TECHNICAL DATA SHEET

THUNDER™ Phospho-AKT pan (S473) TR-FRET Cell Signaling Assay Kit

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BETTER TOOLS. REAL DISCOVERIES.

CATALOG NUMBERS

KIT-AKTS473P-100 (100 tests)
KIT-AKTS473P-500 (500 tests)
KIT-AKTS473P-2500 (2500 tests)
KIT-AKTS473P-5000 (5000 tests)
KIT-AKTS473P-10000 (10000 tests)

Store at -80°C
For research use only.
Not for use in diagnostic procedures.

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PRODUCT DESCRIPTION

This assay kit measures intracellular levels of **phospho-AKT pan (S473)** protein in cell lysates using a simple, rapid and sensitive immunoassay based on the homogeneous (no-wash) THUNDER™ TR-FRET technology. The kit is compatible with both adherent and suspension cells.

SPECIFICITY

This assay kit contains two specific and selective antibodies, one that recognizes **AKT pan** phosphorylated at **Ser473** and another that recognizes an invariant epitope of **AKT pan**.

SPECIES REACTIVITY

Human; Mouse (Swiss-Prot Acc. P31749, P31751, Q9Y243; Entrez Gene Id 207, 208 and 10000).

Other species should be tested on a case-by-case basis.

TR-FRET ASSAY PRINCIPLE

The **Phospho-AKT pan (S473)** assay kit is a homogeneous time-resolved Förster resonance energy transfer (TR-FRET) sandwich immunoassay (Figure 1). The THUNDER™ Cell Signaling assay workflow consists of 3 steps (Figure 2). Following cell treatment, cells are first lysed with the specific Lysis Buffer provided in the kit. Then **Phospho-AKT pan (S473)** in the cell lysates is detected with a pair of fluorophore-labeled antibodies in a simple "add-incubate-measure" format (single-step reagent addition; no wash steps). One antibody is labeled with a donor fluorophore (Europium chelate; Eu-Ab1) and the second with a far-red acceptor fluorophore (FR-Ab2). The binding of the two labeled antibodies to distinct epitopes on the target protein takes place in solution and brings the two dyes into close proximity. Excitation of the donor Europium chelate molecules with a flash lamp (320 or 340 nm) or a laser (337 nm) triggers a FRET from the donor to the acceptor molecules, which in turn emit a TR-FRET signal at 665 nm. Residual energy from the Eu chelate generates light at 615 nm. The signal at 665 nm is proportional to the concentration of **Phospho-AKT pan (S473)** in the cell lysate. Data can be expressed as either the signal at 665 nm or the 665 nm/615 nm ratio.

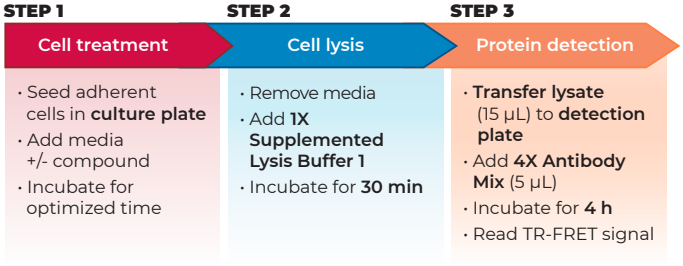

Figure 2 Assay workflow using the 2-plate (transfer) protocol.

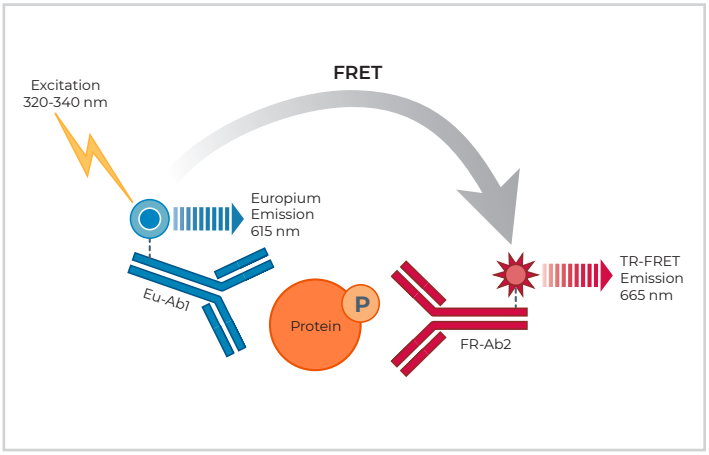

Figure 1 Schematic representation of the TR-FRET cell signaling assay principle.

KIT COMPONENTS

	100 points*	500 points*
Eu-labeled Phospho-AKT pan (S473) antibody (Eu-Ab1)	5 µL	25 µL
Acceptor-labeled Phospho-AKT pan (S473) antibody (FR-Ab2)	20 µL	100 µL
Lysis Buffer 1 (5X)	1 mL	5 mL
Detection Buffer (10X)	50 µL	250 µL
Positive control cell lysate	100 µL	500 µL
Phosphatase Inhibitor Cocktail (100X)	50 µL	250 µL

* The number of assay points is based on an assay volume of 20 µL in half-area 96-well or low-volume 384-well assay plates using the kit components at the recommended concentrations (refer to the User Manual).

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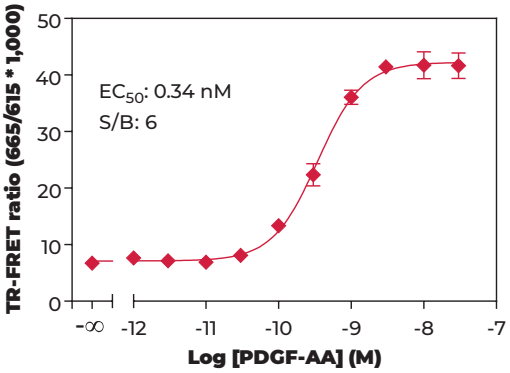
Phospho-AKT pan (S473)

VALIDATION DATA

This assay kit has been validated for the relative quantification of phospho-AKT pan (S473) in NIH3T3, MCF7, Jurkat and HEK293 cell lysates using the 2-plate assay protocol.

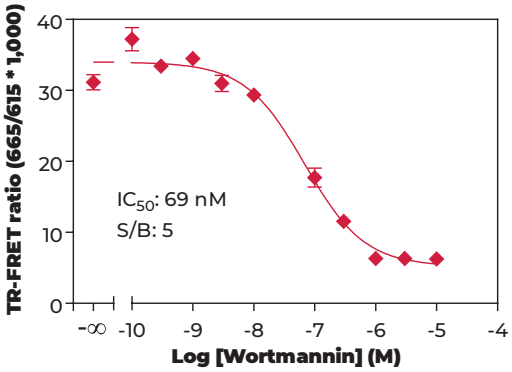
- Adherent cells were cultured 48 hours in a 96-well tissue culture plate, (DMEM + 10% CBS).
- Following cell treatment, the media was removed and cells were lysed with the 1X **Lysis Buffer 1** (50 µL) supplemented with the 100X Phosphatase Inhibitor Cocktail diluted at 1X.
- Following a **30-min** incubation at room temperature (RT) on an orbital shaker (400 rpm), lysates (15 µL) were then transferred to a 384-well assay plate followed by addition of the labeled antibodies Eu-Ab1 and FR-Ab2 (5 µL) for detection of phospho-AKT pan (S473)
- The plate was incubated at RT for **4 hours** and the TR-FRET signal was recorded at 665 and 615 nm (EnVision®; lamp excitation).

STIMULATION OF PHOSPHO-AKT PAN (S473) IN NIH3T3 CELLS



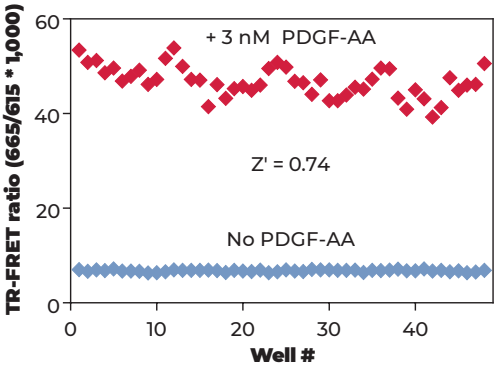
NIH3T3 cells (30,000 cells/well, in triplicate) were incubated with serial dilutions of PDGF-AA for 15 min at RT. Data show that treatment of NIH3T3 cells with PDGF-AA stimulates phosphorylation of AKT pan at S473.

INHIBITION OF PHOSPHO-AKT PAN (S473) IN NIH3T3 CELLS



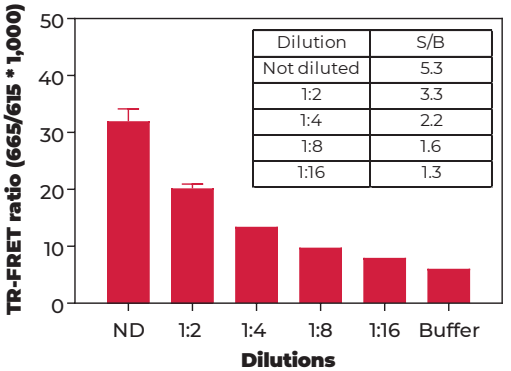
NIH3T3 cells (30,000 cells/well, in triplicate) were incubated with serial dilutions of Wortmannin for 30 min at RT. Cells were then stimulated with 1 nM PDGF-AA for 15 min at RT. Data show that treatment of NIH3T3 cells with Wortmannin inhibits phosphorylation of AKT pan at S473 by PDF-AA.

Z'-FACTOR DETERMINATION IN NIH3T3 CELLS



NIH3T3 cells (30,000 cells/well) were incubated without or with 3 nM of PDGF-AA for 15 min at RT. The Z' factor value was determined using a total of 48 wells for each treatment group. The Z'-factor value of 0.74 indicates that the assay is robust and suitable for HTS.

NIH3T3 CONTROL LYSATE TITRATION (QC TEST)



Dilution	S/B
Not diluted	5.3
1:2	3.3
1:4	2.2
1:8	1.6
1:16	1.3

Quality Control: the Phospho-AKT pan (S473) assay kit is routinely tested against PDGF-AA treated NIH3T3 lysates. NIH3T3 cells were cultured in a T175 flask to 95% confluence and stimulated with 3 nM of PDGF-AA for 15 min at RT. Following cell lysis using 4 mL of 1X Lysis Buffer 1, lysates were serially diluted with 1X Lysis Buffer 1 and tested in triplicate. Data show a linear relationship between lysate dilutions and TR FRET ratio values.

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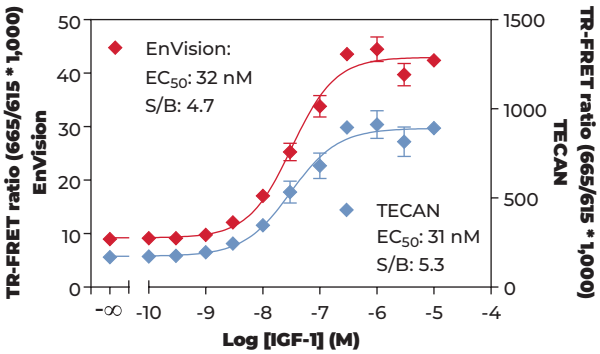
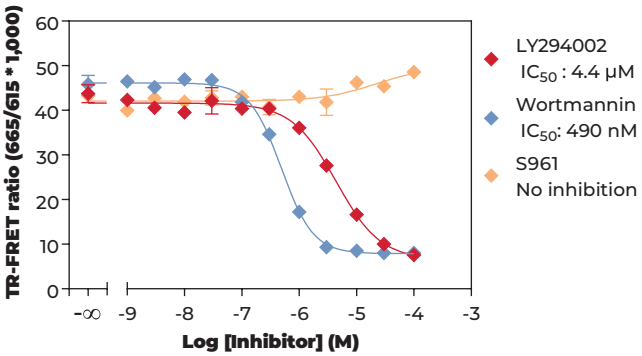
Phospho-AKT pan (S473)

STIMULATION OF PHOSPHO-AKT PAN (S473) IN MCF7 CELLS



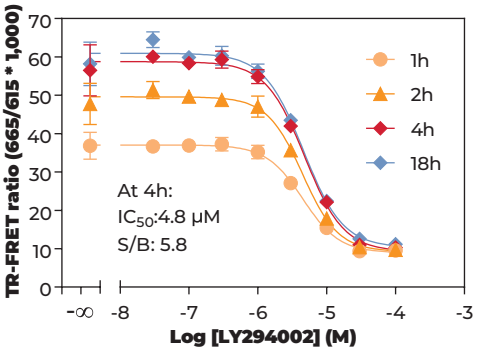
MCF7 cells (60,000 cells/well, in triplicate) were incubated with serial dilutions of IGF-1 for 10 min at RT. Data show that treatment of MCF7 cells with IGF-1 stimulates phosphorylation of AKT pan at S473.

INHIBITION OF PHOSPHO-AKT PAN (S473) IN MCF7 CELLS



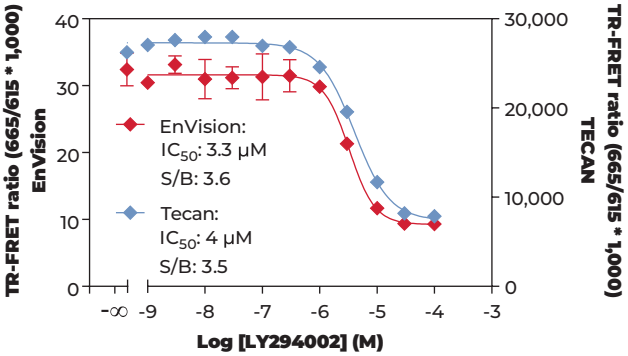
MCF7 cells (60,000 cells/well, in triplicate) were incubated with serial dilutions of either LY294002, Wortmannin or S961 for 30 min at RT. Cells were then stimulated with 1 µM Insulin for 10 min at RT. Data show that treatment of MCF7 cells with LY294002 or Wortmannin, but not S961, inhibits phosphorylation of AKT pan at S473 by Insulin.

INHIBITION OF PHOSPHO-AKT PAN (S473) IN JURKAT CELLS



Jurkat cells (400,000 cells/well, in triplicate) were incubated with serial dilutions of the inhibitor LY294002 for 60 min at 37°C. Data show that treatment of Jurkat cells withLY294002 inhibits basal phosphorylation of AKT pan at S473.

INHIBITION OF PHOSPHO-AKT PAN (S473) IN HEK293 CELLS



HEK293 cells (50,000 cells/well, in triplicate) were incubated with serial dilutions of the inhibitor LY294002 for 30 min at RT. Data show that treatment of HEK293 cells with LY294002 inhibits basal phosphorylation of AKT pan at S473.

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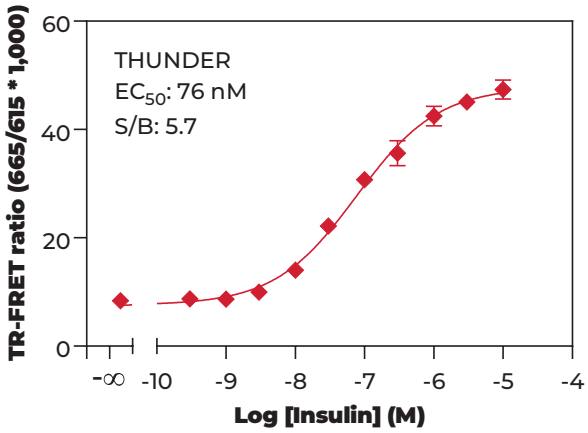
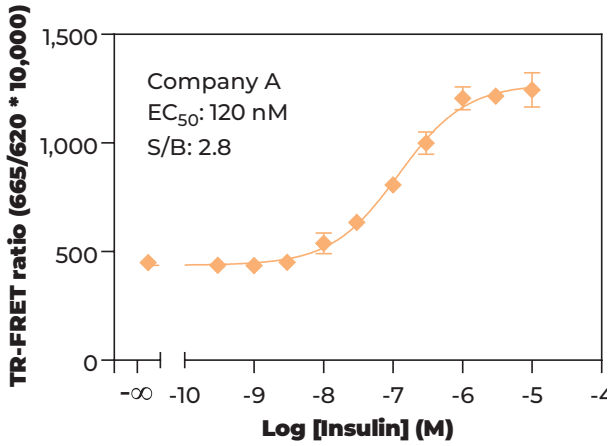
KIT BENCHMARKING – COMPARISON TO OTHER TR-FRET TECHNOLOGIES

As part of assay validation, the THUNDER™ Phospho-AKT (S473) Assay Kit was benchmarked against two competitive TR-FRET assay technologies (Companies A and B). Specifically, the 3 different assays were compared side-by-side for their capacity to detect phospho-AKT pan (S473) stimulation upon MCF7 cell treatment with Insulin using the 2-plate assay protocol. All reagents were prepared according to each manufacturer's recommendations. Following cell treatment, cells were lysed with the corresponding kit's 1X Lysis Buffer supplemented with phosphatase inhibitors. Lysates were then tested on the same white 384-well assay plate and according to the corresponding kit's standard protocol. The plate was read on an EnVision® (lamp excitation) following 4 hours of incubation. Data show that while the 3 TR-FRET assays exhibit comparable sensitivity (EC_{50} values), the THUNDER assay showed the highest S/B ratio.

THUNDER™ PHOSPHO-AKT PAN (S473) ASSAY KIT



COMPANY A PHOSPHO-AKT PAN (S473) ASSAY KIT



COMPANY B PHOSPHO-AKT PAN (S473) ASSAY KIT