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

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Abstract

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.

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 multitargeted inhibitors with unexpected efficacy."[3]

Previous studies have illustrated and described selective cell signaling pathways and demonstrated successful automation of cell-based signal transduction analysis.[4,5] Here, these data are supplemented by automating a novel cell-based ELISA designed to complement endogenous phosphoprotein analysis. Specific for both total and phosphorylated target detection levels, the ELISAOne 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.
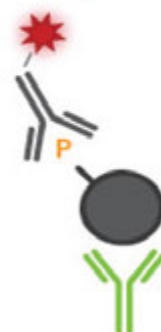
With only one wash step, the ease and efficiency of the assay rival a "mix and read" format typical of high-throughput models. The automation shown here is standard ELISA equipment requiring no specialized modules and is available at a cost accessible to most any laboratory. In addition to introducing 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 ELISAOne 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 ELISAOne assays. Offered in strip format treated with a universal coating, the ELISAOne assay procedure is identical for all targets, allowing custom configuration of parallel target detection on a single microplate. The assay uses 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.
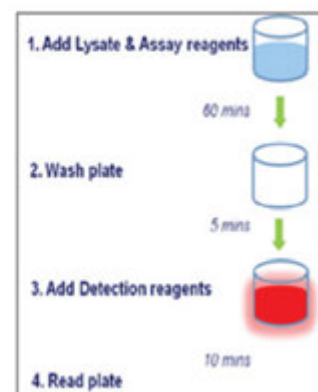

Figure 1. TGR Biosciences ELISAOne Signal Transduction assay principle.

Materials and methods

Materials

- TGR BioSciences ELISAOne phospho-ERK 1/2 (T202/Y204) 96-well kits part number EKT001; phospho-p70S6K (T389) 96-well kits part number EKT010; Phospho-AKT 1/2/3 (p-S473) 96-well kits part number EKT002; Total AKT 1/2/3 96-well kits part number EKT012; ELISAOne Microplates (part number EPL001)

- 5% Gibco Fetal Bovine Serum in Hanks Buffered Saline Solution

- Growth Media (DMEM/F12 Nutrient Mix Life Technologies (part number 11330032); 10% Gibco FBS; 1% Pen/strep, 1% L-Glutamine; 1% ITS)
- ATCC (Number HTB-22) MCF-7 cell line obtained from Sigma
- Life Technologies TrypLE Express (part number 12605028)
- Tocris Bioscience Rapamycin (part number 1292); Wortmannin (part number 1232); PI-103 Hydrochloride (part number 2930); LY294002 Hydrochloride (part number 1130)
- Sigma-Aldrich Insulin, Human Recombinant (part number 91077C)
- Corning 75 and 150 cm^2 canted neck cell culture flasks.

Automation

The Agilent BioTek 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.

Agilent BioTek MultiFlo automated dispenser peristaltic pump cassettes were used for seeding cells in media to assay plates, and all assay reagent dispenses using a low flow rate and the Agilent BioTek liquid handling control (LHC) software. The Agilent BioTek 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.

An Agilent BioTek 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 12 × 8 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.

An Agilent BioTek Synergy HT multimode 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 were calculated for each individual day (intra-assay), and for the multiday 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 in Figure 5 (all).

pg/mL	50% Lysate			10% Lysate			50% Lysate			10% Lysate		
	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- and intra-variability plate map. Agilent BioTek Precision XS automated pipetting system 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.
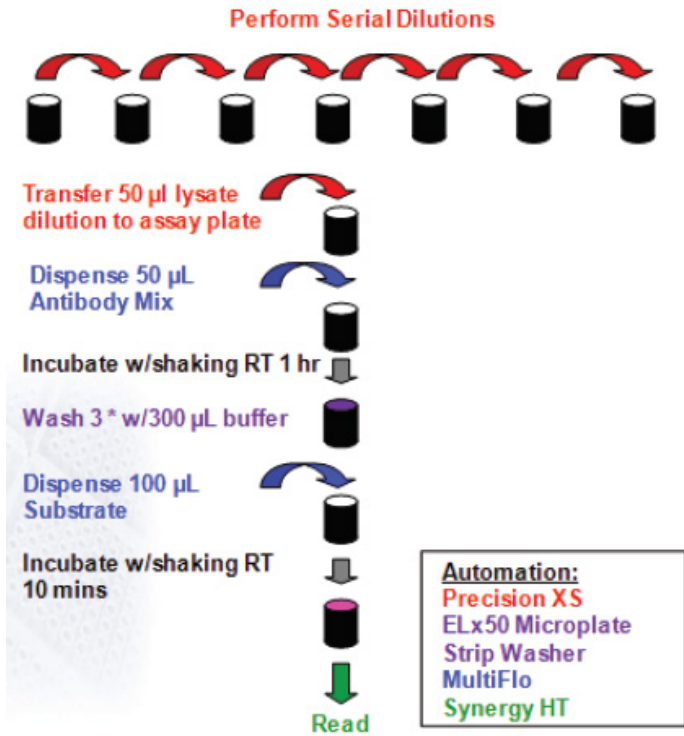
Perform Serial Dilutions

Transfer 50 µl lysate dilution to assay plate

Dispense 50 µL Antibody Mix

Incubate w/shaking RT 1 hr

Wash 3 * w/300 µL buffer

Dispense 100 µL Substrate

Incubate w/shaking RT 10 mins

Automation:
Precision XS
ELx50 Microplate
Strip Washer
MultiFlo
Synergy HT

Read

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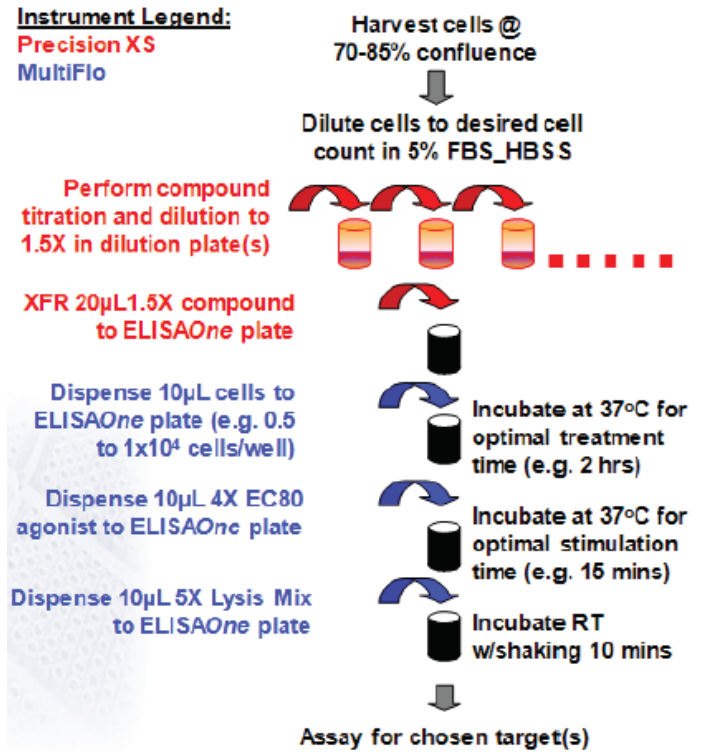

Instrument Legend:
Precision XS
MultiFlo

Harvest cells @ 70-85% confluence

Dilute cells to desired cell count in 5% FBS_HBSS

Perform compound titration and dilution to 1.5X in dilution plate(s)

XFR 20µL 1.5X compound to ELISAOne plate

Dispense 10µL cells to ELISAOne plate (e.g. 0.5 to 1x10⁴ cells/well)

Incubate at 37°C for optimal treatment time (e.g. 2 hrs)

Dispense 10µL 4X EC80 agonist to ELISAOne plate

Incubate at 37°C for optimal stimulation time (e.g. 15 mins)

Dispense 10µL 5X Lysis Mix to ELISAOne plate

Incubate RT w/shaking 10 mins

Assay for chosen target(s)

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 to 6 hours fully automated (dependent on inhibition and stimulation incubation times). The ELISAOne assay protocol followed at the end of this workflow is shown by Figure 3 starting with the Antibody Mix dispense step.

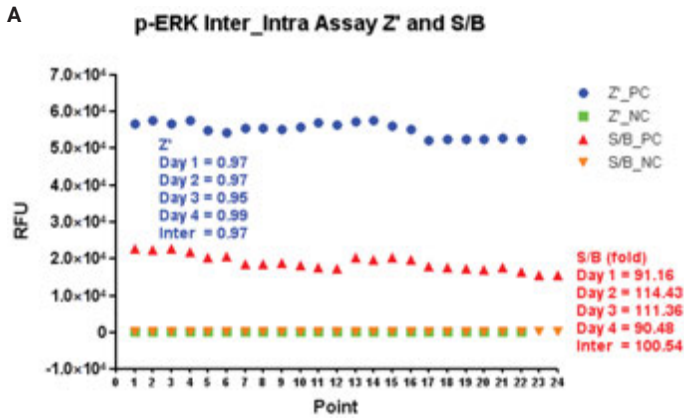# Results and discussion

A

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[6] of 58 in all cases. 0.5 ≤Z <1 is indicative of an excellent assay.
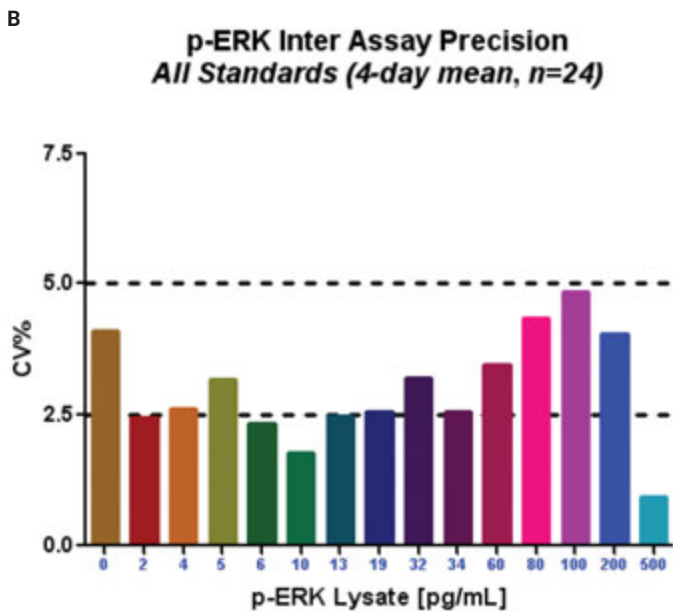
B

Figure 5B. Validation of assay automation, precision. Inter-assay precision of all standards for both titrations calculated to ≤5% CV.
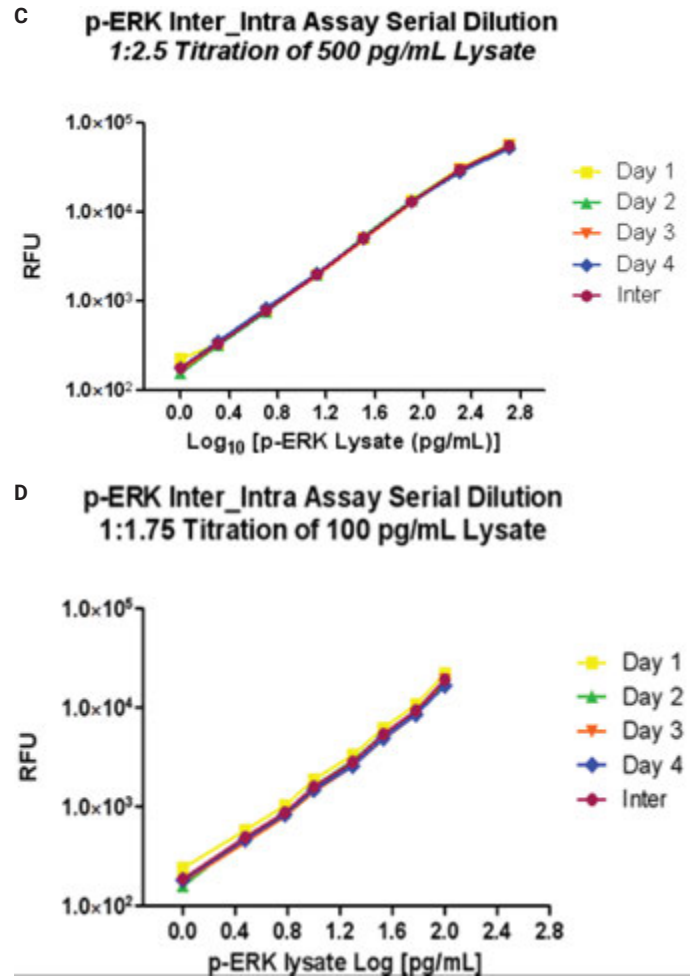
C

D

Figure 5 (C,D). Validation of assay automation and 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.
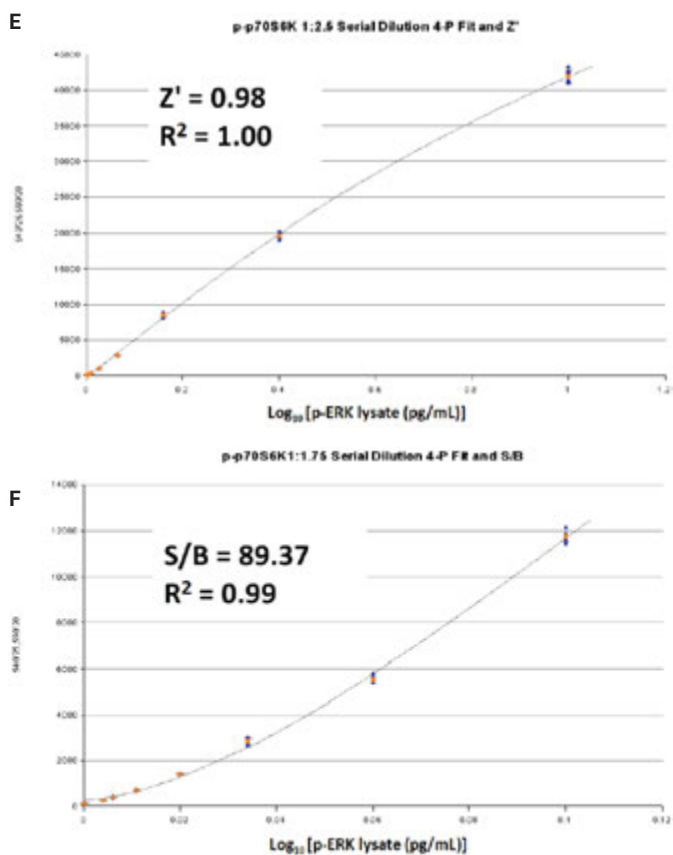
E

p-p70S6K 1:2.5 Serial Dilution 4-P Fit and Z'

Z' = 0.98
R² = 1.00

Log₁₀ [p-ERK lysate (pg/mL)]

F

p-p70S6K1:1.75 Serial Dilution 4-P Fit and S/B

S/B = 89.37
R² = 0.99

Log₁₀ [p-ERK lysate (pg/mL)]

Figure 5 (E,F). Validation of assay automation and accuracy. Intra-assay data for two titrations of p-p70S6 were plotted using a 4-P logistics fit (5 E,F). 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 (nstds = 8, nreps = 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[6] further support robust automated assay performance.

Table 1. 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 % from the 4-P fit data.

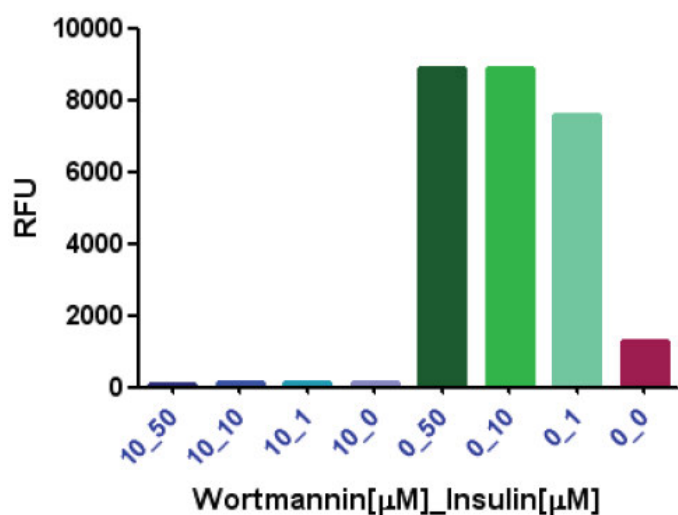| Expected pg/mL	Interpolated pg/mL ±95% CL	% Recovery	CV % (n = 6)
200	198.6 ±21.06	99.31	2.7
32	29.6 ±4.3	92.47	3.7
2	2.81 ±0.31	140.5	2.8

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 ELISAOne 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 to 85% confluence in standard cell culture vials using growth media, harvesting the cells, diluting to the desired cell count in 5% FBS_HBSS media (1×10^4 cells/well), then dispensing diluted cells directly into the wells of a rinsed ELISAOne 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 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.
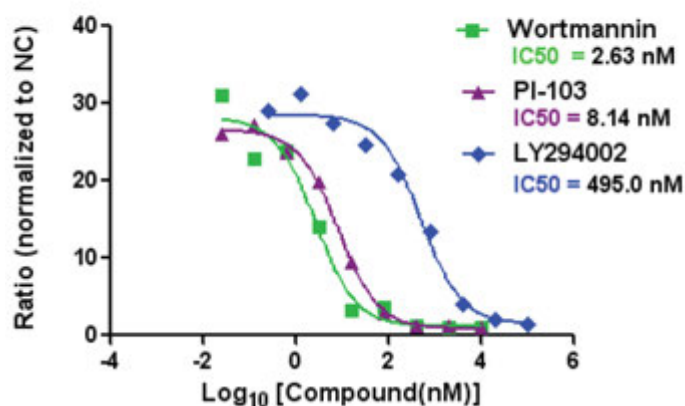
Agonist EC$_{80}$ determination and validation of total vs phosphorylated signaling event for AKT (I + B)

MCF-7 cells were seeded in 20 µL 5% FBS at 1×10^4 cells/well directly into the ELISAOne microplate using the MultiFlo and incubated for 2 hours at 37 °C. Following incubation, 2x ½ log titrations starting at 6 µM (0 to 3 µM final) of insulin were done in 5% FBS and transferred as 20 µL aliquots in triplicate to 6 columns of the ELISAOne plate using the Precision (3 columns for total AKT and the other 3 for phosphorylated AKT). Two columns of the plate were left empty for assay controls. Cells were stimulated with agonist for 15 minutes at 37 °C. 10 µL of kit 5x lysis mix was then added to the plate using the 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.

A

Validation of 'One Well' Assay Procedure
Wortmannin Inhibition and Insulin Stimulation of p-AKT in MCF-7 cells

B

AKT Agonist EC$_{80}$ Determination and Validation of Phosphorylation Event in MCF-7 Cells

C

Multiple Antagonist Dose Response of p-AKT under EC$_{80}$ Agonist Stimulation in MCF-7 Cells
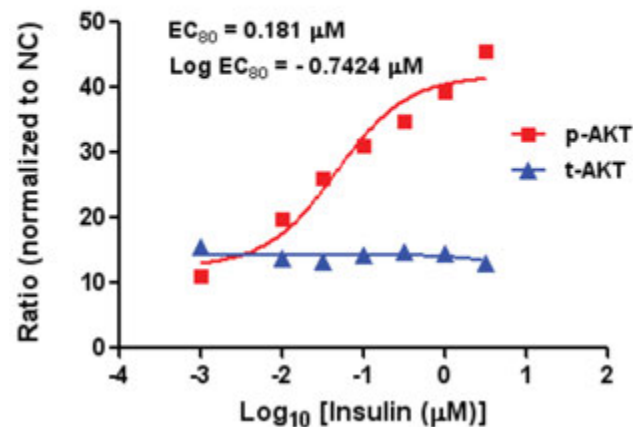

Figure 6 (A, B, C). Pharmacology assessment. (A) Illustrates predicted antagonist shutdown of p-AKT signal regardless of agonist stimulation (1 × 10^4 cells/well, n = 5) verifying effectiveness of the "one well one day" cell treatment procedure (workflow shown by Figure 4). Based on 6A data, max insulin for the EC$_{80}$ dose response was calculated between 10 μM and 1 μM. (B) Illustrates a ½ log dose response curve 0 to 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. (C) Displays the results for 7.7 × 10^3 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.[8,9,10]

p-AKT multiple antagonist dose response for IC_{50} pharmacology assessement

Four target-specific compounds of the PI3K/AKT/mTOR pathway, rapamycin (0 to 200 nM), wortmannin (0 to 10 µM), PI-103 (0 to 10 µM), and LY294002 (0 to 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. Two columns were left untreated for insulin only and cell control wells. Of these, half the replicates were spiked with 4% DMSO and half were not. 20 µL of 1.5x compound titrations at 4% DMSO were dispensed to the ELISAOne plate. Using the Agilent BioTek MultiFlo, 10 µL aliquots of MCF-7 cells at 7.7×10^3/well were then dispensed to the plate resulting in 30 µL of 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 the 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 at 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).

Conclusion

Performance of the instrumentation highlighted here coupled with the vast array of targets for multiple pathway families offered by the ELISAOne 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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