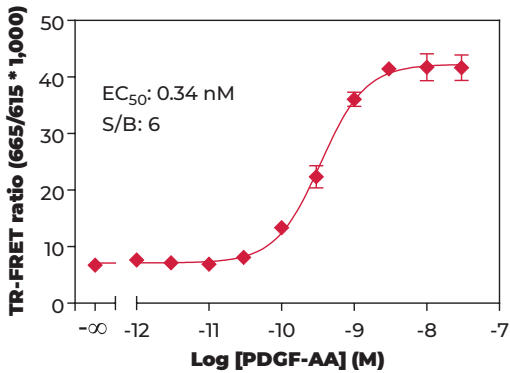


VALIDATION DATA

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.

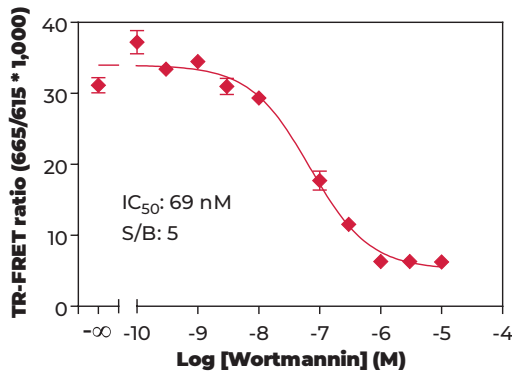
- Adherent cells were cultured 48 hours in a 96-well tissue culture plate, (DMEM + 10% CBS).
- Following cell treatment, the media was removed and cells were lysed with the 1X **Lysis Buffer 1** (50 μ L) supplemented with the 100X Phosphatase Inhibitor Cocktail diluted at 1X.
- Following a **30-min** incubation at room temperature (RT) on an orbital shaker (400 rpm), lysates (15 μ L) were then transferred to a 384-well assay plate followed by addition of the labeled antibodies Eu-Ab1 and FR-Ab2 (5 μ L) 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



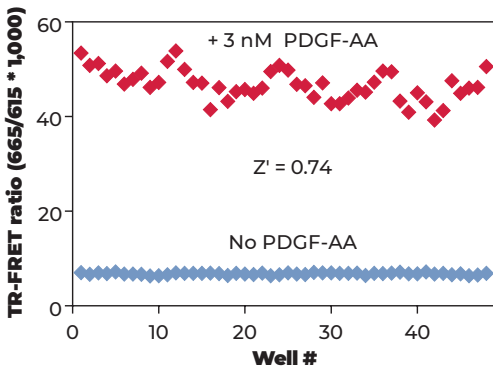
NIH3T3 cells (30,000 cells/well, in triplicate) were incubated with serial dilutions of PDGF-AA for 15 min at RT. Data show that treatment of NIH3T3 cells with PDGF-AA stimulates phosphorylation of AKT pan at S473.

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



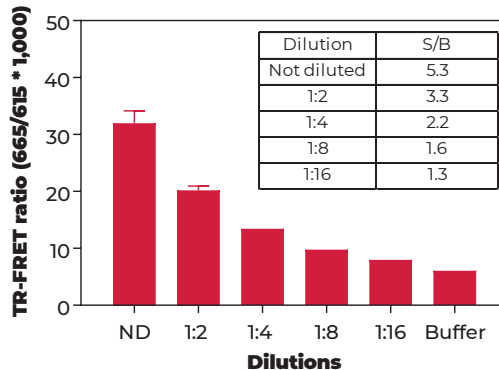
NIH3T3 cells (30,000 cells/well, in triplicate) were incubated with serial dilutions of Wortmannin for 30 min at RT. Cells were then stimulated with 1 nM PDGF-AA for 15 min at RT. Data show that treatment of NIH3T3 cells with Wortmannin inhibits phosphorylation of AKT pan at S473 by PDF-AA.

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.

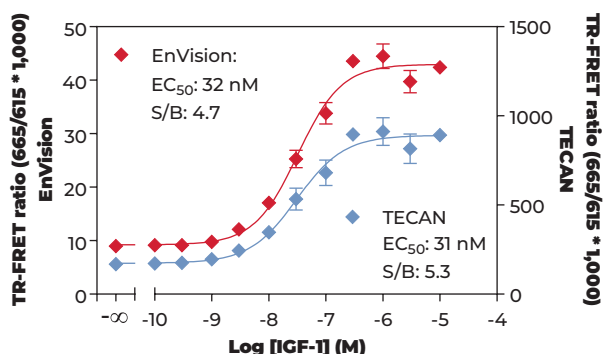
NIH3T3 CONTROL LYSATE TITRATION (QC TEST)



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.

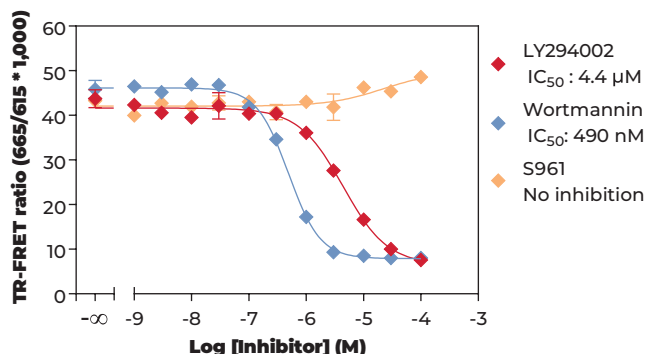


STIMULATION OF PHOSPHO-AKT PAN (S473) IN MCF7 CELLS



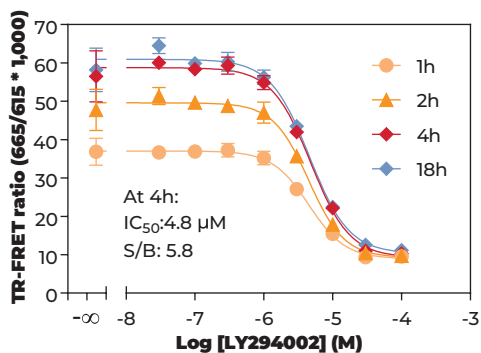
MCF7 cells (60,000 cells/well, in triplicate) were incubated with serial dilutions of IGF-1 for 10 min at RT. Data show that treatment of MCF7 cells with IGF-1 stimulates phosphorylation of AKT pan at S473.

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



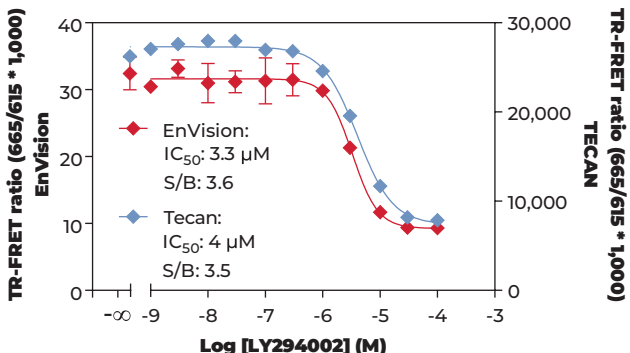
MCF7 cells (60,000 cells/well, in triplicate) were incubated with serial dilutions of either LY294002, Wortmannin or S961 for 30 min at RT. Cells were then stimulated with 1 μM Insulin for 10 min at RT. Data show that treatment of MCF7 cells with LY294002 or Wortmannin, but not S961, inhibits phosphorylation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

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



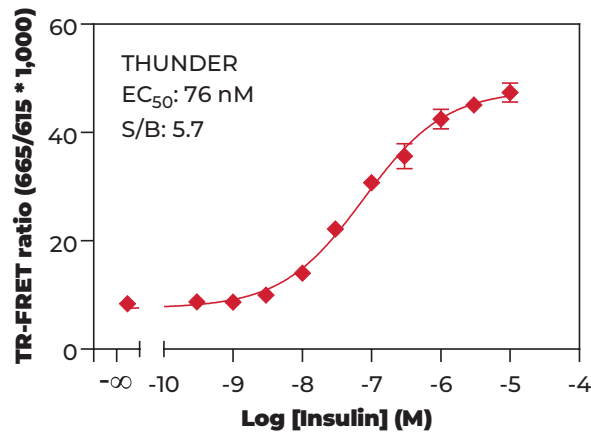
HEK293 cells (50,000 cells/well, in triplicate) were incubated with serial dilutions of the inhibitor LY294002 for 30 min at RT. Data show that treatment of HEK293 cells with LY294002 inhibits basal phosphorylation of AKT pan at S473.



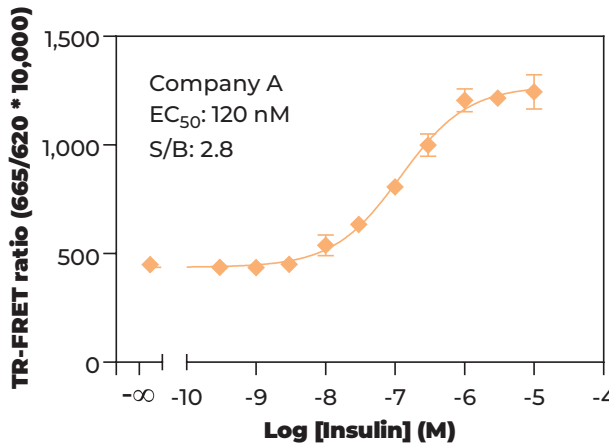
KIT BENCHMARKING – COMPARISON TO OTHER TR-FRET TECHNOLOGIES

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

