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

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Introduction

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 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 levels including seeding cells in microplates; cell media exchanges; serial dilution and transfer steps; assay reagent additions; microplate washing; and, signal detection. Assay performance studies were conducted using lysate kit controls to verify optimal automation performance for S/B, Z', and intra- and inter-assay precision. A 'one well one day' cell based assay protocol was developed and used for pharmacology assessment for agonist/antagonist analysis of the PI-3/AKT/mTOR signaling pathway. Procedures demonstrated here are well suited for validating automation of model system protocol development.

ELISA*One*™ Signal Transduction Assay

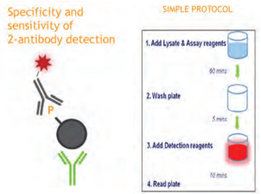

*Figure 1 – TGR BioSciences ELISA*One* Signal Transduction assay principle.*

Over 30 targets in the MAPK; AKT; NF-kB p65; STAT; and Wnt pathway families, in addition to a number of protein normalization tools, are available as ELISA*One* assays. Offered in strip format treated with a universal coating, the ELISA*One* assay protocol is identical for all targets, allowing any combination of parallel target detection on a single microplate. A number of standard protocols for adherent and non-adherent protocols for cell lysate production are included as part of the standard Product Manual. The addition of target-specific capture and detection antibodies in one step, and the need for only a single wash step, allows assay detection in just over an hour. 24-well and 96-well kits allow custom configurations for multi target detection. Shared Lysis, wash buffer, and substrate solutions between target families add to the overall benefits of the assay.

BioTek Instrumentation

Precision™ XS Microplate Pipetting System was used for all serial dilutions and transfers to the assay plate for both the automation validation and pharmacology assessment.

ELx50™ Microplate Strip Washer was used for the assay wash step under command of the onboard NUNC Flat protocol. A 30 second soak was integrated for runs using less than 12 x 8 well strips. Experiments also proved the instrument capable of performing routine cell work including media exchanges and cell washing (data not shown). The compact footprint is advantageous when working under smaller laminar flow hoods.

MultiFlo™ Microplate 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-inch laminar flow hood. The cassettes are fully autoclavable adding to the compatibility of the MultiFlo for cell based work.

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 subsequent experiments. Sensitivity should be determined for each instrument and application independently.

Materials and Methods

Materials

TGR BioSciences ELISA*One* phospho-ERK 1/2 (T202/Y204) 96-well kits Catalog No. EKT001; phospho-p70S6K (T389) 96-well kits Catalog No. EKT010; Phospho-AKT 1/2/3 (p-S473) 96-well kits Catalog No. EKT002; Total AKT 1/2/3 96-well kits Catalog No. EKT012; ELISA*One* Microplates Catalog No. EPL001 • 5% Gibco Fetal Bovine Serum in Hanks Buffered Saline Solution • Growth Media (DMEM/F12 Nutrient Mix Life Technologies Catalog No. 11330032; 10% Gibco FBS; 1% Pen/strep, 1% L-Glutamine; 1%ITS) • MCF-7 cell line obtained from ATCC (Number HTB-22) • TrypLE™ Express (Life Technologies Catalog No. 12605028) • Tocris Bioscience Rapamycin (Catalog No. 1292); Wortmannin (Catalog No. 1232); PI-103 Hydrochloride (Catalog No. 2930); LY294002 Hydrochloride (Catalog No. 1130) • Sigma Aldrich Insulin, Human Recombinant (Catalog No. 91077C) • Corning 75 and 150 cm^2 canted neck cell culture flasks.

Materials and Methods *(continued)*

Method

Validation of Assay Automation

Two serial dilution curves of a 50% and 10% pg/mL concentration of 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 CV, Z', and S/B data was generated for each individual day (intra assay), and over the multi-day period (inter assay). The kit Negative Control was used as the 0 standard in all cases. 500 pg/mL (50%) of control lysate was used for the Z'. 100 pg/mL (10%) 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 workflow to report intra assay data for a second target (data not shown). Automated assay workflow steps are illustrated by Figure 2. Results are shown by Figures 4A, 4B, 4C.

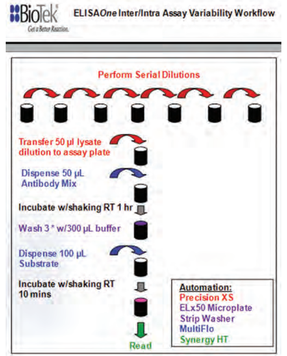

Figure 2 – Inter/Intra Variability Workflow. Four plates were run over a four day period to qualify inter/intra performance of the p-ERK assay fully automated (Figure 4, all). Additional intra assay data was done on another target, p-p70S6K, using the same workflow to compare results of different targets (data not shown).

Verification of Automated Cell Based 'One Well One Day' Assay Protocol via p-AKT Antagonist_Agonist Response

Protocol development was undertaken to validate the viability of an automated cell treatment procedure that could be done directly in the ELISA*One* microplate well in 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 70-80% 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), dispensing diluted cells directly into the wells of a rinsed ELISA*One* microplate followed by a 2 hour serum starve in the presence of 10 µM Wortmannin, a known p-AKT inhibitor, or left untreated to measure agonist only and basal cell response. Cells were then dosed with one of four concentrations of insulin (50, 10, 1, or 0 µM), incubated for 15 minutes, lysed in the well, and assayed for p-AKT in the same plate. The workflow is shown by Figure 3 and results are shown by Figure 5A. This cell treatment protocol was then used for all subsequent pharmacology assessment.

Materials and Methods *(continued)*

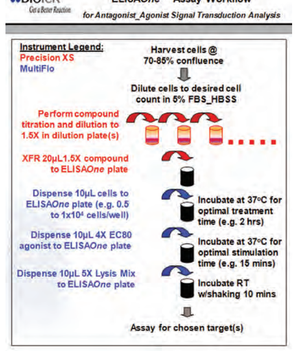

Figure 3 – Fully Automated 'One Well' Cell-Based Assay Workflow. This procedure was developed to replace two overnight cell incubation steps followed by a separate two-hour antagonist incubation. Cell harvest to signal detection was reduced to 4-6 hours fully automated (dependent on inhibition and stimulation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

Agonist EC_{80} Determination and Validation of Total vs Phosphorylated Signaling Event for AKT

MCF-7 cells were seeded in 20 µL 5% FBS @ 1 x 10^4 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 PPXS (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 @ 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, then total and phosphorylated AKT was assayed in parallel using target specific conjugates. The resulting insulin EC_{80} for p-AKT (Figure 5B) was used in subsequent antagonist dose response experiments.

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

Four compounds specific to the PI3K/AKT/mTOR1 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. 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 @ 4% DMSO were dispensed to the ELISA*One* plate. Using MultiFlo 10 µL aliquots of MCF-7 cells @ 7.7 x 10^3 cells/well were then dispensed resulting in 30 µL 1X compound at 2.66% DMSO final. Following a two 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 in quadruplicate, and the plate was assayed for p-AKT. Results supported the low sensitivity of p-AKT to Rapamycin inhibition (data not shown), and potent inhibition of insulin stimulation of p-AKT by the remaining compounds (Figure 5C).

Results

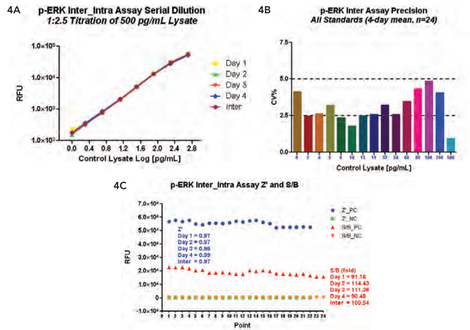

Figure 4 (A,B,C) – Validation of Assay Automation. Two serial dilutions were run for p-ERK over 4 days under full automation. Representative data showing high correlation between runs is shown for the 500 pg/mL lysate titration (4A). Inter assay precision of all standards for both titrations calculates to ≤5%CV (4B). Figure 4C illustrates inter and intra assay data resulting in Z' values consistently ≥0.95 and S/B values greater than 32 fold the published COA[2] of 58 in all cases. A Z' of ≥0.5 is considered indicative of reliable assay performance[1]. Data not shown for p-p70S6 supporting the same results of high correlation of standard points to the fit and tight precision within replicate groups (R^2 ≥0.99 and CV% ≤5.5 for all standards on all runs), a Z' = 0.98 and a S/B of 89 that is 45% higher than the published COA[2] of 62.

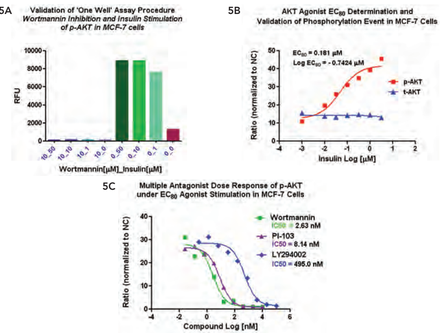

Figure 5 (A,B,C) – Pharmacology Assessment. Predicted shutdown of p-AKT signal by chosen antagonist regardless of agonist concentration is shown by Figure 5A (1x10^4 cells/well, n=5). Based on 5A data, max. insulin for the EC_{80} dose response was calculated between 10 µM and 1 µM. Figure 5B illustrates an insulin ½ log dose response curve from 0-3 µM in triplicate 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 5C shows the results for 7.7 x 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 5B, then assayed for p-AKT detection. All IC_{50} values are well within expected ranges [3,4,5].

Conclusions

- Performance of the component 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.
- Fully automated inter and intra assay results are virtually indistinguishable, and show low variability, high Z' and high S/B values for multiple targets
- Pharmacology data also proves robust assay performance under automation as evidenced by correlation of IC_{50} values for multiple compounds under EC_{80} agonist stimulation to expected ranges
- Individual instrumentation is versatile with data supporting reproducible performance of multiple workflow tasks at both the cell preparation and assay target binding and detection levels
- 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

[1] Zhang, J., et al. A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. *J. Biomol. Screen.* 1999, 4(2), 67-73. | [2] http://www.tgrbio.com/sandwich-immunoassay-elisa-assay-kits/elisa-one-coas.html. | [3] http://www.tocris.com/dispprod.php?ItemId=2181. | [4] http://www.tocris.com/dispprod.php?ItemId=5490. [5] http://www.tocris.com/dispprod.php?ItemId=2022