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

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Overview

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, detection. Assay performance studies were conducted using positive and negative controls to verify optimal automation performance for S/B, Z', and intra- and inter-assay precision. Verification of automation performance for routine cell handling tasks has also been undertaken as part of the initial stages of adapting a model MCF-7/insulin system to probe the phosphorylation of a number of protein kinases endogenously expressed for pharmacology assessment using this assay. Procedures demonstrated here are well suited for validating automation of model system protocol development.
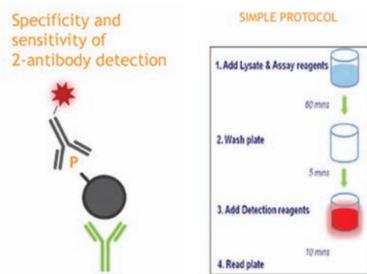
ELISAOne™ Signal Transduction Assay

Figure 1 – TGR BioSciences ELISAOne 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 ELISAOne assays. Offered in strip format treated with a universal coating, the ELISAOne 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, non-adherent, and all-in-one-well 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, allow 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 Sample Processor was used for all assay serial dilutions and transfers to the assay plate for the intra- and inter-variability runs.

ELx50™ Microplate Strip Washer was used for both cell and assay wash steps. Settings were optimized for minimal cell loss during aspiration and buffer dispense. The instrument fits comfortably under a 36-inch laminar flow hood next to the MultiFlo™ Microplate Dispenser (shown Figure 2).

MultiFlo™ Microplate Dispenser peristaltic pump driven 5µL and 10µL cassettes were used for seeding cells in media to tissue culture plates, and all assay reagent dispenses. An 8 point cell serial dilution was dispensed simultaneously to a tissue culture plate using a single cassette (shown Figure 2). The peri pump cassettes are fully autoclavable adding to the versatility of the MultiFlo design.

BioTek Instrumentation (Continued)

Figure 2 – The BioTek ELx50 Microplate Strip Washer (left) and MultiFlo Automated Dispenser (right) fit comfortably side-by-side in a 36-inch laminar flow hood. The MultiFlo is shown in the format used to seed an 8 point serial dilution of cells to a tissue culture plate. Each tube of the peri pump cassette is placed in a separate vial containing a different concentration of cells, and then dispensed to the plate simultaneously with a single command from the Liquid Handling Control™ (LHC™) software.

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, the setting of 46 was used for all experiment detection sensitivity.

Materials and Method

Materials

- TGR BioSciences ELISAOne™ phospho-ERK 1/2 (T202/Y204) 96-well kits Cat # EKT001 /Lot No. E1080
- TGR BioSciences ELISAOne™ phospho-p70 S6K(T389) 96-well kits Cat # EKT010 /Lot No. E1019
- Dulbecco's Phosphate Buffered Saline 1X (DBPS)
- 10% FBS Media (Gibco DMEM/F12 used as base media)
- 0.1% BSA or HSA media (Gibco DMEM/F12 used as base media)
- Serum Free Media (Gibco DMEM/F12 used as base media)
- MCF-7 cell line obtained from ATCC (ATCC Number HTB-22)
- Rapamycin, Tocris Bioscience (Cat # 1292)
- Insulin, Human Recombinant, Sigma Aldrich (Cat # 91077C)
- Corning 96-well Tissue Culture plates
- Corning 150cm² cell culture flasks
- 37°C + 5% CO_2 incubator
- *BioTek Instrumentation as shown (special formats noted here):*
  - **ELx50 Settings-Assay Wash Step:** Onboard NUNC Flat protocol. A 30 second soak was integrated for runs using less than 12 x 8-well strips. **Media Aspiration Step:** Onboard Costar Flat settings with an aspiration height of 029 and an aspiration rate of 2. **Cell Rinse Step:** Onboard Costar Flat settings with a dispense rate of 2. Cells were allowed to soak 30 seconds before aspiration.
  - **MultiFlo Setup:** All dispenses used the low flow rate setting. Unit was controlled using LHC software.

Method

Validation of Assay Automation - Inter-/Intra-Assay Precision

2 serial dilution curves in replicates of 6 were run over a 4-day period 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 (inter-assay), and over the multi-day period (intra-assay). The kit Negative Control was used as the 0 standard for each dilution series. p-p70S6k was also run fully automated using the same plate map and workflow to report inter-assay data for a second target. The assay plate map is shown as Figure 3. Automated assay workflow steps are shown by Figure 4.

Figure 3 – Inter-/Intra-Variability Plate Map. Precision XS performed two 8 point serial 1:2.5 and 1:1.75 titrations and then transferred 50µL/well in replicates of 6 to the assay plate. The 1:2.5 dilution was a 50% start concentration of the control lysate, and the 1:1.75 dilution was a 10% start concentration of the control lysate. The 10% lysate preparation was used for S/B calculations as it could be compared to the COA.
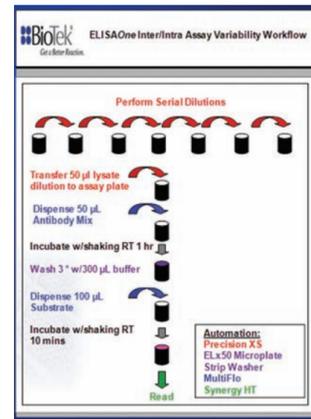
Materials and Method (Continued)

Figure 4 – Inter/Intra Variability Workflow. 4 plates were run over a 4 day period to qualify inter/intra performance of the assay fully automated. Results are shown by Figure 6 (all). Separately, additional inter assay data was done on another target, p-p70S6K, using the same protocol to compare results of different targets under full automation. Results shown by Figure 7 (all).

% Inhibition and S/B Baseline Data Generation Using Cell Serial Dilution

An 8 point dilution of MCF-7 cells was used to determine baseline signal inhibiton response for insulin stimulated phosphorylation levels of p70S6K. %inhibition and S/B of p-p70 levels from agonist and antagonist treated cells were determined for each dilution to gauge reproducibility of automated cell seed counts in subsequent experiments. Cell treatment workflow is shown by Figure 5, and represents the protocol for Run 1 data in Figure 8.

Validation of Automated Cell Media Exchange Steps

Three MCF-7 cell treatment protocols were followed to determine variability of % inhibition and S/B response for zero, one, and two automated aspiration steps during cell lysate preparation. The two aspiration step protocol introduced a cell wash step, dispensing 100 µL of DPBS with a 30 second soak following the overnight media aspiration and before dispense of the treatment step. Cell treatment workflows for 0 and 2 automated aspiration steps are shown by Figure 5, and represent the protocols for Runs 2 and 3 data in Figure 8.
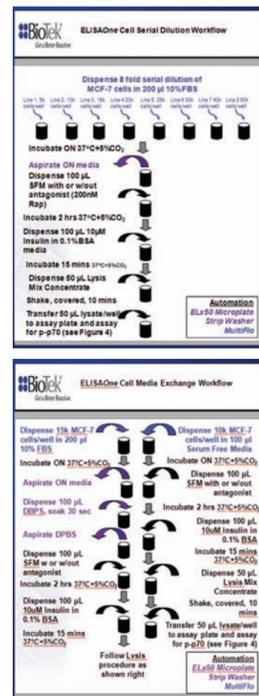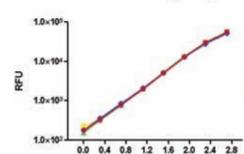

Figure 5 – Top: protocol for 8 point cell serial dilution from 5 to 50k cells/well in 10% FBS media dispensed simultaneously to tissue culture plate. One aspiration step from the cell plate was employed following an overnight incubation. Bottom: protocols for a 0 and 2 aspiration step cell treatment procedure during MCF-7 lysate preparation.
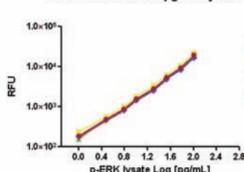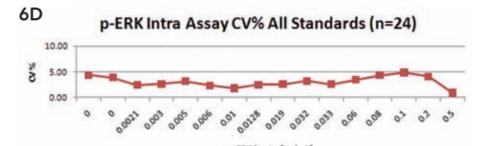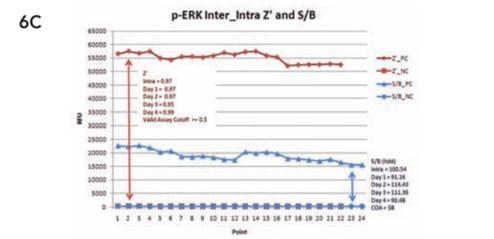
Results

Figure 6 (A, B) – Data shows high correlation and repeatability for both titrations. Curves track nicely 100 fold for 0-100 pg/mL (6B) and 5-500 pg/mL (6A).

Results (Continued)

p-ERK Inter Assay Precision (RFU CV%) 1:2.5 Titration						p-ERK Inter Assay Precision (RFU CV%) 1:1.75 Titration				
pg/mL n=6	Day 1	Day 2	Day 3	Day 4		pg/mL n=6	Day 1	Day 2	Day 3	Day 4
500	0.84	1.00	1.52	0.41		100	4.82	3.25	6.06	5.28
200	4.47	2.17	2.65	6.76		60	2.86	2.86	2.25	5.34
80	4.31	3.48	2.90	6.64		34	2.61	1.63	2.24	3.78
32	2.22	2.10	2.37	4.38		19	3.34	0.98	1.88	4.06
13	1.23	1.12	3.24	4.31		10	2.13	1.16	1.30	2.55
5	2.48	1.43	3.01	3.75		6	2.57	1.89	2.44	2.48
2	5.35	1.58	2.01	2.74		4	2.09	5.32	2.65	2.36
0	4.87	2.52	5.89	4.23		0	4.74	5.77	5.42	3.56

Figure 6 (C,D,E) – Excellent inter- and intra-assay performance is supported by Z' values consistently ≥ 0.951, RFU CV% values for all standards ≤ 5% (intra 6D) or ≤ 6% (inter 6E), and S/B values 30+ fold higher than the COA[2] in all cases.
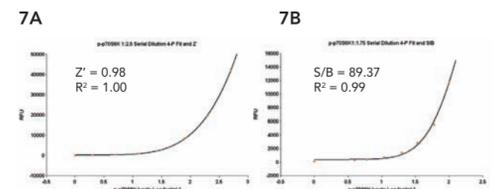

Figure 7 (A,B) – Inter-assay data for p-p70S6K data also shows excellent assay performance as indicated by a high Z' value; S/B 12+ fold higher than the COA[2]; 4-P Fit R² values ≥ 0.99; high correlation of the standard points to the fit; and low deviation within replicate groups.
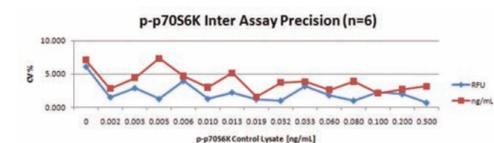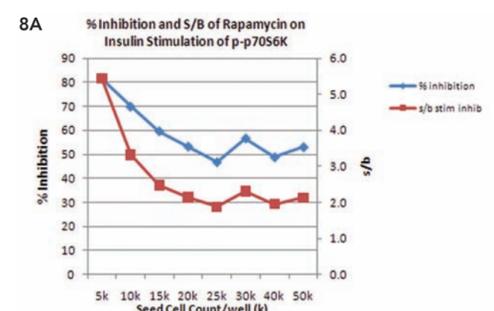

Figure 7 (C) – High precision for p-p70S6K inter-assay data is shown here for both RFU and back-calculated pg/mL CV% values. Most points are well below 5%, with the others all ≤ 7.5%. 10% is considered a standard cutoff for ELISA detection values. Back-calculated values were reduced from the 4-P fit algorithm shown by Figure 7(A,B).

Figure 8 (A,B) – Data shown comparing two different cell counts and three different cell aspiration protocols. Figure 5 illustrates the workflows utilized. Automation of cell seeding remains constant. Correlation and reproducibility of quality indicators (%Inhibition and S/B for agonist/antagonist) confirms acceptable instrument performance of routine cell handling tasks.

	Cell Count	% Inhibition	S/B	Asp Steps
Run 1	15k	60.0	2.5	1
Run 2	15k	65.4	2.9	2
Run 1	10k	70.0	3.3	1
Run 3	10k	78.4	4.6	0

Conclusions

Component based automation highlighted here can be confidently introduced at any level of the ELISAOne protocol adding to the flexibility of this robust assay:

1. Fully automated inter- and intra-assay results are virtually indistinguishable, and show low variability, high Z' and high S/B values for multiple targets.

2. Reproducible % inhibition values between different cell seed counts and cell lysate preparation protocols support successful integration of automation for routine cell handling tasks.

3. Individual instrumentation is versatile, the data supporting repeatable performance of multiple workflow tasks at both the cell lysate preparation level and the assay target detection level.

4. 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