Automation of a Novel Cell-Based ELISA for Cell Signaling Pathway Analysis

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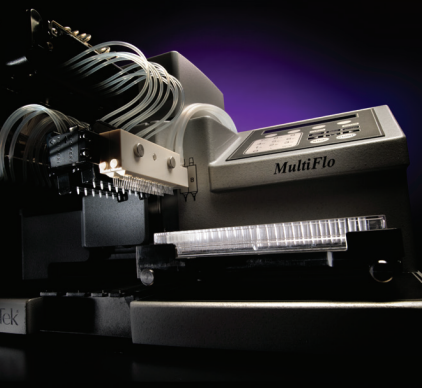

Monitoring and quantifying cell signaling pathways is critical for understanding the behavior of cell processes and many disease states. Protein kinases involved in these cellular cascades play many diverse biological roles including normal growth and development; their aberrant behavior is linked to a number of infirm states including cancer. A novel one wash step ELISA assay has been developed for detection of cell based signal transduction events. In addition to offering simplicity, speed, and flexibility, the technology is a highly sensitive technique that lends itself to automation at multiple workflow levels. To confirm optimal assay performance under full automation studies were conducted to demonstrate sound intra- and inter-assay precision, accuracy, S/B, and Z'. Subsequently, verification of a 'one well one day' cell-based assay protocol was developed allowing treatment and lysis of the cells directly in the ELISAOne microplate. Pharmacology assessment using this procedure was then undertaken for agonist/antagonist analysis of the PI3/AKT/mTOR signaling pathway using MCF-7 cells and multiple antagonist compounds.

Key Words:

AKT
PI3
mTOR
PI-103
LY294002
Wortmannin
Rapamycin
ERK
p70S6
Signal Transduction
Kinase Inhibitors
Kinase Signaling
Phosphorylation Events
Cell Signaling Pathway Analysis
Phospho Protein Analysis
Phospho Proteomics
ELISAOne

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Introduction

Protein expression, modification, and synergy in cell signaling pathways is a vast and diverse area of research. One example is shown by studies demonstrating support for the idea that combinatorial inhibition of cell signaling pathway targets may be a more effective means of disease state therapeutics than selective inhibition. Prototypically, but not exclusively, cancer models are used and multiple pathway targets and compound inhibitors are run as a panel[1,2]. Evidence found using this paradigm can be quite significant:

"To define targets critical for cancers driven by activation of PI3 kinase, we screen a panel of potent and structurally diverse drug-like molecules that target this enzyme family. Surprisingly, a single agent (PI-103) effected proliferative arrest in glioma cells, despite the ability of many compounds to block PI3 kinase signaling through its downstream effector, Akt. The unique cellular activity of PI-103 was traced directly to its ability to inhibit both PI3 kinase α and mTOR.... Moreover, the lead inhibitor that we report [PI-103] highlights how the conserved nature of the PI3 kinase catalytic domain can be exploited to discover multi targeted inhibitors with unexpected efficacy." [Cancer Cell 9, 341-349, May 2006 ©2006 Elsevier Inc. DOI 10.1016/j.ccr.2006.03.029]

In previous studies BioTek has illustrated and described selective cell signaling pathways and demonstrated successful automation of cell based signal transduction analysis[3,4]. Here we supplement this data by automating a novel cell based ELISA designed to complement endogenous phosphoprotein analysis. Specific for both total and phosphorylated target detection levels, the ELISA*One*™ assay format allows quantification and normalization data in parallel (Figure 6B). Screening targets using compound panels (Figure 6C), or determining optimal induction concentrations for agonist stimulation (Figure 6A, 6B) are all applications highlighted here. The assay is also well suited for downstream validation of results derived from phospho-proteomic global analysis techniques.

With only one wash step, the ease and efficiency of the assay rival a 'mix and read' format typical of high-throughput models. The automation shown here is standard ELISA equipment requiring no specialized modules and is available at a cost accessible to most any laboratory. In addition to introducing automation to the assay workflow, the benefits of the test were enhanced by the development of an abbreviated cell handling protocol that could be done quickly and efficiently over the course of a typical work day using very few consumables.

From cell and assay prep through to target detection this workflow enabled multiple compound dose responses of p-AKT inhibition in MCF-7 cells to be done within a single ELISA*One* microplate in a time-to-result of less than 6 hours.

Assay Principle

Over 30 targets in the MAPK; AKT; NF-kB p65; STAT; and Wnt pathway families are available as ELISA*One*™ assays. Offered in strip format treated with a universal coating, the ELISA*One*™ assay procedure is identical for all targets, allowing custom configuration of parallel target detection on a single microplate. The assay utilizes a single solution phase delivery of capture and detection antibodies, one wash step, a substrate development step, and either absorbance or fluorescence detection. Shared Lysis, wash, and substrate solutions between target families add to the overall advantages of the assay.

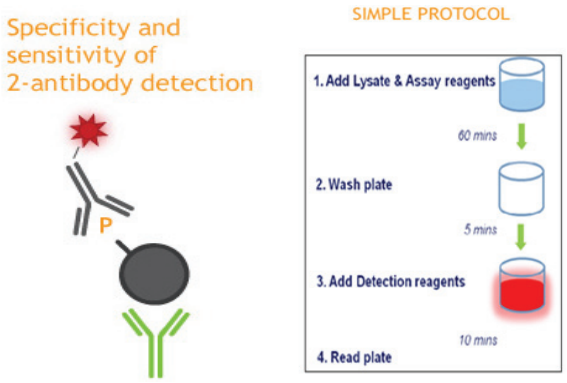


Figure 1. TGR Biosciences ELISA*One*™ Signal Transduction assay principle.

Materials and Methods

Materials

▪ TGR BioSciences ELISA*One*™ phospho-ERK 1/2 (T202/Y204) 96-well kits Cat# EKT001; phospho-p70S6K (T389) 96-well kits Cat# EKT010; Phospho-AKT 1/2/3 (p-S473) 96-well kits Cat# EKT002; Total AKT 1/2/3 96-well kits Cat# EKT012; ELISA*One*™ Microplates Cat# EPL001 ▪ 5% Gibco Fetal Bovine Serum in Hanks Buffered Saline Solution ▪ Growth Media (DMEM/F12 Nutrient Mix Life Technologies Cat # 11330032; 10% Gibco FBS; 1% Pen/strep, 1% L-Glutamine; 1%ITS) ▪ ATCC (Number HTB-22) MCF-7 cell line obtained from Sigma ▪ TrypLE™ Express (Life Technologies Catalog # 12605028) ▪ Tocris Bioscience Rapamycin (Cat # 1292); Wortmannin (Cat #1232); PI-103 Hydrochloride (Cat # 2930); LY294002 Hydrochloride (Cat # 1130) ▪ Sigma Aldrich Insulin, Human Recombinant (Cat # 91077C) ▪ Corning 75 and 150 cm^2 canted neck cell culture flasks

Automation

BioTek's Precision™ XS Automated Pipetting System was used for all serial dilutions and transfers to the assay plate for both the analytical method validation and pharmacology assessment.

BioTek's MultiFlo™ Automated Dispenser peri pump cassettes were used for seeding cells in media to assay plates, and all assay reagent dispenses using a low flow rate and the BioTek Liquid Handling Control (LHC) software. The MultiFlo and ELx50™ Washer fit comfortably side-by-side in a 36" Laminar Flow Hood. Cassettes are fully autoclavable adding to the compatibility of the MultiFlo for cell-based work.

BioTek's ELx50™ Microplate Strip Washer was used for the assay wash step via the onboard NUNC Flat protocol. A 30 second soak was integrated for runs using less than 12x8 well strips. The instrument also performed routine cell culture work including media exchanges and cell washing (data not shown). The compact footprint is advantageous when working under smaller Laminar Flow hoods.

BioTek's Synergy™ HT Multi-Mode Microplate Reader was used to detect Raw Fluorescence Units using a filter set of 540/25 for excitation and 590/20 for emission. Following an Auto Sensitivity test on a high well for the assay, a setting of 46 was used for all subsequent experiments. Sensitivity should be determined for each instrument and application independently.

Method

Validation of Automation

Two serial dilution curves of a 50% (1:2.5) and 10% (1:75) pg/mL concentration of positive control lysate were run over a 4-day period in replicates of 6 and assayed for p-ERK. Curve fits for each dilution were plotted, and Z', S/B, and precision data was calculated for each individual day (intra assay), and for the multi-day period (inter assay). The kit Negative Control was used as the 0 standard in all cases. 500 pg/mL of control lysate was used for the Z'. 100 pg/mL of control lysate was used for the S/B as it could be compared to the Certificate of Analysis (COA) to verify expected performance. p-p70S6K was also run fully automated using the same map and workflow to report intra assay precision and accuracy data for a second target. The assay map and automated workflow are illustrated by Figures 2 and 3 respectively. Results are shown by Figure 5 (all).

	50% Lysate			10% Lysate			50% Lysate			10% Lysate		
pg/mL	1	2	3	4	5	6	7	8	9	10	11	12
A	500	500	500	100	100	100	500	500	500	100	100	100
B	200	200	200	60	60	60	200	200	200	60	60	60
C	80	80	80	34	34	34	80	80	80	34	34	34
D	32	32	32	19	19	19	32	32	32	19	19	19
E	13	13	13	10	10	10	13	13	13	10	10	10
F	5	5	5	6	6	6	5	5	5	6	6	6
G	2	2	2	3	3	3	2	2	2	3	3	3
H	0	0	0	0	0	0	0	0	0	0	0	0

Figure 2. Inter/Intra Variability Plate Map. Precision XS performed two 8-point serial titrations of control lysate (orange 1:2.5, green 1:1.75), then transferred 50 µL/well in replicates of 6 to the assay plate.

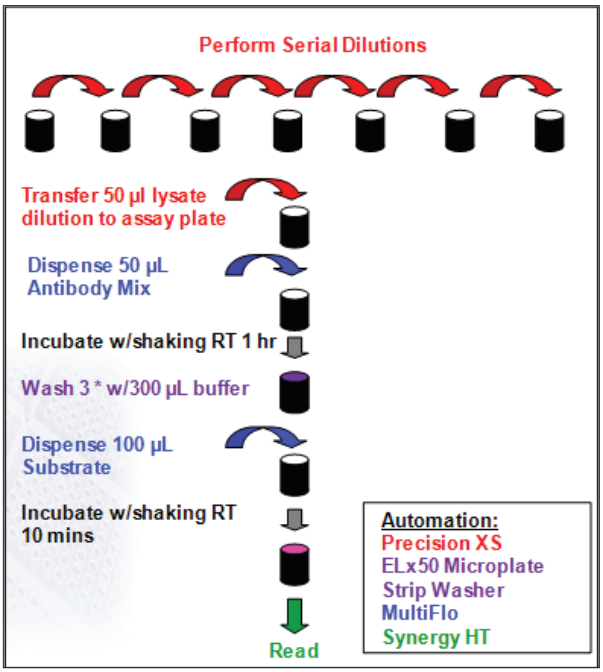


Figure 3. Inter/Intra Variability Workflow. 4 plates were run over a 4 day period to qualify inter/intra performance of the p-ERK assay fully automated (Figure 5, A-C). Additional intra assay data was done on another target, p-p70S6K, using the same workflow to compare results of different targets (Figure 5D).

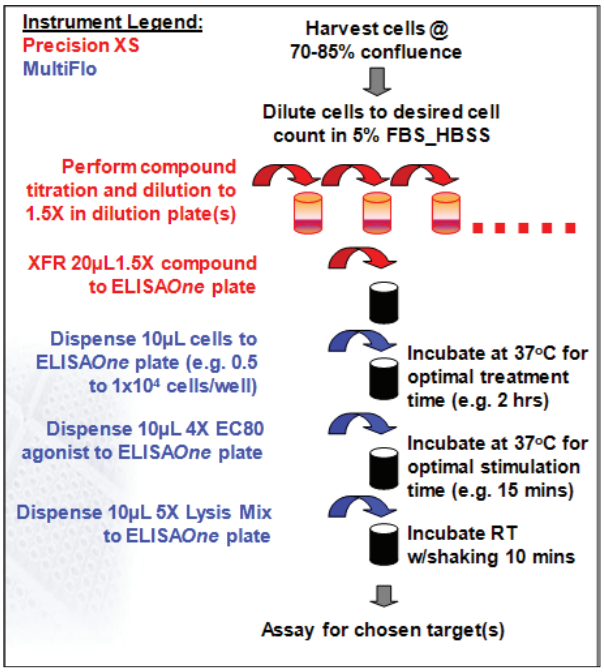


Figure 4. Fully Automated 'One Well One Day' Cell-Based Assay Workflow. This procedure was developed to replace 2 overnight cell incubation steps followed by a separate 2 hour antagonist treatment. Cell harvest to signal detection was reduced to 4-6 hours fully automated (dependent on inhibition and stimulation incubation times). The ELISA*One* assay protocol followed at the end of this workflow is shown by Figure 3 starting with the Antibody Mix dispense step.

Development and Automation of Cell-Based 'One Well One Day' Assay Protocol via p-AKT Antagonist_ Agonist Response (I + B)

Protocol development was undertaken to design and verify an automated cell seeding and treatment procedure that could be done directly in the ELISA*One* microplate in less than one day, eliminating the combination of overnight incubations for the cell culture transfer and serum starvation steps followed by separate compound treatment steps. This was achieved by culturing MCF-7 cells to approximately 70-85% confluence in standard cell culture vials using growth media, harvesting the cells, diluting to the desired cell count in 5% FBS_HBSS media (1x10^4 cells/well), then dispensing diluted cells directly into the wells of a rinsed ELISA*One* microplate. This was followed by a 2 hour serum starve in the presence of 10 µM Wortmannin, a known p-AKT inhibitor, or in the absence of antagonist to measure agonist only and basal cell response. After dosing cells with one of 4 concentrations of insulin (50, 10, 1, or 0 µM), followed by a 15 minute incubation, cells were lysed and then assayed for p-AKT in the same plate. The workflow is shown by Figure 4 and results are shown by Figure 6A. This cell treatment protocol was then used for all subsequent pharmacology assessment.

Agonist EC_{80} Determination and Validation of Total vs Phosphorylated Signaling Event for AKT (I + B)

MCF-7 cells were seeded in 20 µL 5% FBS @ 1x10^4 cells/well directly into the ELISA*One* microplate using MultiFlo™ and incubated for 2 hours @ 37°C. Following incubation, 2X ½ log titrations starting at 6 µM (0-3 µM final) of insulin were done in 5% FBS and transferred as 20 µL aliquots in triplicate to 6 columns of the ELISA*One* plate using Precision™ (3 columns for total AKT and the other 3 for phosphorylated AKT). 2 columns of the plate were left empty for assay controls. Cells were stimulated with agonist for 15 mins @ 37°C. 10 µL of kit 5X lysis mix was then added to the plate using MultiFlo™, followed by a 10 minute RT shaking incubation. Positive and negative kit controls for each target were added in quadruplicate. Total and phosphorylated AKT was assayed in parallel using solution-phase target specific conjugates. The resulting insulin EC_{80} for p-AKT (Figure 6B) was used in subsequent antagonist dose response experiments.

p-AKT Multiple Antagonist Dose Response for IC_{50} Pharmacology Assessement

4 target-specific compounds of the PI3K/AKT/mTOR pathway - Rapamycin (0-200 nM), Wortmannin (0-10 µM), PI-103 (0-10 µM), and LY294002 (0-100 µM) – were serial diluted 1:5 in 100% DMSO to 37.5X, then diluted to 1.5X in 5% FBS_HBSS to 4% DMSO. 2 columns were left untreated for insulin only and cell control wells. Of these, ½ the replicates were spiked with 4% DMSO and ½ were not. 20 µL of 1.5X compound titrations @ 4% DMSO were dispensed to the ELISA*One* plate. Using MultiFlo™, 10µL aliquots of MCF-7 cells @ 7.7 x10^3 /well were then dispensed to the plate resulting in 30 µL 1X compound at 2.66% DMSO final. Following a 2 hour incubation at 37°C, 10 µL/well of 4X EC_{80} insulin was dispensed using MultiFlo™. Cells were stimulated with agonist for 15 minutes at 37°C. 10 µL/well of 5X lysis mix was added and the microplate was shaken @ RT for 10 minutes. 50 µL of kit controls were dispensed per well in quadruplicate, and the plate was assayed for p-AKT. Results supported the low sensitivity of p-AKT to Rapamcyin inhibition (data not shown), and potent inhibition of insulin stimulation of p-AKT by the remaining compounds (Figure 6C).

Results

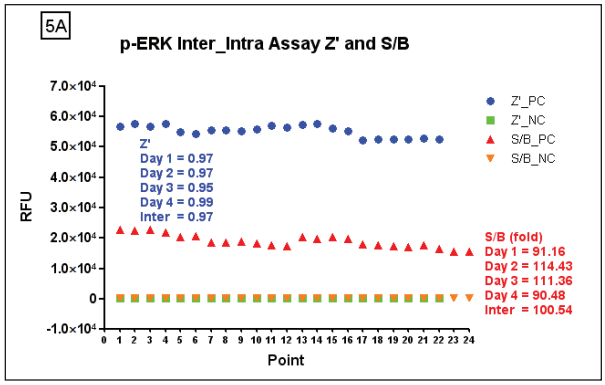


Figure 5A. Automation of Assay Validation, Quality Assessment. Inter and intra assay data resulting in Z' values >= 0.95 and S/B values greater than 32 fold the published COA[5] of 58 in all cases. 0.5 <= Z < 1 is indicative of an excellent assay[6].

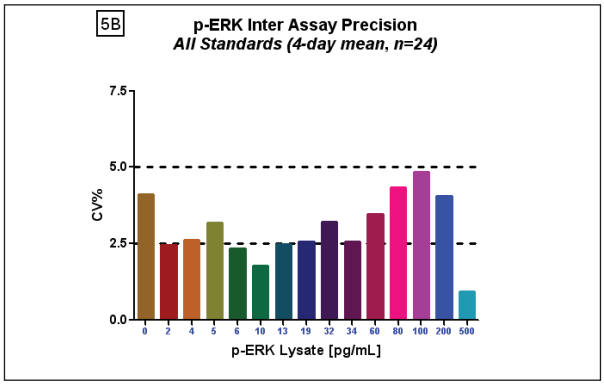


Figure 5B. Validation of Assay Automation, Precision. Inter assay precision of all standards for both titrations calculated to <=5% CV.

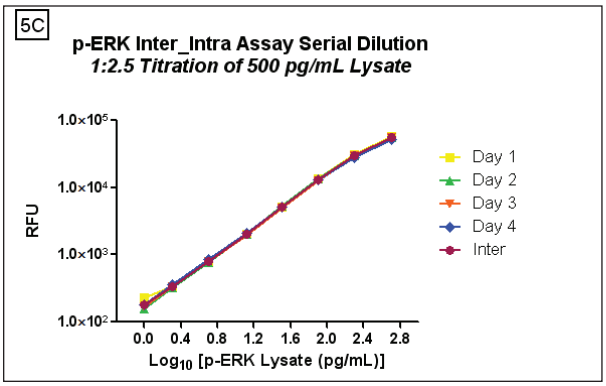
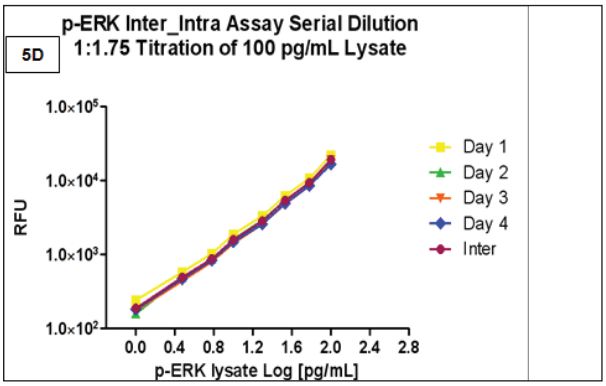
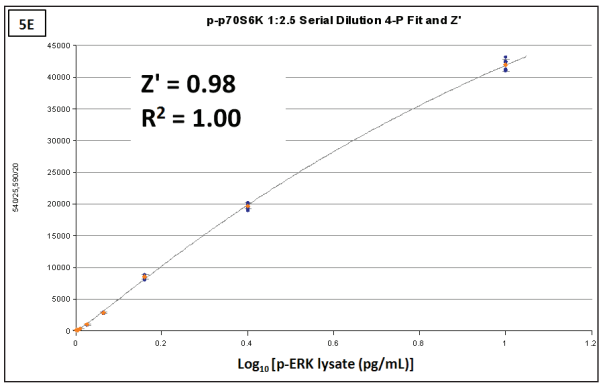


Figure 5 (C,D). Validation of Assay Automation, Repeatability. Two serial dilutions were run for p-ERK over 4 days under full automation. Representative data showing high correlation between and within runs is shown for both the 500 pg/mL (5C) and 100 pg/mL lysate (5D) titrations. Plate map shown by Figure 2 and workflow by Figure 3.





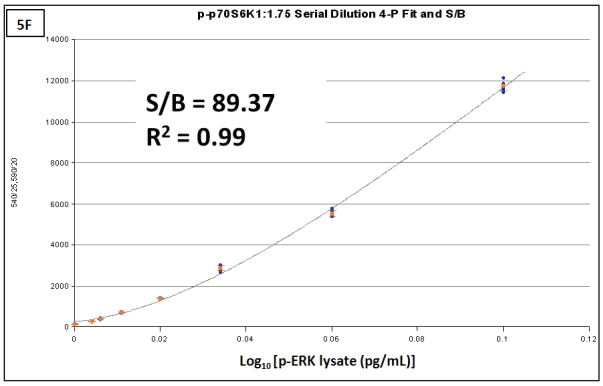
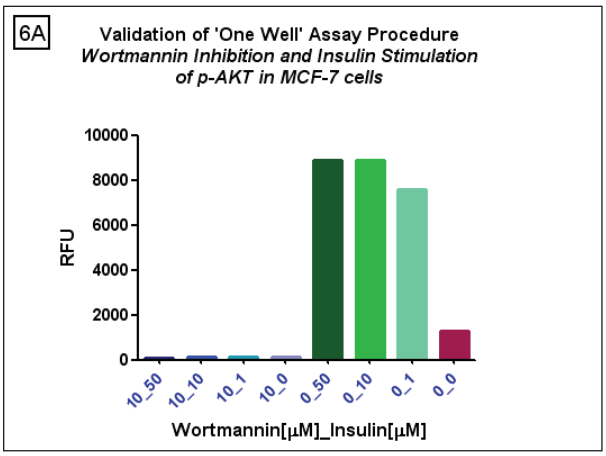
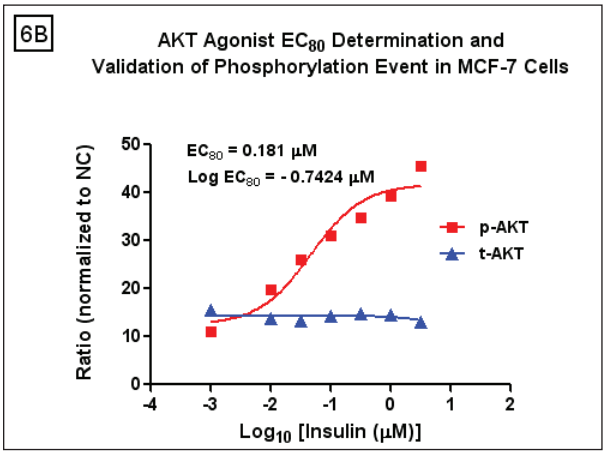


Table 1

Expected pg/mL	Interpolated pg/mL +/- 95% CL	% Recovery	CV% (n=6)
200	198.6 +/- 21.06	99.31	2.7
32	29.6 +/- 4.3	92.47	3.7
2	2.81 +/- 0.31	140.5	2.8

Figure 5 (E,F) / Table 1. Validation of Assay Automation, **Accuracy.** Intra assay data for two titrations of p-p70S6 were plotted using a 4-P logistics fit (5 E,F). Representative accuracy for the 1.25 titration (selected because it shows the broadest range) is shown for 3 orders of magnitude as calculated by % Recovery and CV % (Table 1) from the 4-P fit data. The 200, 32, and 2 pg/mL standards returned interpolated concentrations of 198.6, 29.6, and 2.81 pg/mL respectively at less than 4% CV. The average CV % for interpolated concentrations of all standards (n_{stds}=8, n_{reps}=6) for the 1.25 titration was 4.22 %, and for the 1.75 titration it was 3.9 %. High correlation of standard points to the fit (both R^2 >= 0.99), a Z' = 0.98 and an S/B 45% higher than the published COA[5] further support robust automated assay performance.





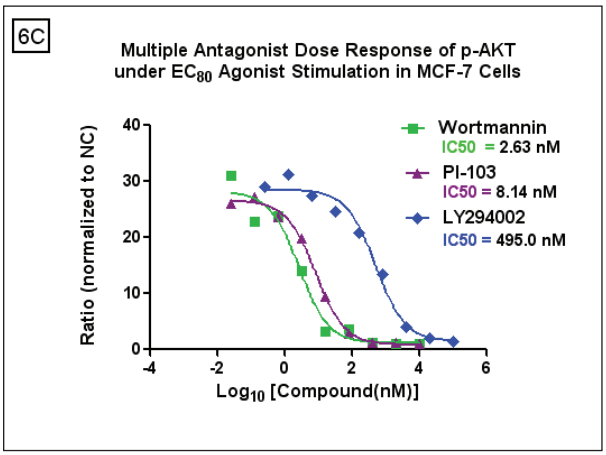


Figure 6 (A, B, C). Pharmacology Assessment. Figure 6A illustrates predicted antagonist shutdown of p-AKT signal regardless of agonist stimulation (1x104 cells/well, n=5) verifying effectiveness of the 'one well one day' cell treatment procedure (workflow shown by Figure 4). Based on 6A data, max insulin for the EC_{80} dose response was calculated between 10 µM and 1 µM. Figure 6B illustrates a ½ log dose response curve 0-3 µM insulin (n=3) run in parallel for both p-AKT and t-AKT. Total AKT levels remain constant as expected, while agonist stimulation of phosphorylated AKT returned an EC_{80} of 0.181. Figure 6C (below) displays the results for $7.7x10^3$ MCF-7 cells/well treated for 2 hours with multiple compounds titrated 1:5, stimulated with EC_{80} of agonist determined from Figure 6B data, then assayed for p-AKT. All IC_{50} values are well within expected ranges[7,8,9].

Conclusions

Performance of the instrumentation highlighted here coupled with the vast array of targets for multiple pathway families offered by the ELISA*One*™ assay suite add significant value to signal transduction analysis. This collaboration can prove especially useful in studies requiring measurement of both total and phosphorylated levels for single or multiple kinase targets analyzed with a panel of compounds running in customizable configurations using the same assay technology.

Fully automated inter and intra assay results show low variability and suitable recovery over 3 orders of magnitude indicating that even in the low pg/mL concentration range precision and accuracy are not sacrificed. High Z' and S/B values for multiple targets solidify performance confidence. Pharmacology data also support confident assay performance under automation as evidenced by correlation of IC_{50} values for multiple compounds to expected ranges.

Data supports repeatable performance of multiple workflow tasks at both the cell preparation and assay target binding and detection levels highlighting the versatility of component instrumentation. Automation can be comfortably placed side by side within standard laminar flow hoods and is easy to clean and sterilize, adding to the benefits of automating cell work.

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