

## Abstract

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. Activation of GPCRs can result in stimulation of numerous signaling networks, several of which have been established to result in phosphorylation of Extracellular Regulated Kinase 1/2 (ERK 1/2). As a result, measurement of ERK 1/2 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 the cell line of interest. Here we demonstrate that the assay can be performed in a homogeneous format and adapted to automation. Details regarding workflow, assay performance, and application to GPCR antagonist identification will be illustrated.

## Introduction

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 3). As such 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 ERK2 Cellular Assay that combines BacMam-mediated gene delivery of a GFP-ERK2 sensor with LanthaScreen® Cellular Assay technology for the measurement of intracellular phospho-ERK2 levels.

The BacMam ERK2 Cellular Assay transduces live cells using BacMam ERK2 virus, which results in expression of a GFP-ERK2 fusion protein (Figure 3). When these 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 (Figure 5). 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 5). Therefore, the phospho-ERK2 sensor uses a homogeneous TR-FRET immunoassay to detect a specific post-translational modification of the ERK2 protein in a cell-based format.

## BioTek Instrumentation

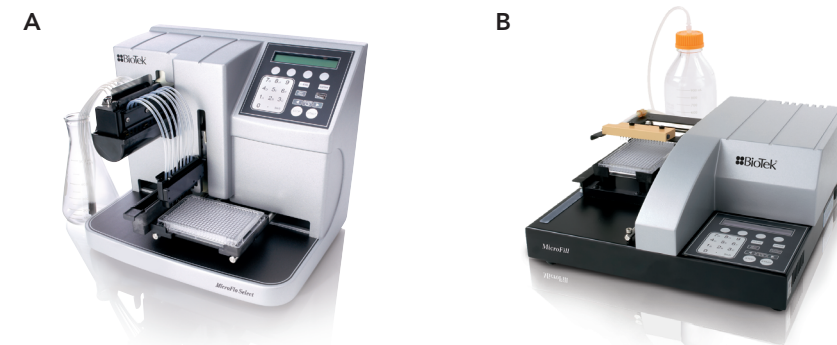


Figure 1 – MicroFlo Select and MicroFill Reagent Dispensers.

(A) The MicroFlo Select offers fast, accurate reagent dispensing capabilities through the use of its peristaltic pump, with volumes ranging from 1-3000 µL/well. The instrument was used to add agonist, as well as dispense Tb-AntiERK2 antibody Lysis buffer to the 384-well cell plates. (B) The MicroFill provides accurate reagent dispensing using a syringe pump with an autoclaveable fluid path.

## BacMam Delivery Method

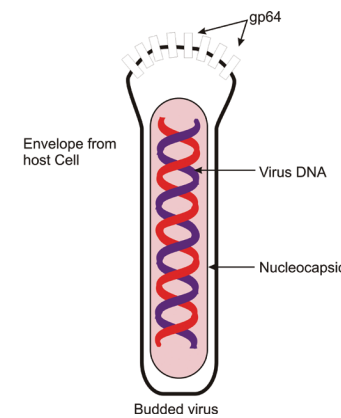


Figure 2 – BacMam virus.

- Baculovirus is an insect cell virus
- Non-replicating in mammalian cells
- Biosafety level-1
- High, CMV-driven expression
- Non-toxic to cells
- Easy to use; just add virus
- Transient expression for 5 days
- Transduces hard-to transfect cells
  - Primary cells
  - Stem cell
- Tolerates large inserts up to 38 kb

## GPCR-mediated phospho-ERK2 Activation

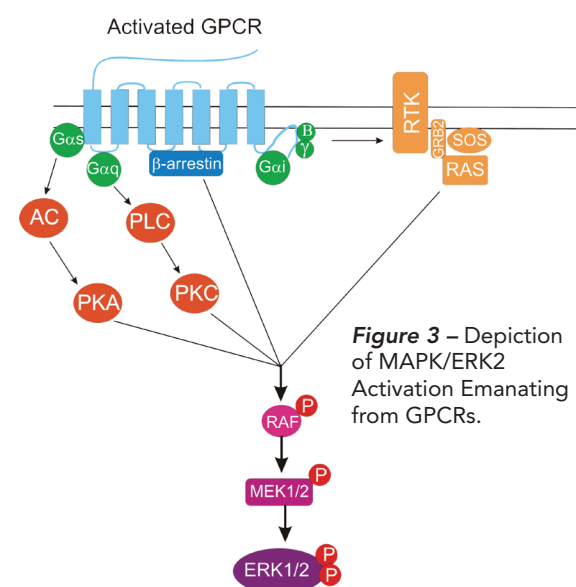


Figure 3 – Depiction of MAPK/ERK2 Activation Emanating from GPCRs.

Ligand binding can result in phosphorylation of ERK2 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.

## LanthaScreen® Cellular Assay Schematic

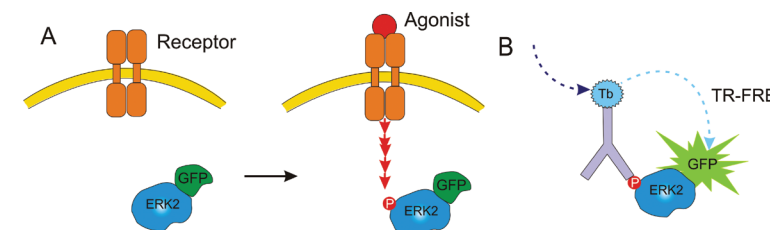


Figure 4 – LanthaScreen® cellular assay schematic.

(A) Schematic depiction of agonist-mediated Receptor activation leading to ERK2 phosphorylation. Live cells expressing GFP-ERK2 fusion protein are stimulated to promote ERK phosphorylation. (B) Following stimulation, a 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 fluorophore Tb to GFP, leading to an increase in TR-FRET signal. The result is a lysate-based immunoassay in which GFP serves as a FRET acceptor for the Tb-labeled phospho-ERK2 antibody donor.

## LanthaScreen® Assay Procedure

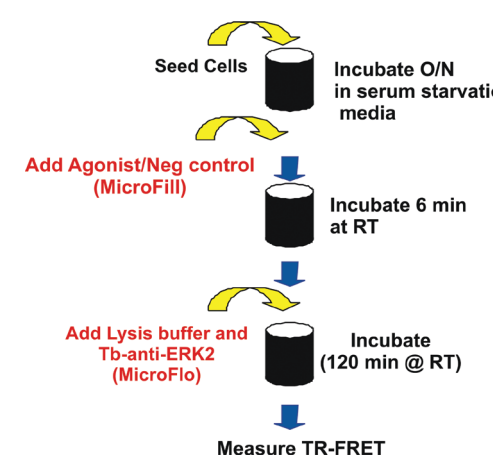


Figure 5 – Automated LanthaScreen® assay procedure.

A cell line stably expressing a GPCR was 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.

## Uniformity of Dispense

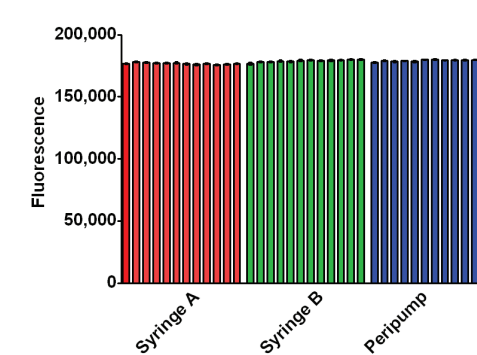


Figure 6 – Dispense uniformity of reagent dispensers.

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 point represents the mean of 16 wells.

## Emission Ratio Comparison

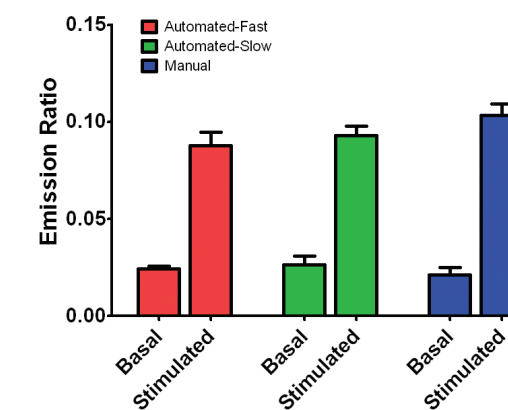


Figure 7 – Comparison of Automated and Manual Procedures using HTR1A GeneBLazer® CHO cells that were transduced with BacMam ERK2 reagent.

	MicroFlo		Manual
	Fast Dispense	Slow Dispense	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.

## ERK2 Response to EGF Stimulation

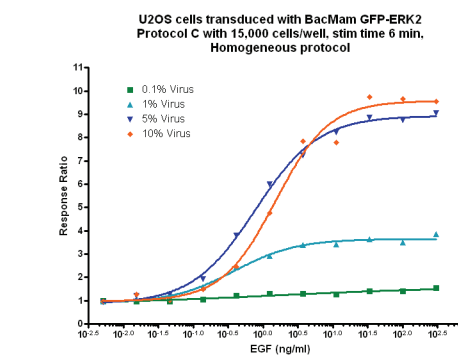


Figure 8 – U2OS cells transduced with BacMam ERK2 virus at four different concentrations. The following day cells were stimulated with EGF for 6 minutes followed by lysis with Cellular LanthaScreen® Lysis buffer containing Tb-anti-phospho-ERK2 antibody. TR-FRET signal was determined and data normalized to the response ratio.

## D2 Dopamine Receptor Antagonist ERK2 Screening

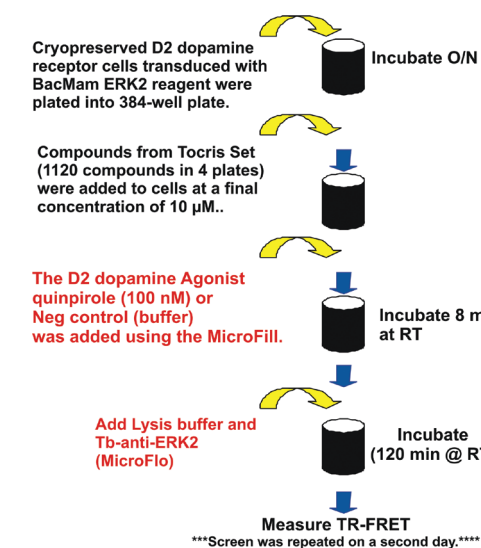


Figure 9 – D2 dopamine antagonist screen procedure.

## Screening Results

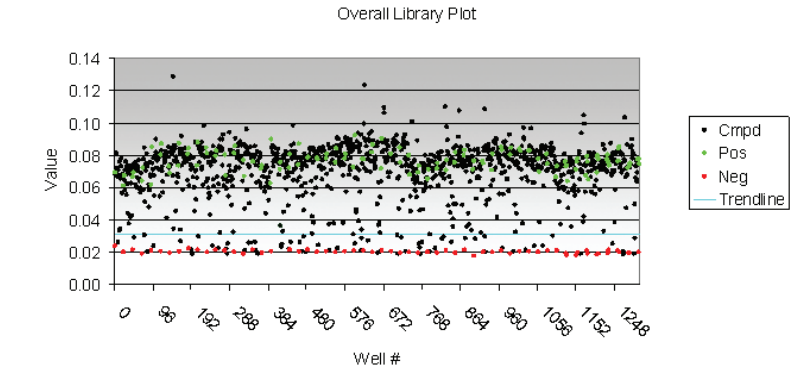


Figure 10 – Library screening plot. Phospho-ERK2 values from a single screen, with cut off value indicated. Eventual results indicating multiday inhibitors are depicted as red data points.

Day 1	
Plate	Z'
1	0.61
2	0.53
3	0.59
4	0.61

- 67/1120 Compounds scored as inhibitors in preliminary screen
- All D2 antagonists were detected

Follow-up Screen	
Plate	Z'
1	0.50
2	0.65
3	0.63
4	0.70

- 55/1120 compounds were inhibitors on both days
- 20/55 were reported dopamine receptor inhibitors
- 4/55 were reported MEK inhibitors
- All D2 antagonists were detected

Table 2 – Screening statistics. Compounds which showed inhibitory activity below the cutoff on both days were scored as "hits". For each screen, cutoff was set at 6% (67/1120 compounds). The Toctris library is biased towards GPCRs and ion channels, so this hit rate is reasonable.

## Conclusions

- The available dispenser options from BioTek are capable of providing equivalent amounts of LanthaScreen® reagent.
- Use of the MicroFlo and MicroFill Dispensers for the ERK2 cellular LanthaScreen® assay produced lower errors than manually performing the assay, in a side-by-side experiment.
- Use of the MicroFlo and MicroFill Dispensers for an ERK2 cellular LanthaScreen® assay produced a slightly lower response ratio than when the assay was performed manually, but Z' values were comparable through improved precision.
- There is a slight performance advantage to using Fast Dispense speed with the MicroFlo when adding Tb-antibody containing lysis buffer.
- The MicroFlo and MicroFill Dispensers are capable of automating the addition of agonist and the addition of a LanthaScreen® cellular lysis buffer containing Tb-labeled-antibody.
- Use of the instrument and assay combination can provide meaningful biological information.
- 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.

