Application Note

Automated Processing of a Homogeneous Phospho-ERK1/2 Assay for Use as a Generic GPCR Sensor

Homogeneous LanthaScreen® Cellular Assay

Paul Held and Peter Banks, BioTek Instruments, Inc. Winooski, VT
Kristin Huwiler, Matthew Robers, and Bonnie Hanson, Life Technologies, Madison, WI

Activation of GPCRs can result in stimulation of numerous signaling networks, many of which have been established to result in phosphorylation of Extracellular-signal Regulated Kinase 1/2 (ERK1/2). As a result, measurement of ERK1/2 phosphorylation can serve as a surrogate for GPCR activation. We have combined BacMam-mediated gene delivery of a GFP-ERK2 sensor with LanthaScreen® Cellular Assay technology for a time-resolved fluorescence resonance energy transfer (TR-FRET) based measurement of intracellular phospho-ERK2 levels. Together these technologies enable a flexible platform for measuring GPCR activation in any cell line of interest. Here we demonstrate that the assay can be performed in a homogeneous format and adapted to automation.

Introduction

Drugs targeting members of the G-protein coupled receptor (GPCR) super-family represent the core of modern medicine, accounting for the majority of the best-selling drugs and roughly 40% of all prescription pharmaceuticals on the market today. Cell membrane receptors such as receptor tyrosine kinases (RTK) and G-protein coupled receptors (GPCR) mediate extracellular signals from the cell membrane to the nucleus by way of several different messenger networks. The signals are interpreted and different cellular responses are elicited depending on the duration and intensity of the signal. The extracellular-signal regulated kinase (ERK) has been shown to be a central mediator in transmitting these growth and differentiation signals from cell surface receptors from a number of different pathways (Figure 1).

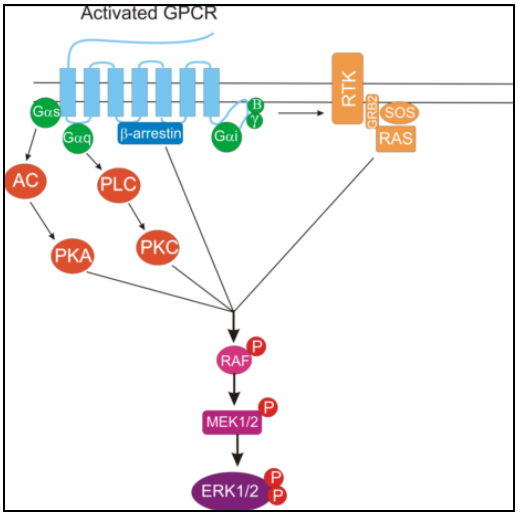

Figure 1. Depiction of MAPK/ERK1/2 activation emanating from GPCRs.

Ligand binding can result in phosphorylation of ERK1/2 by numerous mechanisms. Binding to Receptor Tyrosine Kinases (RTKs) induces recruitment of docking proteins. Ras proteins then become activated, resulting in the activation of RAF kinase, which initiates the phosphorylation cascade of MEK1/2 and ERK1/2. The MAPK/ERK pathway can also be activated by GPCR agonists.

These mechanisms can involve secondary messenger-dependent protein kinases, recruitment of beta-arrestin, or transactivation of receptor tyrosine kinase activity [1].

The phospho-activation of ERK1/2 can serve as a surrogate marker for G-protein dependent and G-protein independent signals. However, until recently HTS-compatible technologies for measuring ERK activation have been lacking. We have produced a BacMam ERK1/2 Cellular Assay that combines BacMam-mediated gene delivery of a GFP-ERK2 sensor with LanthaScreen® Cellular Assay technology for the measurement of intracellular phospho-ERK1/2 levels.

The BacMam virus is a modified baculovirus construct. Baculovirus is an insect cell virus that is non-toxic to mammalian cells, while at the same is incapable of replicating in them. The virus construct has a CMV expression regulatory element that provides high levels of transient expression for approximately 5 days. The virus construct, which tolerates large inserts (up to 38kb), is also capable of transducing hard to transfect cell lines such as primary or stem cells (Figure 2).

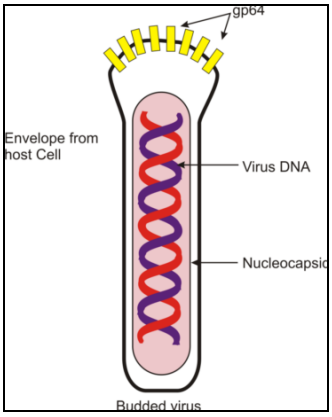

Figure 2. BacMam virus

The BacMam ERK1/2 Cellular Assay transduces live cells using BacMam ERK2 virus, which results in expression of a GFP-ERK2 fusion protein (Figure 3A). When the cells expressing the GFP-ERK2 are stimulated to phosphorylate ERK2 and subsequently lysed, the Terbium (Tb)-labeled detection antibody binds the dually phosphorylated (Thr185, Tyr187) moiety on ERK2. This association allows energy transfer to occur between the excited state Tb fluorophore and Green Fluorescent Protein (GFP), leading to an increase in TR-FRET signal (Figure 3B).

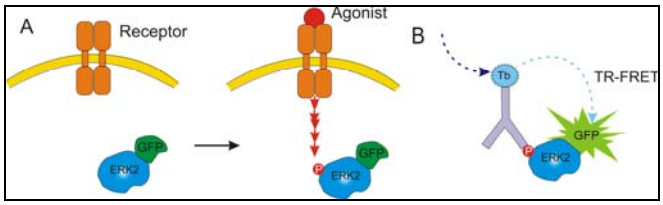

Figure 3. LanthaScreen® Cellular Assay Schematic. (**A**) Live cells expressing GFP-ERK2 fusion protein are stimulated to promote ERK phosphorylation. (**B**) LanthaScreen cellular lysis solution containing a Tb-labeled anti-phospho-ERK2 [pThr185/pTyr187] antibody is added to the cells. Binding of the antibody provides the close association necessary for energy transfer from the excited donor fluorophor Tb to GFP, leading to an increase in TR-FRET signal.

Therefore, the phospho-ERK1/2 sensor uses a homogeneous TR-FRET immunoassay to detect a specific post-translational modification of the ERK2 protein in a cell-based format.

Materials and Method

The ERK1/2 phosphorylation assays were carried out as described by Huwiler *et. al.* [2] modified such that the media was not removed prior to the addition of lysis buffer. Briefly, cell lines stably expressing a GPCR were transduced with BacMam ERK2 reagent and cryopreserved. The BacMam-treated cells were thawed the day before the experiment, washed in low serum (0.1%) assay media, seeded into 384-well plates (25 µL media/well), and serum starved overnight. Media with or without agonist was added (5 µL) separately using the syringe pump of the MicroFill and incubated at RT for 6-8 minutes, depending on the GPCR cell line. Cells were then lysed directly (without media aspiration) by the addition of (6 µL) concentrated lysis buffer containing LanthaScreen® Tb-anti-ERK2 [pThr185/pTyr187] antibody using the MicroFlo peristaltic pump and TR-FRET signals measured (Figure 4).

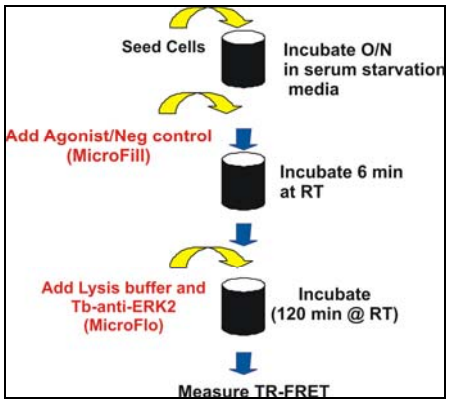

Figure 4. Automated LanthaScreen® Assay Procedure

For the D2 dopamine antagonist compound screening, cryopreserved D2 dopamine cells transduced with BacMam ERK2 were plated in 384-well plates (15,000 cells/well) and incubated overnight in assay media. The following day, the Tocris compound set (1120 compounds) was added to the cells at a final concentration of 10 µM. The D2 dopamine agonist was then added to the cells using the MicroFill at a final concentration of 100 nM. Cells were incubated for 8 minutes at RT prior to the addition of lysis buffer containing the Tb-anti-ERK2 [pThr185/pTyr187] antibody using the MicroFlo and the TR-FRET signal determined 2 hours later. The screen was then repeated on a second day. Compounds which showed inhibitory activity below the cutoff on both days were scored as “hits”. For each screen the hit rate was set to 6% (76/1120 compounds).

Results

Initial experiments tested the ability of the syringe pump of the MicroFill or the peristaltic pump of the MicroFlo to dispense the ERK1/2 lysis buffer. Figure 5 demonstrates that either dispenser is capable of dispensing low volumes (5 µL) of the viscous lysis buffer accurately and precisely when using fluorescein as a tracer. Little difference in signal regardless of the dispenser was observed along with %CVs that were less than 2% (data not shown).

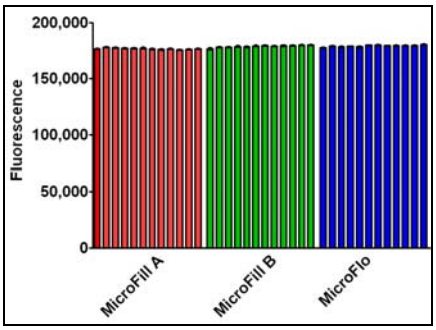

Figure 5. Dispense Uniformity of Reagent Dispensers. The different dispenser modules were used to dispense 5 µL of lysis buffer containing fluorescein dye into 192 wells of a 384-well microplate. The fluorescence was determined using a Synergy 2 Multi-Mode Microplate Reader. Each data points represent the mean of 16 wells.

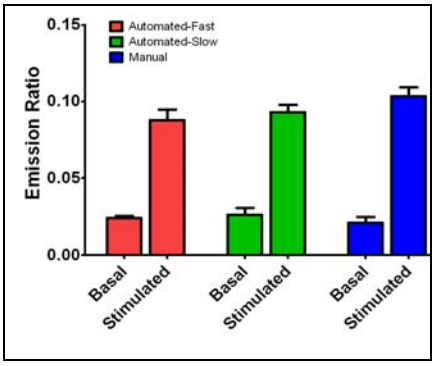

Figure 6. Comparison of Automated and Manual Procedures. Using HTR1A GeneBLAzer® CHO cells that were transduced with BacMam ERK2 reagent, reagents were added manually of by using a MicroFill and MicroFlo Dispensers.

The dispensers were compared to manual pipetting for their ability to elicit an agonist pERK1/2 response. The MicroFill was used to dispense 5 µL of either serum, which was used as the agonist or media, used as the basal control, followed by the addition of lysis buffer containing Tb-anti-ERK2 antibody using a MicroFlo. As shown in Figure 6, the use of dispensers to automate the reagent dispenses in the LanthaScreen assay produces comparable results to that obtained with manual reagent pipetting (Figure 6). Concurrently, slow and fast automated dispense-rates were compared and found to equivalent. A statistical comparison shows that the automated methods have a slightly smaller response ratio, but have equivalent z' factors (Table 1).

	MicroFlo		
	Fast Dispense	**Slow Dispense**	**Manual Dispense**
Z' factor	**0.62**	**0.57**	**0.64**
Response Ratio	**3.62**	**3.51**	**4.84**

Table 1. Statistical Comparison between Automated and Manual Liquid handling.

A typical experiment where the MicroFlo was used to add lysis buffer containing Tb-anti-ERK2 antibody is shown in Figure 7. U2OS cells were transduced with different concentrations of BacMam virus and then stimulated by different concentrations of EGF. In these experiments the cellular response is as expected for increasing amounts of virus. Only the cells that received virus responded to EGF stimulation and the response was viral load dependant (Figure 7).

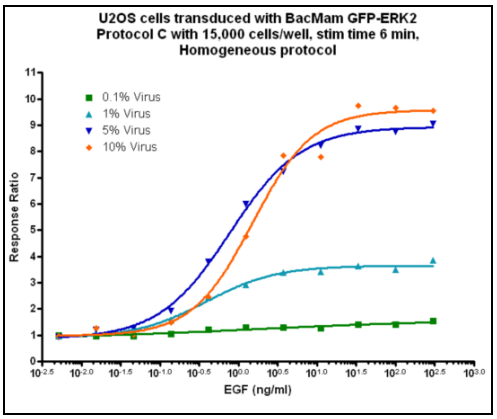

Figure 7. U2OS cells transduced with BacMam ERK2 virus at four different concentrations. The following day cells were stimulated with EGF for 6 min. followed by lysis with Cellular LanthaScreen® Lysis buffer containing Tb-anti-phospho-ERK2 antibody. TR-FRET signal was determined and data normalized to the basal response ratio.

Using the MicroFill and MicroFlo to automate the reagent additions a compound library screen for D2 dopamine antagonists was performed. A typical scatter plot of the data is presented in Figure 8. Response ratios were determined for the 1120 compounds tested and a cut off value was determined such that 6% of the compounds (76/1120) were scored as having inhibitory activity. The Tocris library is biased towards GPCR and ion channel binding compounds, so the 6% cut off criteria was deemed acceptable. The screen was then repeated a second time. As seen in Figure 8, most compounds either did not specifically interact with the D2 dopamine receptor or were known agonists. These compounds had emission ratio (GFP/Tb) of 0.06-0.08. The cut off value that scored 6% of the compounds, which was approximately 0.03, has also been plotted (Trendline). The screen was then repeated and only compounds that were below the cut off for both screens were deemed to be true inhibitors (Figure 8).

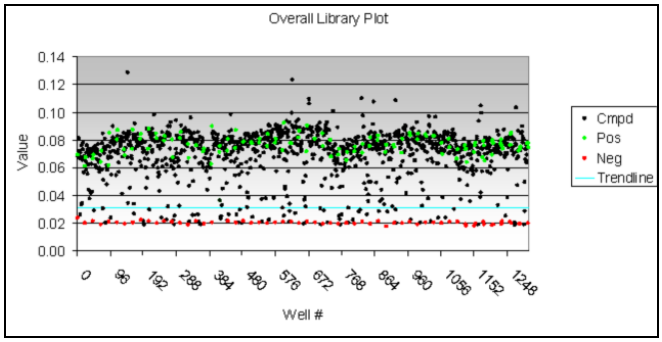

Figure 8. Library Screening Plot. Phospho-ERK1/2 values from a single screen, with cut off value indicated. Eventual results indicating multiday inhibitors are depicted as red data points.

The statistics from the plate controls for both the initial screen and the repeat screen indicate the robustness of the assay when using automated dispensers to add reagents. The Z'-factor statistics are all 0.5 or greater for all of the plates tested (Table 2).

	Z' factor	
Plate	Day 1 Screen	Repeat Screen
1	0.61	0.50
2	0.53	0.65
3	0.59	0.63
4	0.61	0.70

Table 2. Screening Statistics. Z'-factor calculations generated from control wells for each 384-well plate.

Of the 67 compounds were scored as hits after the first screen all of the know D2 antagonists were identified. With the follow up repeat-screen, 55 compounds identified as hits from the initial screen were also identified as inhibitors on both days. Of these 55 compounds identified, 20 were reported dopamine receptor inhibitors and 4 were known MEK inhibitors. In both screens all of the known D2 antagonist inhibitors were identified.

Discussion

These data indicate that the available dispenser options, such as the MicroFill and MicroFlo, from BioTek are capable of automating the addition of reagents for the LanthaScreen® ERK2 cellular assay. The dispensers are capable of accurately and precisely providing the appropriate amounts of reagent. In side-by side comparison, the use of the MicroFlo and MicroFill Dispensers for the ERK1/2 LanthaScreen® cellular assay produced lower errors than manually performing the assay, as indicated by lower standard deviations. While the use of the MicroFlo and MicroFill Dispensers for an ERK1/2 LanthaScreen® cellular assay produced a slightly lower response ratio than when the assay was performed manually, the Z' values were comparable through improved precision.

The use of this instrument and assay combination can provide meaningful biological information. The ability to use the BacMam system to transiently transduce numerous cells lines with ERK2-GFP chimeric genes allows the investigator to study a wide variety of studies using the ERK1/2 LanthaScreen assay. For example, biologically focused compound libraries can be easily and accurately screened for D2 antagonist activity using the MicroFill and MicroFlo Dispensers in combination with the LanthaScreen® cellular phospho-ERK2 assay. These cell-based assays require the addition of reagent in a timely fashion after a short incubation. The use of highly accurate and precise automated dispensers makes the running considerably less arduous and more accurate than if attempted with manual pipettors.

References

[1] Luttrell, D.K. and L.M. Luttrell (2003) Signaling in Time and Space: G Protein-coupled Receptors and Mitogen-acitivated Protein Kinases. Assay Drug Dev. Technol. 1:327-338.

[2] Huwiler, K.G., T. Machleidt, L. Chase, B. Hanson, and M.B. Robers (2009) Characterization of Serotonin 5-hydroxytryptamine-1A Receptor Activation Using a Phospho-extracellular-signal Regulated Kinase 2 Sensor. Anal. Biochem. 393:95-104.

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