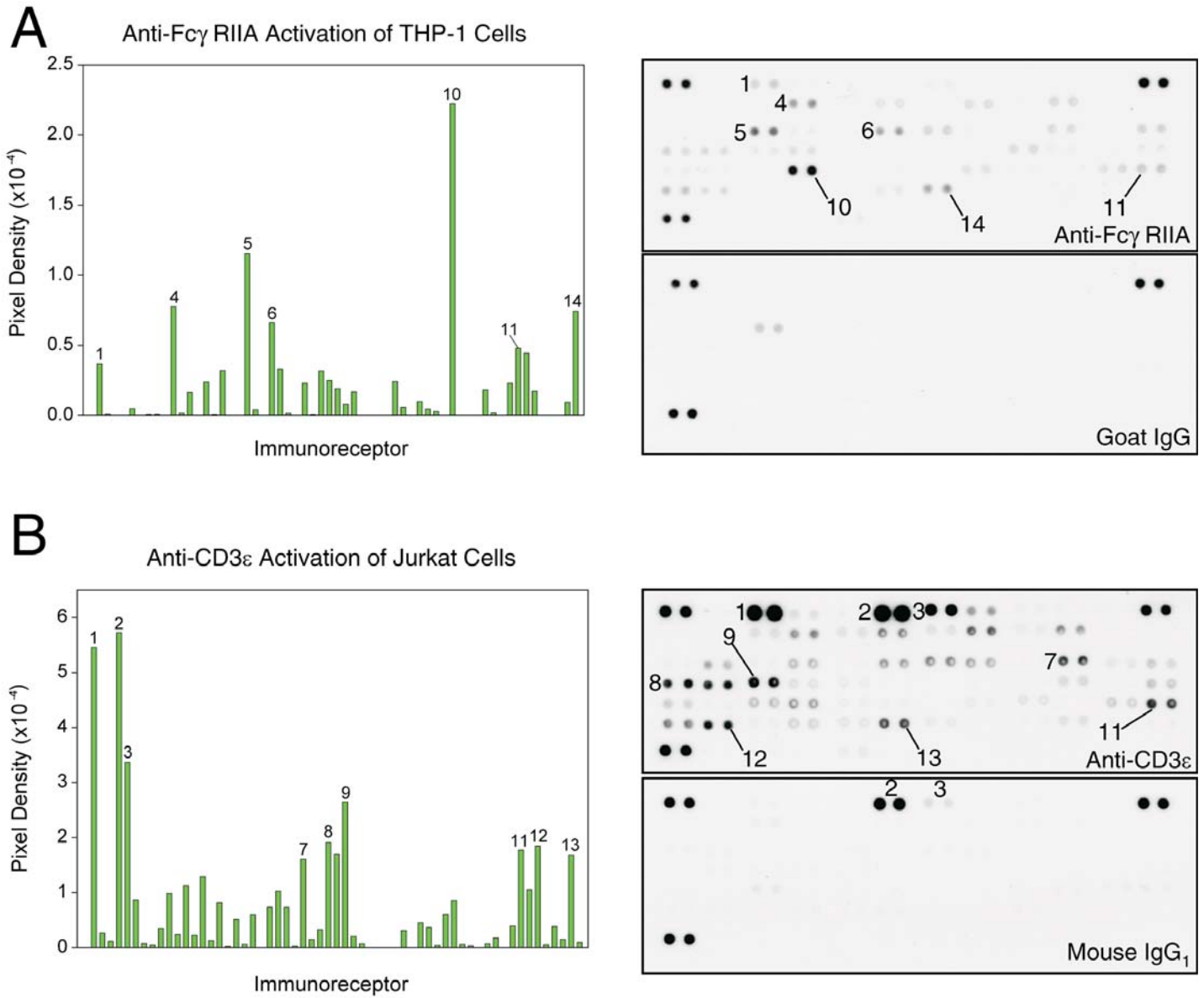


Figure 1: The Human Phospho-Immunoreceptor Array detects multiple tyrosine phosphorylated immunoreceptors in lysates prepared from cells activated by antibody-mediated cross-linking of cell-surface immunoreceptors. (A) THP-1 cells were incubated with goat anti-Fc γ RIIA antibody (R&D Systems, Catalog # AF1875) or normal goat IgG (R&D Systems, Catalog # AB-108-C) followed by incubation with a donkey anti-goat IgG antibody (DAG; R&D Systems, Catalog # AF109) for five minutes [Maresco, D.L. *et al.* (1999) *J. Immunol.* **162**:6458]. (B) Jurkat cells were incubated with a mouse monoclonal anti-CD3 ϵ antibody (R&D Systems, Catalog # MAB100; clone UCHT1) or mouse IgG $_1$ isotype control (R&D Systems, Catalog # MAB002) followed by incubation with a goat anti-mouse antibody (GAM; R&D Systems, Catalog # AF007) for 5 minutes. Arrays were incubated with 100 μ g of lysate. Array signals from scanned X-ray film images of the anti-immunoreceptor activated samples were analyzed using image analysis software. Selected spots in the figure are indicated in the table below. The profiles of IgG controls are similar to those observed in untreated cells (data not shown).

Location	Receptor
1	2B4/SLAMF4
2	CD3 ϵ
3	CD5
4	CEACAM-1
5	Fc γ RIIA
6	FcRH2/IRTA4
7	ILT3/CD85k
8	ILT6/CD85e
9	KIR2DL4
10	PD-1
11	Siglec-7
12	Siglec-10
13	TREM-2
14	TREML1/TLT-1

SPECIFICITY

Figure 2.

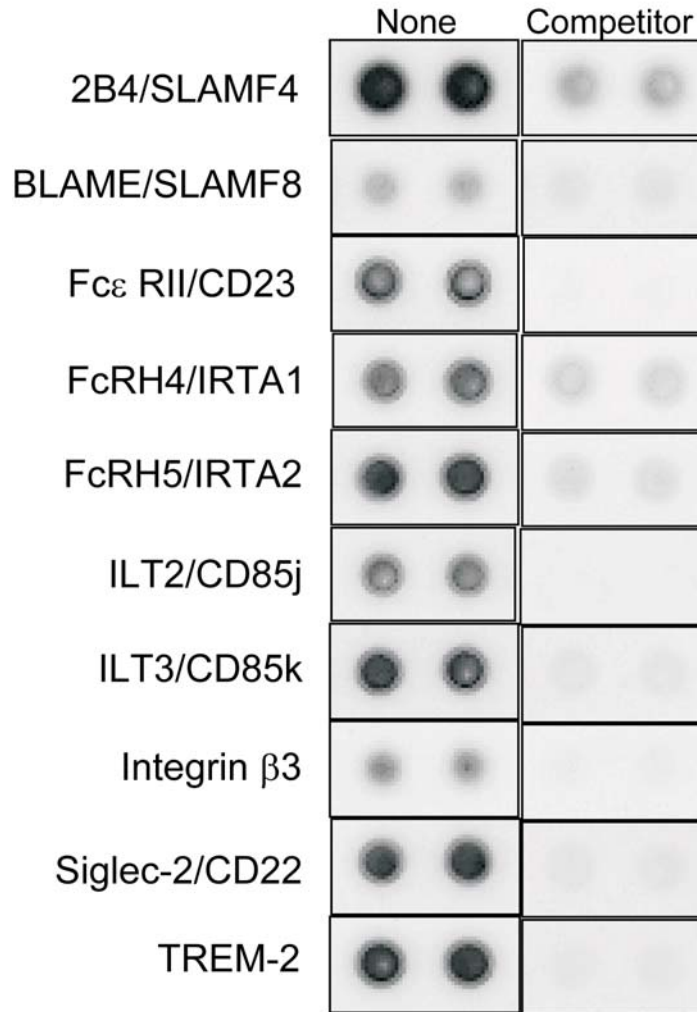


Figure 2: The Human Phospho-Immunoreceptor Array is specific for immunoreceptors as shown by receptor competition. Raji cells were treated with 1 mM Pervanadate for 5 minutes. 10 µg of recombinant human immunoreceptor extracellular domains were added to 75 µg of lysate and analyzed using the Human Phospho-Immunoreceptor Array. Competition of a particular immunoreceptor was observed only with the corresponding recombinant soluble receptor.

APPENDIX

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

Coordinate	Receptor	Coordinate	Receptor	Coordinate	Receptor
A1, A2	PY-Control*	C11, C12	FcRH2/IRTA4	E13, E14	SHP-1
A3, A4	Bt-Control**	C13, C14	FcRH4/IRTA1	E15, E16	SHP-2
A5, A6	2B4/SLAMF4	C15, C16	FcRH5/IRTA2	E17, E18	Siglec-2/CD22
A7, A8	BLAME/SLAMF8	C17, C18	ILT2/CD85j	E19, E20	Siglec-3/CD33
A9, A10	BTLA	C19, C20	ILT3/CD85k	E21, E22	Siglec-5
A11, A12	CD3 ϵ	C21, C22	ILT4/CD85d	E23, E24	Siglec-7
A13, A14	CD5	C23, C24	ILT5/CD85a	F1, F2	Siglec-9
A15, A16	CD6	D1, D2	ILT6/CD85e	F3, F4	Siglec-10
A17, A18	CD28	D3, D4	Integrin β 3/CD61	F5, F6	SIRP- β 1
A19, A20	CD84/SLAMF5	D5, D6	KIR2DL4	F7, F8	SLAM/CD150
A21, A22	Bt-Control	D7, D8	LAIR-1	F9, F10	TREM-1
A23, A24	PY-Control	D9, D10	LAIR-2	F11, F12	TREM-2
B5, B6	CD229/SLAMF3	D11, D12	LMIR1/CD300A	F13, F14	TREML1/TLT-1
B7, B8	CEACAM-1	D13, D14	LMIR2/CD300C	F15, F16	Goat IgG
B9, B10	CLEC-1	D15, D16	LMIR3/CD300LF	F17, F18	Mouse IgG ₁
B11, B12	CLEC-2	D17, D18	LMIR6/CD300LE	F19, F20	Mouse IgG _{2A}
B13, B14	CRACC/SLAMF7	D19, D20	MDL-1/CLEC5A	F21, F22	Mouse IgG _{2B}
B15, B16	CTLA-4/CD152	D21, D22	NKp30/NCR3	F23, F24	Mouse IgG ₃
B17, B18	DCIR/CLEC4A	D23, D24	NKp44/NCR2	G1, G2	PY-Control
B19, B20	Dectin-1/CLEC7A	E1, E2	NKp46/NCR1	G3, G4	Bt-Control
C1, C2	DNAM-1	E3, E4	NKp80/KLRF1	G5, G6	Rat IgG ₁
C3, C4	Fc ϵ RII/CD23	E5, E6	NTB-A/SLAMF6	G7, G8	Rat IgG _{2A}
C5, C6	Fc γ RIIA	E7, E8	PD-1	G9, G10	Rat IgG _{2B}
C7, C8	Fc γ RIIIA/B	E9, E10	PECAM/CD31	G11, G12	PBS
C9, C10	FcRH1/IRTA5	E11, E12	SHIP-1		

*Phospho-Tyrosine Positive Control

**Biotinylated Control

NOTES

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