

TransBinding Phosphotyrosine/PI3K Assay Kit

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Product User Manual
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(TransBinding NFkB Kit UM052505)

1. INTRODUCTION

Tyrosine phosphorylation responds to a myriad of signals such as growth factors and hormones from their microenvironment. The phosphorylation is mediated by protein tyrosine kinases (PTKs), many of which are receptor tyrosine kinases (RTKs) that are expressed on the cell surface. RTKs consist of an extracellular ligand binding domain, a single transmembrane domain, and an intracellular kinase domain. Ligand-induced activation of RTKs leads to autophosphorylation at tyrosine residues, which creates binding sites to recruit Src homology (SH2) domain-containing proteins and to activate different signaling pathways. One such important molecule is PI3K. Its SH2 domains bind to active RTK and initiates the activation of its downstream molecule Akt. PI3K/Akt pathway plays a number of important functions, including cell survival, cell cycle, and transcription activation. RTKs, through their involvement of tyrosine kinase activity play an important role in human cancer development.

Panomics TransBinding™ Phosphotyrosine/PI3K Assay Kit will enable the researcher to detect the activation of RTKs and the binding to PI3K. The ELISA-based kits combine a fast, user-friendly format with a sensitive and specific quantitative assay. A SH2 domain of PI3K has been immobilized on the 96-well plate. Activated RTKs will bind to this protein domain. The bound RTKs or other phosphotyrosine proteins are detected by biotinylated antibody against phosphotyrosine residues. HRP-conjugated streptavidin provides sensitive colorimetric readout easily quantified by spectrophotometry. The whole assay will complete in less than 3.5 hours. The 96-well plate with individual strips of 8 wells is suitable for manual use or high-throughput screening applications. TransBinding™ Phosphotyrosine/PI3K Assay Kit has many applications including the study of drug potency, inhibitor or activator proteins, and/or protein structure/function in the signaling pathway.

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2. SCHEMATIC DIAGRAM

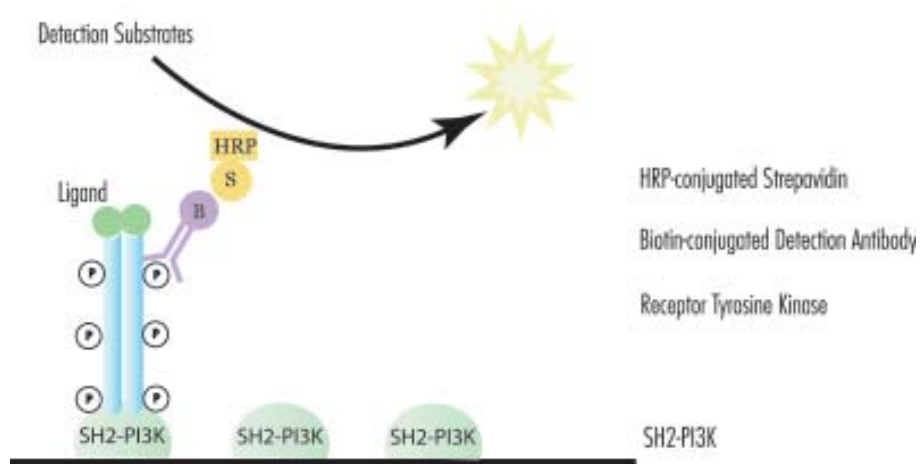


Figure 1: Panomics TransBinding Kits are ideal for detection of the activation of receptor tyrosine kinase (RTKs) and the binding to phosphoinositide 3-kinase (PI3K). The kits includes a 96-well plate that has been coated with the SH2 domain of PI3K (SH2-PI3K). The activated RTKs from total cell extracts specifically bind to the SH2-PI3K. By using an antibody that is directed against phosphotyrosine residue or against RTK, the complex bound to the SH2-PI3K is detected. Addition of a streptavidin conjugated to horseradish peroxidase (HRP) provides sensitive colorimetric readout that is easily quantified by spectrophotometry.

2. MATERIALS PROVIDED

See product labels for appropriate storage conditions.

Box: (Store at 4°C except Detection Antibody)

- 1000X Detection antibody (10 µl) (store at -20°C upon arrival)
(biotin-conjugated anti-phosphotyrosine antibody)
- 200X Streptavidin-HRP conjugate (60 µl)
- 1X Dilution Buffer (30 ml)
- 10X Wash Buffer (60 ml)
- Substrate Solution (10 ml)
- Stop Solution (10 ml)
- Plate sealer (1 each)

Sealed Bag: (Store at 4°C)

- SH2-PI3K coated 96-well assay plate

3. ADDITIONAL MATERIALS REQUIRED

- Microplate spectrophotometer capable of reading at 450 nm (655 nm as optional reference wavelength)
- Multi-channel pipettor reservoirs
- Rocking platform shaker
- Centrifuge
- Sonicator
- 1X Cell Lysis Buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X 100, 2.5 mM Sodium pyrophosphate, 1 mM β Glycerophosphate, 1 mM Na_3VO_4 , 1 $\mu\text{g/ml}$ Leupeptin)
- 1X Protease inhibitor cocktail (Sigma)
- PMSF

4. PREPARING BUFFERS AND SAMPLES

4.1 Preparation of Whole Cell Lysate

A single 10 mm³ plate of cells at 90% confluence are required per assay. This should yield between 1-5 mg of protein. The final concentration of the cell lysate should be 0.5 - 4 mg/ml.

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

4.1.2 Add ice-cold 1X Cell Lysis Buffer (see Section 3) to cells (250 µl per 10⁷ cells).

Note *The Protease inhibitor cocktail (1X Final concentration) and PMSF (1 mM final concentration) should be added to the 1X Cell Lysis Buffer immediately prior to use.* Scrape adherent cells off tissue culture dish with a plastic cell scraper, transfer cell suspension to a 1.5 ml microfuge tube. Maintain all components on ice.

4.1.3 Sonicate cell suspension for 5-10 sec to shear DNA and reduce sample viscosity.

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

4.1.5 Dilute 5-10 µl of whole cell lysate (approx. 5-20 µg) with 1X Dilution Buffer to 50 µl and add the diluted sample to one well

4.2 Preparation of 1X Wash Buffer

Prepare the amount of 1X Wash Buffer required for the assay as follows: For every 100 ml of 1X Wash Buffer required, dilute 10 ml of 10X Wash Buffer with 90 ml distilled water. Mix gently to avoid foaming. The 1X Wash Buffer may be stored at 4°C for one week. The Tween 20 contained in 10X Wash Buffer may form clumps, so resuspend the buffer by vortexing for 2 minutes prior to use.

4.3 Preparation of 1X Detection Antibody

Prepare the amount of 1X Detection Antibody required for the assay by diluting the supplied stock solution with 1X Dilution Buffer.

4.4 Preparation of 1X HRP-conjugated Streptavidin

Prepare the amount of 1X HRP-conjugated Streptavidin required for the assay by diluting the supplied stock solution with 1X Dilution Buffer.

5. ELISA PROTOCOL

- 5.1. Prepare the total cell lysates and 1X Wash Buffer as described above in the section 4 PREPARING BUFFERS AND SAMPLES. Multi-channel pipettor reservoirs may be used for dispensing the buffers and samples.
- 5.2. Determine the appropriate number of microwell strips required for testing samples, controls and blanks. If less than 8 wells in a strip need to be used, cover the unused wells with a portion of the plate sealer while you perform the assay. The content of these wells is stable at room temperature if kept dry and, therefore, can be used later for a separate assay. Store the unused strips in the pouch at 4°C. Use the strip holder for the assay.
- 5.3. Rinse each well once with 200 µl of 1X Wash Buffer. Tap the inverted plate 3 times on absorbent paper towels.
- 5.4. We recommend using 5-20 µg of whole cell extract per well. Dilute the sample with 1X dilution buffer to 50 µl.
- 5.5. Sample wells: Add 50 µl of diluted samples per well
Blank wells: Add 50 µl of dilution buffer per well.
- 5.6. Use the provided plate sealer to cover the plate. Incubate for 1 hour at room temperature with mild agitation (100 rpm on a rocking platform).
- 5.7. Wash each well 3 times with 200 µl of 1X Wash Buffer. Tap the inverted plate 3 times on absorbent paper towels.
- 5.8. Add 100 µl of diluted Detection Antibody (1:1000 dilution in 1X Antibody Dilution Buffer) to wells.
- 5.9. Cover the plate and incubate for 1 hour at room temperature with mild agitation (100 rpm on a rocking platform).
- 5.10. Wash each well 3 times with 200 µl of 1X Wash Buffer. Tap the inverted plate 3 times on absorbent paper towels.

- 5.11 Add 100 μ l of diluted Streptavidin-HRP Conjugate (1:200 dilution in 1X Antibody Dilution Buffer) to all wells being used.
- 5.12 Cover the plate and incubate for 1 hour at room temperature with mild agitation (100 rpm on a rocking platform).
- 5.13 During this incubation, place the developing substrate solution to room temperature.
- 5.14 Wash each well 3 times with 200 μ l of 1X Wash Buffer. Tap the inverted plate 3 times on absorbent paper towels.
- 5.15 Add 100 μ l of room-temperature Substrate Solution to all the wells being used.
- 5.16 Incubate 5-15 minutes at room temperature protected from direct light. Monitor the blue color development in the sample and positive control wells until it turns medium to dark blue. Do not overdevelop.
- 5.17 Add 100 μ l of Stop Solution. The blue solution will turn yellow once the reaction has been stopped.
- 5.18 Read absorbance on a spectrophotometer within 5 minutes at 450 nm with a reference wavelength of 655 nm. Blank the plate reader according to the manufacturer's instructions using the blank wells. If necessary, the samples can be diluted with distilled water before measurement.
- 5.19 Typical results are shown in APPENDIX section.

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TROUBLESHOOTING GUIDE

No or weak signal

- Check all reagents was added in the proper order
- Sodium azide inhibits the HRP reaction, use provided buffers
- Check plate reader settings
- Substrate solution should be at room temperature before detection
- Not enough cellular extract per well

High background in all wells

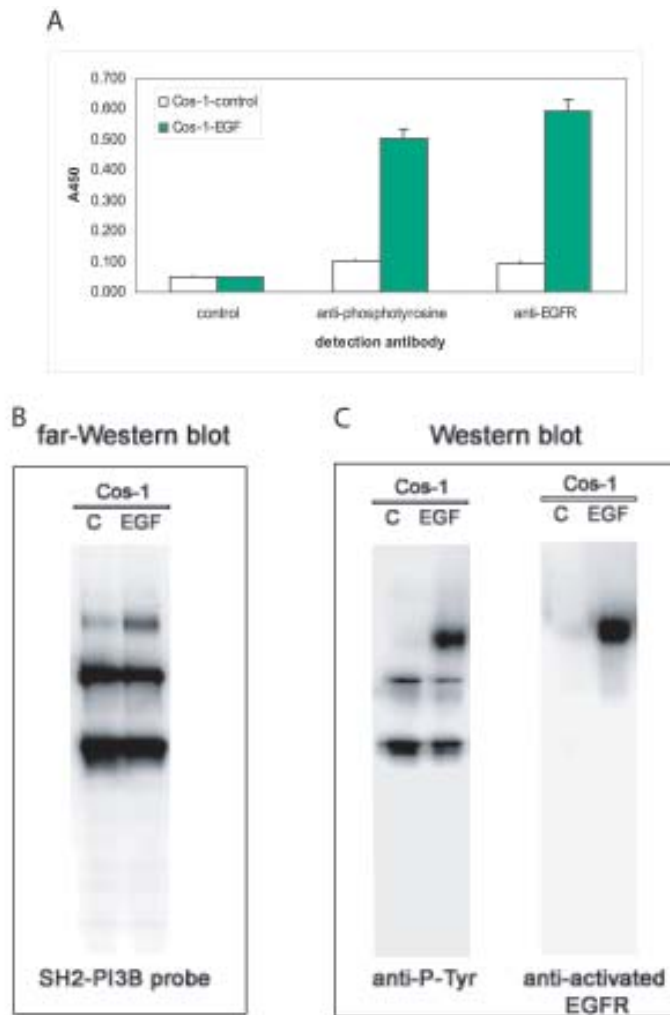
- Developing time was too long
- Incorrect antibody dilutions
- Make sure all wells are filled with Wash Buffer in washing step
- Avoid cross-contamination between wells
- Too much cellular extract per well

10. REFERENCES

1. Schlessinger, J. and Lemmon, M.A. (2003) *Science's STKE* 1991.
2. Koch, C.A. *et al.* (1991) *Science* 252: 668-674.
3. Pawson, T. *et al.* (2001) *Trends in Cell Biology* 11: 504-511.
4. Yaffe, M.B. (2002) *Nature Reviews Molecular Cell Biology* 3: 177-186.

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APPENDIX:



Detection of receptor tyrosine kinase activation in ELISA-based assay format using TransBinding™ Phosphotyrosine/PI3K Assay Kit and TransBinding™ EGFR/PI3K Assay Kit. A. The increased levels of tyrosine phosphorylated proteins and tyrosine phosphorylated EGFR are captured by SH2 domain of PI3K and detected with specific antibodies. The total cell lysates from control- and EGF treated Cos-1 was used at 5 µg per well. B. SH2 domain of PI3B binds to tyrosine phosphorylated proteins in far-Western assay. C. EGF promotes protein tyrosine phosphorylation of Cos-1 in Western blot assay.

