TranSignal™ Phosphotyrosine Profiling Array

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Product User Manual
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1. ARRAY OVERVIEW

Panomics' TranSignal™ Phosphotyrosine Array includes 38 different human SH2 domains that bind to their targeted tyrosine phosphorylated proteins. The binding is regulated by phosphorylation of tyrosine residues. These arrays are made using the recombinant conserved binding sites of individual SH2 domain proteins fused with glutathione-S-transferase (GST). Proteins are affinity-purified and immobilized onto a membrane. Each SH2 domain on the arrays is spotted in duplicate.

For a complete map and list of the domains included on the array, see Appendix B.

2. INTRODUCTION & BACKGROUND

SH2 Domain Structure & Function

A key to understanding cellular signal transduction is clarifying how proteins interact with one another. Protein-protein interactions are often mediated by non-catalytic, conserved domains. One of these domains is the SH2 domain.

The SH2 domain was first identified in the retroviral oncoprotein v-FPS as a non-catalytic kinase domain important for phosphotyrosine signaling (1). SH2 domains are defined by conserved region of approximately 100 amino acid residues that is required for multi-protein complex formation (2). While SH2 domains do not have kinase activity, these domains regulate the function of catalytic kinase domains and are involved in mediating tyrosine kinase interactions with cellular substrates. Interaction between proteins with SH2 domains and their binding partners is direct, specific, and phosphotyrosine dependent. These interactions facilitate the recruitment of tyrosine kinase-associated proteins and lead to the activation of downstream signaling cascades (3-5).

Proteins that contain SH2 domains are present in a variety of cellular locations and perform many biochemical functions. For example, SH2 domains can be found in enzymes, adaptor proteins, regulatory subunits of signaling proteins, scaffold proteins, transcription factors and oncogenic proteins. These proteins play critical roles by acting as adaptors between receptors and downstream signaling molecules, transmitting signals within a cell and regulating the kinase activity of specific proteins (3-5).

Changes in protein phosphorylation are a major conduit of information for cellular responses, and defects in SH2 domain-dependent signaling are often directly or indirectly shown to be involved in human diseases (5).

Valuable Tool for Studying Signal Transduction Pathways

Signal transductions that regulate cell proliferation and differentiation are often transmitted via activation of tyrosine kinases, and increased activity of tyrosine kinases is often seen in human diseases.

We have developed a method that uses SH2 domain binding to highlight specific "fingerprints" of tyrosine phosphorylation of proteins, in cells. Such patterns are specific to cancer cell types, or the prescence of cell stimuli, such as growth factors. Using this method, classification of primary tumors or tumor-derived cell lines can be undertaken, as well as aiding the identification of clinically relevant parameters for the treatment of disease.

Because of the binding specificity of the SH2 domain to phosphorylated tyrosine residues a specific pattern of tyrosine phosphorylation can be elucidated. This provides a significant advantage over the anti-phosphotyrosine antibody in signal transduction and molecular diagnostic research. The use of SH2 domains for tyrosine phosphorylation profiling focuses on specific pathways and thus avoid binding preference to dominant populations of tyrosine kinases.

Panomics offers SH2 domain arrays that can be used directly with cell lysates, for profiling tyrosine phosphorylations in cells. The proteins bound to the SH2 domains are then detected with biotinylated anti-phosphotyrosine antibodies OR antibody specific to tyrosine kinases, and then visualized with a HRP-based chemiluminescene detection system for highly sensitive, specific and convenient detection.

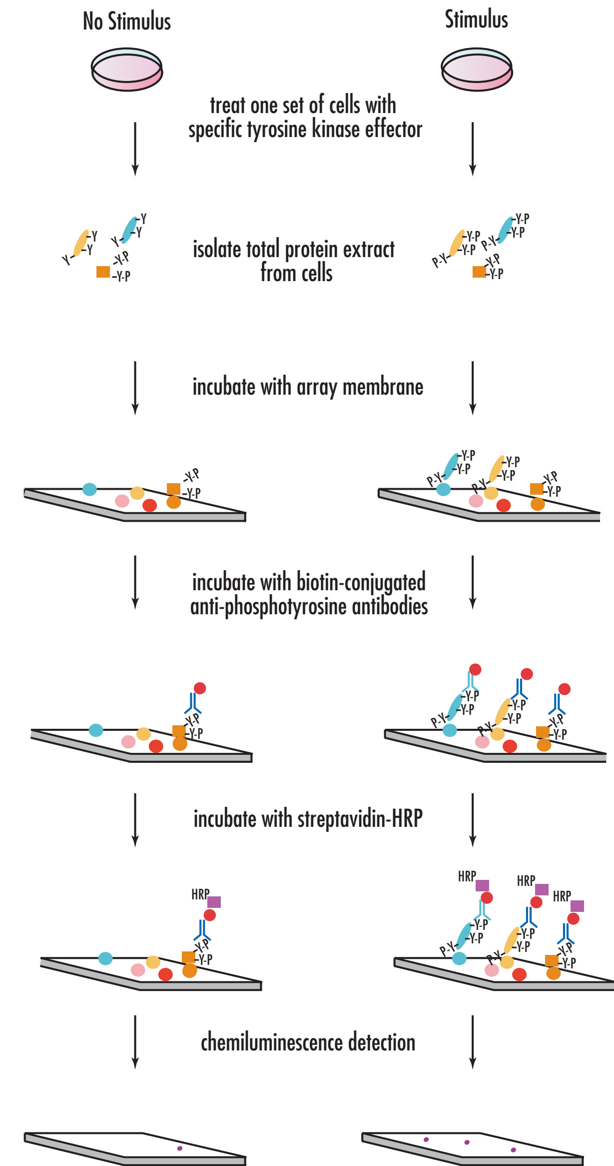

Figure 1: Flow chart of the TranSignal™ Tyrosine Phosphorylation Array assay.

3. MATERIALS PROVIDED

STORAGE CONDITONS: Upon receipt, check each component's label for appropriate storage conditions.

- **TranSignal SH2 Domain Array** (2 each; 3 cm wide x 6 cm long)
- **1X Blocking Buffer** (15 ml)
- **1X Antibody Dilution Buffer** (15 ml)
- **1000X Streptavidin-HRP Conjugate** (15 µl)
- **20X Wash Buffer** (20 ml)-dilute to 1X with dH_20
- **Detection Buffer A** (600 µl)
- **Detection Buffer B** (600 µl)
- **Four-Well Tray**
- **500X Detection Antibody** (Biotin-conjugated anti-phosphotyrosine antibody, 15 µl)
- **2X Cell Lysis Buffer (300 µl)**

Sufficient quantities of each buffer are provided for two assays.

4. ADDITIONAL MATERIALS REQUIRED

- **1X PBS**
- **Microcentrifuge**
- **Orbital shaker**
- **Hyperfilm™ ECL** (Amersham, Cat.# RPN1674K) or equivalent

OR

- **Chemiluminescence imaging system** (e.g., FluorChem™ from Alpha Innotech Corp.)

5. OVERVIEW & GENERAL CONSIDERATIONS

5.1 Handling array membranes:

5.1.1 Always use forceps to handle membranes by the edge only.

5.1.2 Always place array membranes protein-side up. To do this, use the notched corner (top-right when longer side of membrane is horizontal) for orientation.

5.1.3 Never allow array membranes to dry during experiments.

5.1.4 Stripping of the array membranes is not recommended.

5.2 Incubation:

5.2.1 To avoid drying, completely cover membranes with sample or buffer during incubation steps.

5.2.2 Perform all incubation and wash steps under gentle agitation.

5.2.3 Avoid causing buffer foaming during incubation steps.

6. PREPARATION OF CELL LYSATE

For adherent cells, a single 10 cm plate of cells at ~ 90% confluence are required per membrane. For suspension cells, each membrane will require 10^6 - 10^7 cells. This total yield for either cell type should be between 1-5 mg of protein.

6.1 After appropriate cell treatments, wash adherent cells with ice-cold PBS, and drain. Wash suspension cells with ice-cold PBS and centrifuge at 2000 rpm for 5 min, to pellet the cells.

6.2 **For adherent cells:** Add 100 µl of ice-cold 2x Cell Lysis Buffer to the washed cells on each 10 cm plate. If you need to use more than one plate of a smaller diameter, be sure to divide the 100 µl of 2x Cell Lysis Buffer among the num ber of plates. Using more than 100 µl may result in an elevated background intensity during later chemiluminescent detection. Scrape cells off tissue culture dish with a plastic cell scraper and transfer the cell suspension to 1.5 mL microcentrifuge tubes. Maintain all components on ice.

For suspension cells: Resuspend the cell pellet in 250 µl of ice-cold 2x Cell Lysis Buffer, and transfer resuspended cells to a 1.5 mL microcentrifuge tube.

6.3 Using an ice and water mixture to keep the tubes cool, lyse the cells each tube by sonicating for 2-3 seconds and placing the tube in the ice for 10-15. Repeat this sonication and cooling process for 2 more times.

6.4 Centrifuge tubes at 10,000 RPM for 5 min at 4°C, and transfer supernatant (your cell lysate) to clean, 1.5 mL microcentrifuge tubes.

6.5 Determine protein concentration of cell lysates. The lysate can be used immediately or stored at -80°C for further use.

7. INCUBATION

In this Section, you will incubate cell lysate with the array membrane. For all steps requiring incubation, please make sure that the membrane is fully submerged in the buffer. Note that the array membranes have a notch at the top right-hand corner for orientation purposes.

Note: *Be sure that the membrane is fully submerged in assay buffer at all times. Never let the membrane dry out.*

7.1 Place each membrane into the provided four-well tray containing 5 ml of 1X Wash Buffer for 30 min. If necessary, trim the membrane to fit the well (Do not cut the protein spots).

7.2 Discard the Wash Buffer and add 5 ml of 1X Blocking Buffer. **Make sure that the membrane is fully submerged in buffer.**

7.3 Place the tray on a shaker and incubate at room temperature for approximately 1 to 2 hours, or until each membrane appears uniformly wetted and no dry spots are visible.

7.4 Dilute the cell lysates, prepared in section 6, in 3.0 ml with 1X PBS, per membrane used.

7.5 Remove 1X Blocking Buffer from the membranes, and rinse the membranes once in 1X Wash Buffer.

7.6 Add the diluted cell lysates to the membranes, place the lid on the 4-well tray and incubate overnight with gentle shaking at 4°C.

7.7 After incubation, wash the membrane three times with 5 ml of 1X Wash Buffer for 10 min (each wash) at room temperature. Remove Wash Buffer.

7.8 For each membrane, dilute 6 µl of the Detection Antibody in 3 ml of

Antibody Dilution Buffer and add this mixture to one well on the tray.

7.9. Add each membrane to separate wells containing the diluted Detection Antibody, place the tray on a shaker, and incubate for 2 hr at room temperature.

7.10 After incubation, wash the membrane three times with 5 ml of 1X Wash Buffer for 10 min (each wash) at room temperature. Decant the wash and proceed to the next incubation step.

7.11 Add 4 µl Streptavidin-HRP to 4 ml 1X Wash Buffer, per membrane used.

7.12 Transfer 4 ml of the diluted Streptavidin- HRP to each well of the 4-well tray, place the tray on a shaker and incubate for 1 hr at room temperature.

7.13 After incubation, wash the membrane three times with 5 ml of 1X Wash Buffer for 10 min (each wash) at room temperature.

8. DETECTION

Important note: Do not let the membrane dry out during detection.

8.1 Prepare the detection solution immediately before use by mixing equal amounts of Detection Buffers A and B—e.g., 300 µl of Detection Buffer A and 300 µl of Detection Buffer B, per 1 membrane.

8.2 Using forceps to hold the cut corner, carefully remove each membrane from its tray. Drain the excess Wash Buffer from the membrane by touching the edge against tissue. Place protein- side up by orienting the notch to the top, right-hand corner on a clean plastic sheet.

8.3 Pipet the mixed Detection Buffers onto the membrane. Ensure that the buffer mixture is evenly distributed over the membrane without air bubbles.

8.4 Incubate for 5 min at room temperature.

8.5 Remove excess substrate by holding the membranes with forceps and touching the edge against tissue. Place the membrane between two plastic sheets and gently press on the top sheet to remove air bubbles.

8.6 Expose the membranes using either Hyperfilm™ ECL or a chemiluminescence imaging system, such as the FluorChem™ imager from Alpha Innotech Corp. In either case, we recommend that you

try several different exposures of varying lengths of time (e.g., 30 sec–5 min).

9. TROUBLESHOOTING GUIDE

Problem	Cause	Recommendation
Weak or no signal	Ligand or protein is not binding to the SH2 domains.	Ensure that the ligand or protein is tyrosine-phosphorylated.
High background	Antibody concentration is too high.	Further dilute the antibody.
	Nonspecific interactions with antibodies or other reagents used in the assay.	Check signal using a zero standard (i.e. PVDF membrane alone). High background is usually the result of insufficient blocking. Try longer incubation with the blocking buffer.
		Dilute the Detection Buffer.
Uneven background	Membrane dried out during incubation.	Keep the membrane fully submerged in solution during all incubation steps.
	Volume of blocking solution, sample, or antibody is too low.	Increase the volume to make sure that the membrane is fully submerged during incubation.
	Membrane is not evenly covered by detection buffer.	Increase the volume of detection buffer to make sure that the membrane surface is fully covered.
	Air bubbles on membrane surface during detection.	Remove air bubbles from membrane surface.

10. REFERENCES

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2. Koch, C.A. *et al.* (1991) *Science* 252: 668-674.

3. Pawson, T. *et al.* (2001) *Trends in Cell Biology* 11: 504-511.

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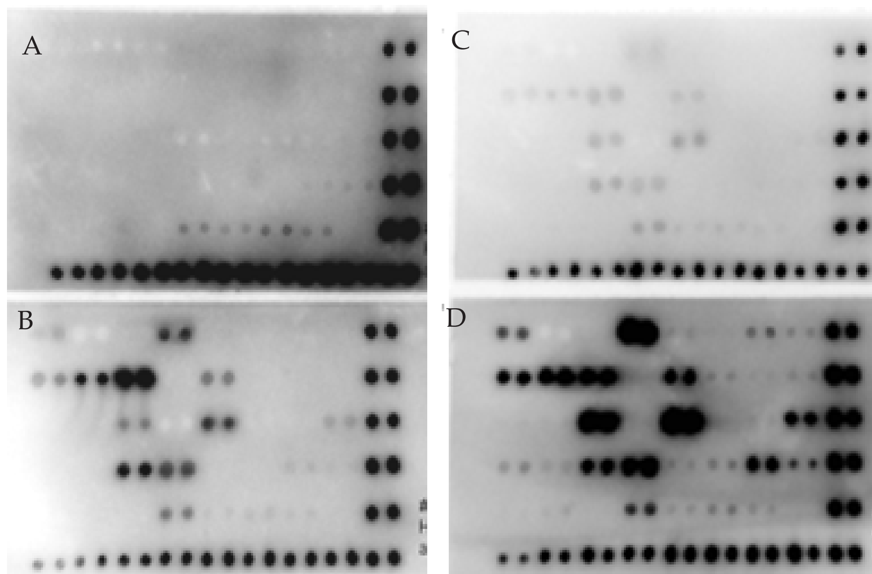
5. Yaffe, M.B. (2002) *Nature Reviews Molecular Cell Biology* 3: 177-186.

APPENDIX A: Typical Results for Phosphotyrosine Profiling Array



Typical results obtained with the TranSignal Phosphotrosine Profiling Array . *Cell lysate from untreated Hela (A), A431 (C) and from EGF treated Hela (B) and EGF treated A431 (D) was incubated with the array. The Tyrosine phosphorylated proteins were detected using a biotinylated anti-phosphotyrosine antibody and Streptavidin-HRP, as detailed in the protocol. The images were acquired using FluorChem™ imager (from Alpha Innotech). Spots with stronger intensities indicate higher binding affinity of the ligand of interest to SH2 Domain(s).*

APPENDIX B: Schematic diagram of the Phosphotyrosine Profiling Array

	1 2	3 4	5 6	7 8	9 10	11 12	13 14	15 16	17 18
A	Abl1	BRDG1	BTK	CRKL	CSK	EAT2	FES	FGR	Pos
B	Fyn	GRAP	GRB2	GRB14	HCK	LCK	Lyn	MATK	Pos
C	NCK1	NCK2	P85A-D1	P85A-D2	P85B-D1	P85B-D2	PLCg1-D1	PLCg1-D2	Pos
D	PTPN11-D1	PTPN11-D2	RASGAP1-D1	RASGAP1-D2	RaLP	SHC1	SHC2	SHC3	Pos
E	Src	STAP2	TNS	YES	ZAP70-D1	ZAP70-D2	GST		Pos
F	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos

Schematic diagram of the TranSignal Phosphotyrosine Profiling Array. *The SH2 domain proteins on the array are spotted in duplicate at 100 ng. Positive marker has been spotted along the bottom (row F) and in duplicate along the right side (column 17, 18) of the membrane. These spots are intended for alignment. Note that the notch is at the top right-hand corner.*

Protein name	Array Position	Accession No.	FULL NAME
ABL1	A1, A2	NP_955595	v-abl Abelson murine leukemia viral oncogene homolog 1
BRDG1	A3, A4	NP_036240.	Docking protein BRDG1 (BCR downstream signaling 1)
BTK	A5, A6	NP_000052.	Bruton's tyrosine kinase
CRKL	A7, A8	NP_005198.	CrRK (V-crk avian sarcoma virus CT10)-like protein
CSK	A9, A10	NP_004374.	Tyrosine-protein kinase CSK (C-SRC kinase) (CYL)
EAT2	A11, A12	NP_444512	SH2 domain-containing molecule EAT2
FES	A13, A14	NP_001996.	Proto-oncogene tyrosine-protein kinase FES/FPS (C-FES)
FGR	A15, A16	NP_005239	Gardner-Rasheed feline sarcoma viral (v-fgr) oncogene homolog
FYN	B1, B2	NP_002028	FYN oncogene related to SRC, FGR, YES
GRAP	B3, B3	NP_006604	GRB2-related adaptor protein
GRB2	B5, B5	NP_002077.	Growth factor receptor-bound protein 2 (ASH protein)
GRB14	B7, B8	NP_004481.	Growth factor receptor-bound protein 14 (GRB14 adapter protein).
HCK	B9, B10	NP_002101.	Hemopoietic cell kinase isoform p61HCK
LCK	B11, B12	NP_005347	lymphocyte-specific protein tyrosine kinase
LYN	B13, B14	NP_002341	v-yes-1 Yamaguchi sarcoma viral related oncogene homolog
MATK	B15, B16	NP_647611.	Megakaryocyte-associated tyrosine-protein kinase
NCK1	C1, C2	NP_006144	NCK adaptor protein 1
NCK2	C3, C4	NP_003572.	NCK adaptor protein 2
P85A-D1	C5, C6	NP_852664	phosphoinositide-3-kinase, regulatory subunit, polypeptide 1 (p85 alpha), SH2 domain #1
P85A-D2	C7, C8	NP_852664	phosphoinositide-3-kinase, regulatory subunit, polypeptide 1 (p85 alpha), SH2 domain #2
P85B-D1	C9, C10	NP_005018	phosphoinositide-3-kinase, regulatory subunit, polypeptide 2 (p85 beta), SH2 domain #1
P85B-D2	C11, C12	NP_005018	phosphoinositide-3-kinase, regulatory subunit, polypeptide 2 (p85 beta), SH2 domain #2
PLCG1-D1	C13, C14	NP_002651.	1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase gamma 1 (PLC-gamma-1), SH2 domain #1
PLCG1-D2	C15, C16	NP_002651.	1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase gamma 1 (PLC-gamma-1), SH2 domain #2
PTPN11-D1	D1, D2	NP_035332	protein tyrosine phosphatase, non-receptor type 11, SH2 domain #1
PTPN11-D2	D3, D4	NP_035332	protein tyrosine phosphatase, non-receptor type 11, SH2 domain #2
RaLP	D5, D6	NP_976224.	Rai-like protein RaLP.
RASGAP1-D1	D7, D8	NP_002881	RAS p21 protein activator (GTPase activating protein) 1, SH2 domain #1
RASGAP1-D2	D9, D10	NP_002881	RAS p21 protein activator (GTPase activating protein) 1, SH2 domain #2
SHC1	D11, D12	NP_003020	SHC (Src homology 2 domain containing) transforming protein 1
SHC2	D13, D14	XP_375550	SHC transforming protein 2 (SH2 domain protein C2) (Protein Sck).
SHC3	D15, D16	NP_058544.	SHC transforming protein 3 (SH2 domain protein C3) (Neuronal Shc) (N-Shc) (Protein Rai).
SRC	E1, E2	NP_005408	v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)
STAP2	E3, E4	NP_060190.	signal-transducing adaptor protein-2
TNS	E5, E6	NP_072174.	Tensin
YES	E7, E8	NP_005424.	v-yes-1 Yamaguchi sarcoma viral oncogene homolog 1
ZAP70-D1	E9, E10	NP_001070	zeta-chain (TCR) associated protein kinase 70kDa, SH2 domain #1
ZAP70-D2	E11, E12	NP_001070	zeta-chain (TCR) associated protein kinase 70kDa, SH2 domain #2
GST	E13, E14	AAA57098	Glutathione S-transferase