Representative Data for

BacMam ERK2 [pThr185/pTyr187] Cellular Assay

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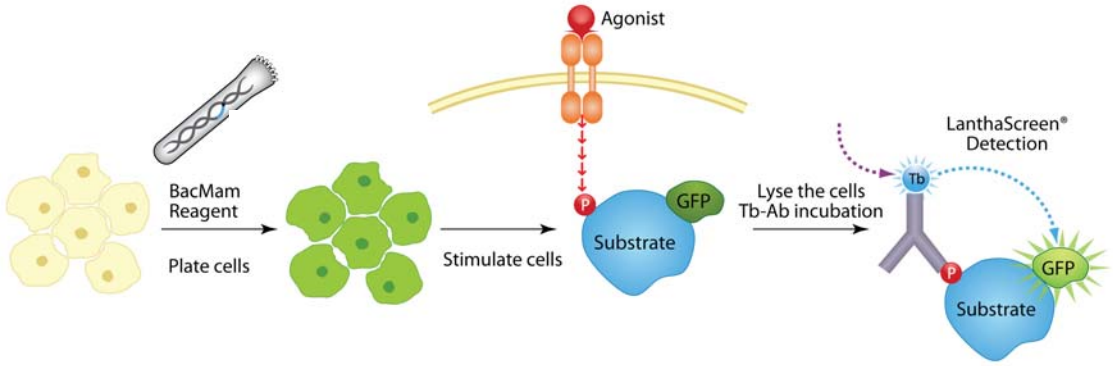
Overview

Introduction to BacMam. LanthaScreen® Cellular Assays can now be used together with a gene delivery method known as BacMam for the transient expression of the desired GFP-substrate fusion in many cell lines of interest. The BacMam delivery system utilizes modified baculovirus as a vehicle to deliver and express target genes in mammalian cells. This technology has several advantages over commonly used transient methods for heterologous gene expression, including: 1) high transduction efficiency across a broad range of cell types, including primary and stem cells; 2) little-to-no observable cytopathic effects; 3) reproducible and titratable target gene expression; 4) the virus is non-replicating in mammalian cells, therefore it is designated as Biosafety Level 1 (BSL1); and 5) compatible with simultaneous delivery of multiple genes. Thus, the combination of the LanthaScreen® Cellular Assay technology and BacMam-mediated gene delivery provides a fast, convenient, and robust method for interrogating specific signal transduction events in a cell background of choice. Please see Kost, et al for more information pertaining to BacMam gene expression in cells.



Schematic illustration of the workflow of BacMam-enabled Cellular Assay using LanthaScreen® Technology. Cells are treated with BacMam virus encoding a GFP-fusion protein and plated in a 384-well assay plate. 24-48 hours post-transduction, the cells are stimulated to induce the specific post-translational modifications (such as phosphorylation as shown) of the GFP-substrate fusion. Cells are then lysed in the presence of terbium-labeled anti-modification specific antibody and TR-FRET from terbium to GFP can be detected.

BacMam-enabled ERK2 [pThr185/pTyr187] Cellular Assay using LanthaScreen® technology

The BacMam-enabled ERK2 Assay is designed for the interrogation of the mitogen-activated protein kinase pathway (MAPK). Within this pathway, Extracellular Regulated Kinase-2 (ERK2) phospho-activation is known to be mediated via receptor tyrosine kinases as well as G-protein-coupled receptors (GPCRs) agonism. BacMam-enabled ERK2 assay provides a broadly applicable robust assay platform for the analysis of numerous signal events in variety of cell types.

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The validation of the BacMam ERK2 Cellular Assay was performed in a 384-well format using U-2 OS as well as CHO cells stably expressing the 5HT1A receptor. Following stimulation with EGF (U-2 OS) or 5-HT (CHO 5HT1A), the phosphorylation status of GFP-ERK2 was then measured in lysates using LanthaScreen® Tb-anti-ERK2 [pThr/pTyr 185/187] antibody as a detection reagent. Included are representative results using this assay, including an example of the assay optimization process and examples of assays performed using various cell backgrounds. Please see Huwiler, K., et al for more data for GPCR applications.

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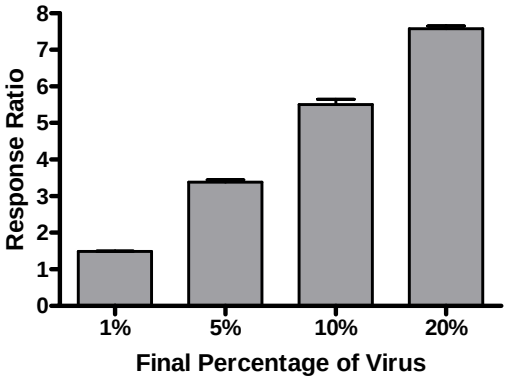
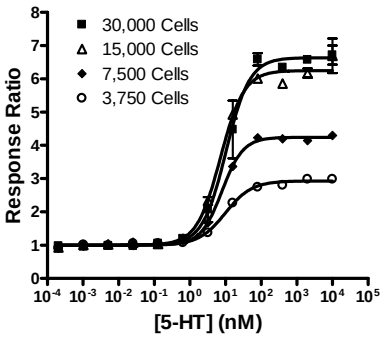
1. Representative assay optimization and validation for CHO-K1 5-HT1A cells (via HTR1A activation)

Figure 1a — Optimization of BacMam ERK2 Transduction



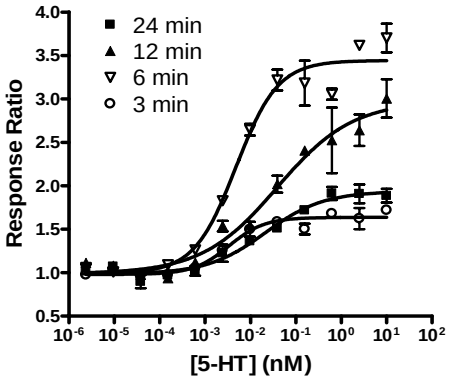
Measurement of 5-HT induced ERK2 phosphorylation using the BacMam ERK2 [pThr185/pTyr187] Cellular Assay. CHO-K1-5-HT1A cells (Invitrogen, K1712) were transduced with BacMam ERK2 reagent at the indicated concentrations (v/v) for 4 hrs at room temperature in the dark. Cells were then incubated with the BacMam Enhancer solution (Invitrogen, PV5835) for 22 hrs at 37°C/5%CO_2. Cells were then resuspended in low-serum assay medium (OPTI-MEM + 0.1% dFBS) at a density of 0.75 x 10^6 cells/mL. 20 µL was then added to each well of a white, tissue culture treated 384-well plate (Corning #3570). This corresponds to approximately 15,000 cells per well. Following 4 hr incubation, cells were left untreated or treated with 0.3 µM 5-HT for 6 min (final total volume in a well was 30 µL). Cells were lysed with 6 µL/well of fully-supplemented 6 x lysis buffer containing 12 nM Tb-anti-ERK2 [pThr185/pTyr187] antibody and equilibrated for 3 hr at room temperature in the dark. Following lysis, TR-FRET was measured on a BMG Labtech PHERAstar plate reader. Emission ratios represent the time-gated fluorescence intensity at 520 nm / 490 nm. Response ratios are calculated as the emission ratio of 5-HT stimulated sample divided by that of unstimulated sample. (n=6)

Figure 1b – Optimization of cell number per well



Variation in the number of cells per well can affect the assay window. CHO-K1 cells stably expressing 5-HT1A receptor transduced with BacMam ERK2 were seeded in 384-well assay plate at the indicated number of cells per well and serum-starved overnight (in OptiMEM + 0.1% serum) before being treated with serial dilutions of agonist (5-HT) for 6 minutes. Cells were then lysed by addition of lysis buffer containing LanthaScreen® Tb-anti-ERK2 [pThr185/pTyr187] antibody. Following equilibration, TR-FRET signals were measured on a BMG PHERAStar Fluorescence Plate Reader. Emission ratios represent the time-gated fluorescence intensity at 520 nm / 490 nm. Response ratios are calculated as the emission ratio of 5-HT stimulated sample divided by that of unstimulated sample.

Figure 1c — Optimization of cell stimulation time



Optimal stimulation time determination. CHO-K1 cells stably expressing 5-HT1A receptor were transduced with BacMam ERK2 (5%, v/v), seeded and serum starved in 384-well plate as described in Figure 1a. Cells were stimulated with 5-HT for 24, 12, 6, or 3 minutes prior to cell lysis. Maximal assay window (response ratio) was achieved at 6 minutes.

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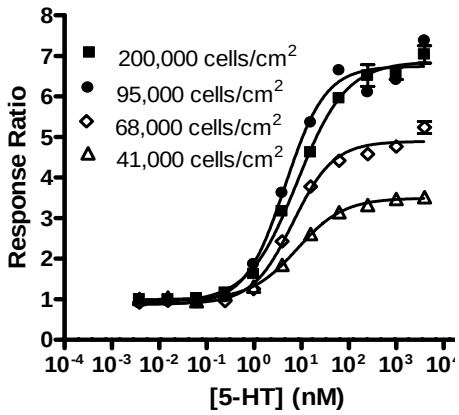
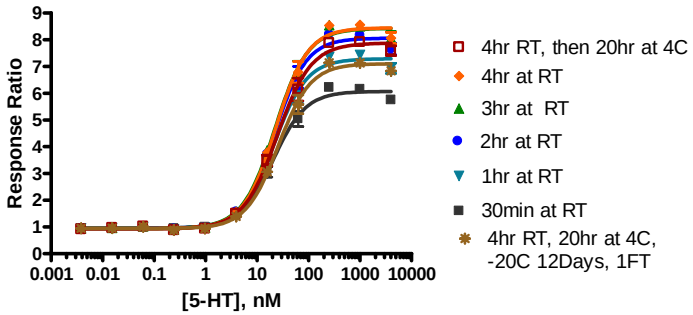
Figure 1d — Optimization of cell harvesting density prior to the assay set up



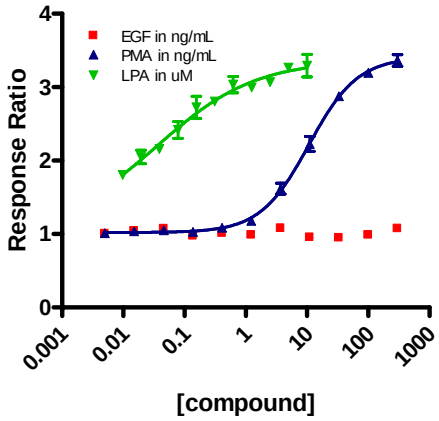
Cell harvesting density testing. CHO-K1 cells stably expressing 5-HT1A receptor grown to indicated cell density were transduced with BacMam ERK2 and seeded in a 384-well plate as described in Figure 1a. Cells were stimulated with indicated amount of 5-HT for 6 minutes. This graph shows greater cell confluence (>95,000 cells/cm^2) led to increased assay window.

Figure 1e – Assay equilibration time



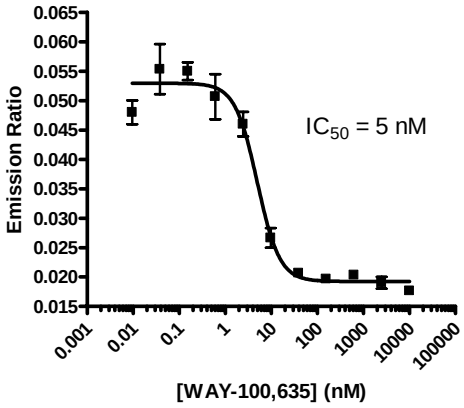
Assay equilibration time optimization Assay was set up as described in Figure 1a legend. Cells were stimulated with indicated amount of 5-HT for 6 minutes. After adding the 6x lysis buffer containing 12 nM Tb-anti-ERK2 [pThr185/pTyr187] antibody, the assay was equilibrated for the indicated amount of times at room temperature in the dark prior to reading the plate.

Figure 1f -- Alternative ligand / agonist panel



Alternative agonist testing. CHO-K1 cells stably expressing 5-HT1A receptor were transduced with BacMam ERK2 (3%), seeded and serum starved in 384-well plate as described in Figure 1a. Cells were stimulated with indicated ligands for 6 minutes prior to cell lysis.

Figure 1g— Inhibition of 5-HT-induced ERK2 activation



Measurement of 5HT1A activation using the Lanthascreen® ERK2 Cellular Assay: Inhibitor profile. BacMam ERK2 transduced CHO-K1 5-HT1A cells were plated in 384-well assay plate and serum-starved overnight. On the day of the assay, cells were preincubated 15 min with serial-diluted inhibitor compound prior to stimulation with of 5-HT for 6 minutes at room temperature (final [5-HT] = 30 nM). TR-FRET readout was performed. Error bars represent the average of at least 3 data points +/- SE.

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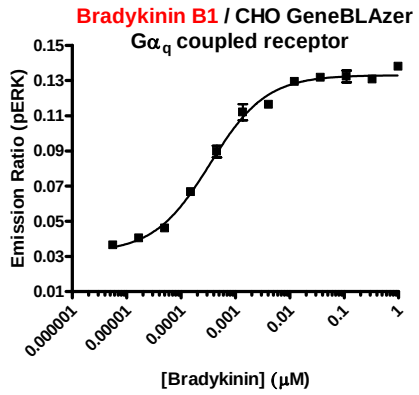
2. Validation data for ERK2 being a generic GPCR readout

Figure 2a — Measurement of $G\alpha_q$-coupled GPCR activity



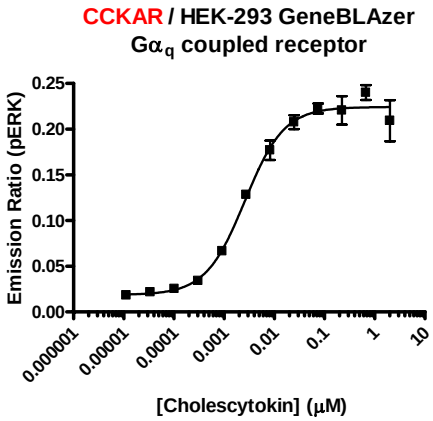


Figure 2b — Measurement of $G\alpha_{i/o}$-coupled GPCR activity

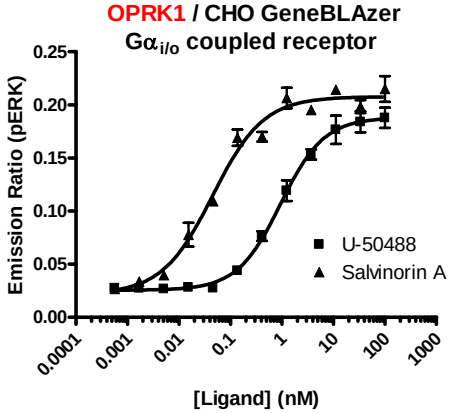
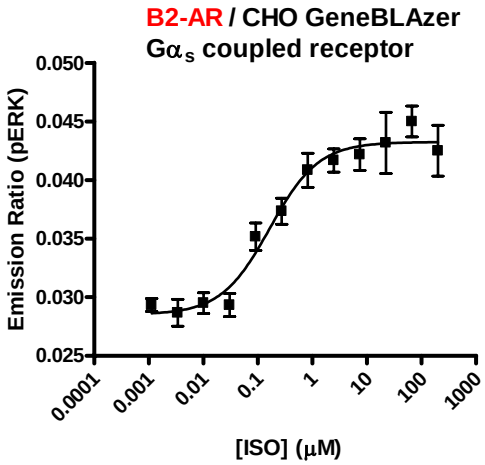


Figure 2c — Measurement of $G\alpha_s$-coupled GPCR activity



Ga_q, $Ga_{i/o}$, and Ga_s coupled Receptor Activation Analyzed using BacMam ERK2 GPCR sensor

GeneBLAzer® cell lines stably expressing a GPCR of interest were transduced by plating overnight in presence of BacMam ERK2 virus. BacMam-treated cells were then seeded in 384-well plates and serum-starved overnight (in OptiMEM + 0.1% serum) before being treated with serial dilutions of agonist for 7 minutes. Cells were then lysed by addition of lysis buffer containing LanthaScreen® Tb-anti-ERK2 [pThr185/pTyr187] antibody. Following an equilibration at room temperature, TR-FRET signals were measured on a BMG PHERAStar Fluorescence Plate Reader. Emission ratios represent the time-gated fluorescence intensity at 520 nm / 490 nm.

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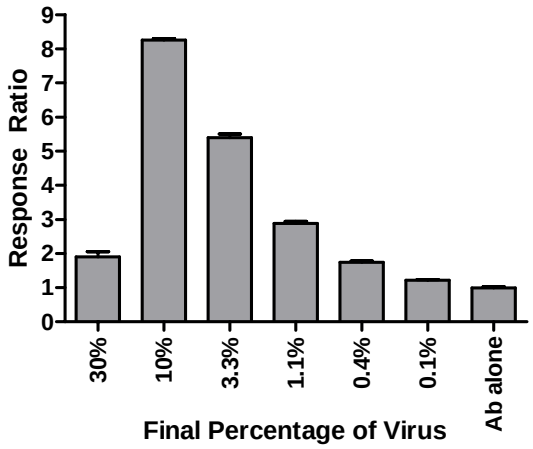
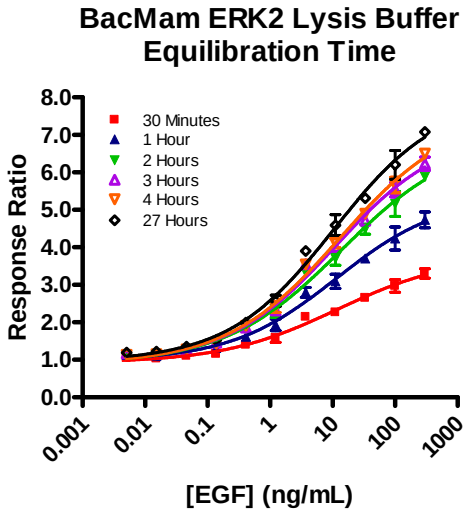
3. Representative assay optimization and validation for U-2 OS cells (via EGF/MAPK pathway)

Figure 3a — Optimization of BacMam ERK2 transduction



Measurement of EGF induced ERK2 phosphorylation using the BacMam ERK2 [pThr185/pTyr187] Cellular Assay.
U-2 OS cells were suspended in low-serum assay medium at a density of 0.6 x 10^6 cells/mL and mixed with variable concentrations of BacMam ERK2 Reagent. 20 uL was then added to each well of a white, tissue culture treated 384-well plate (Corning). This corresponds to approximately 12,000 cells per well. Following a 22 hour incubation, cells were stimulated with EGF (100ng/mL final) to a final volume of 30 uL and incubated for 6 min. Following treatment, cells were lysed with 6 uL/well of fully-supplemented lysis buffer containing 12 nM Tb-anti-ERK2 [pThr185/pTyr187] Antibody and equilibrated for 3 hours at room temperature. TR-FRET signal was measured on a BMG Labtech PHERAstar plate reader. Response ratio was calculated as the emission ratio of EGF-stimulated sample divided by the emission ratio of unstimulated sample.

Figure 3b – Assay equilibration time



Measurement of ERK2 Phosphorylation using the BacMam ErK2 [pThr185/pTyr187] Cellular Assay. U-2 OS cells were harvested, resuspended in assay medium and mixed with BacMam ERK2 Reagent to achieve 3.3% virus (v/v) concentrations. The mix was applied to designated wells of a 384-well plate, yielding a cell density of ~12,000 cells per well. Following a 24 hour incubation, cells were treated for 6 minutes with a serial dilution of EGF. Cells were lysed by adding fully-supplemented 6X lysis buffer containing 12 nM Tb-anti-ERK2 [pThr185/pTyr187] Antibody and equilibrated for the designated times at room temperature before TR-FRET was measured on a BMG Labtech Pherastar plate reader.

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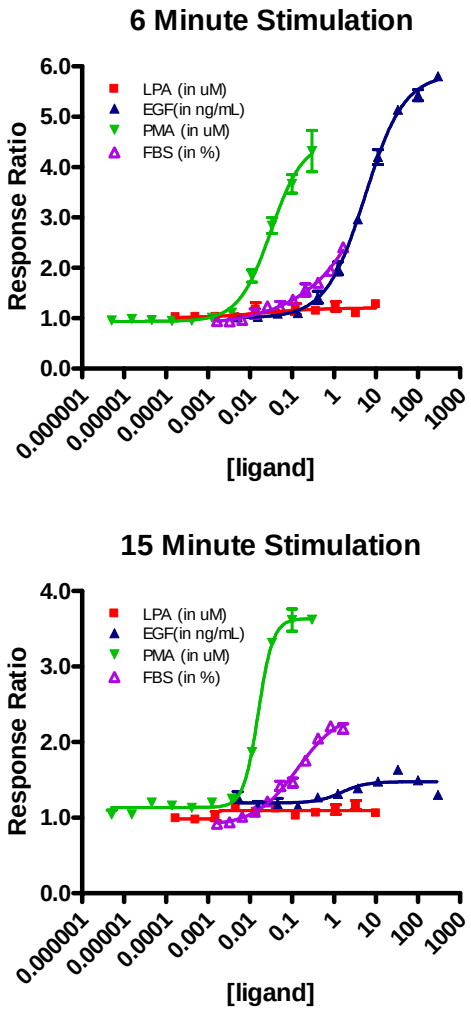
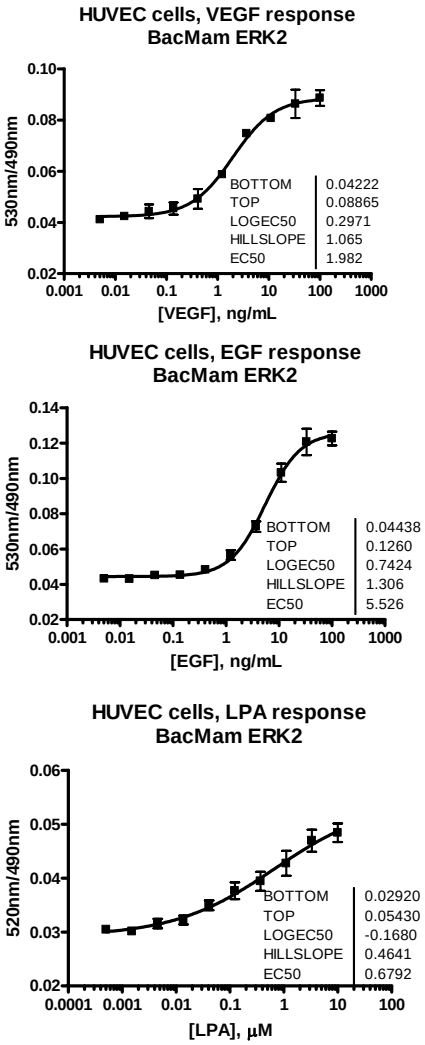
Figure 3c – Alternate agonist profiles



Measurement of ERK2 Phosphorylation using the BacMam ErK2 [pThr185/pTyr187] Cellular Assay. U-2 OS cells were harvested, resuspended in assay medium and mixed with BacMam ERK2 Reagent to achieve 3.3% virus (v/v) concentrations. The mix was applied to designated wells of a 384-well plate, yielding a cell density of ~12,000 cells per well. Following a 24 hour incubation, cells were treated for 6 and 15 minutes with a serial dilution of the indicated ligand. Cells were lysed by adding fully-supplemented 6X lysis buffer containing 12 nM Tb-anti-ERK2 [pThr185/pTyr187] Antibody and equilibrated for 3 hours at room temperature before TR-FRET was measured on a BMG Labtech Pherastar plate reader.

4. Assay Portability (Other cell backgrounds tested)

Figure 4a ERK2 Phosphorylation in HUVEC cells



ERK2 Phosphorylation in HUVEC cells HUVEC cells (Invitrogen, C0035C) were pretransduced with BacMam GFP-ERK2, plated in low-serum assay medium onto a 384-well assay plate and incubated overnight. Cells were then stimulated with various concentrations of VEGF, EGF or LPA for 10 minutes. 6 x lysis buffer containing 12 nM Tb-anti-ERK2 [pThr185/pTyr187] was added to the cells and incubated at room temperature for 2 hours. TR-FRET emission ratios were determined on a BMG PHERAstar fluorescence plate reader (excitation at 340 nm, emission 520 nm and 490 nm)

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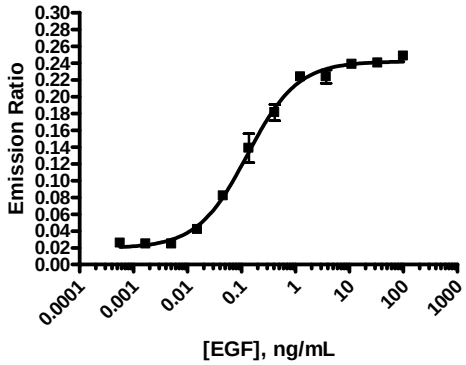
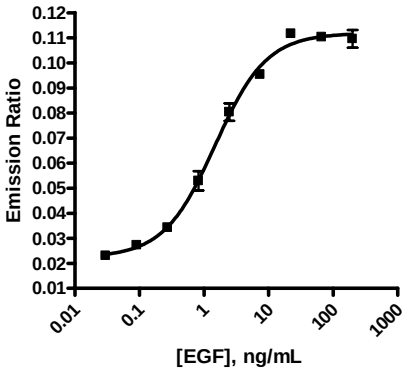
Figure 4b Human dermal fibroblasts



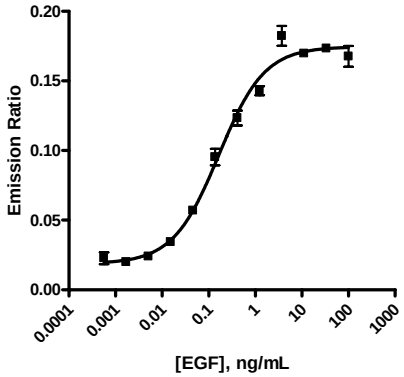
ERK2 Phosphorylation in human dermal fibroblast cells Human Dermal Fibroblasts (Invitrogen, C0045C) were harvested, resuspended in assay medium and mixed with BacMam ERK2 Reagent to achieve 5% virus (v/v) well. Following a 24 hour incubation, cells were treated for 6 minutes with a serial dilution of EGF. Cells were lysed by adding fully-supplemented 6X lysis buffer containing Tb-anti-ERK2 [pThr185/pTyr187] Antibody and equilibrated for the designated timers at room temperature before TR-FRET was measured on a BMG Labtech Pherastar plate reader.
concentrations. The mix was applied to designated wells of a 384-well plate, yielding a cell density of ~10,000 cells per

Figure 4c Adipose-derived stem cells



ERK2 Phosphorylation in Human Adipose-derived stem cells. Human Adipose-derived stem cells were transduced with BacMam GFP-ERK2, and serum starved in low-serum assay medium in a 384-well assay plate overnight. Cells were then stimulated with various concentrations of EGF for 8 minutes. 6 x lysis buffer containing 12 nM Tb-anti-ERK2 [pThr185/pTyr187] was added to the cells and incubated at room temperature for 2 hours. TR-FRET emission ratios were determined on a BMG PHERAstar fluorescence plate reader (excitation at 340 nm, emission 520 nm and 490 nm)

Figure 4d Human mammary epithelial cells



ERK2 Phosphorylation in Human mammary epithelial stem cells. Human mammary epithelial cells (Invitrogen, A10565) were pretransduced with BacMam GFP-ERK2, plated in low-serum assay medium onto a 384-well assay plate and incubated overnight. Cells were then stimulated with various concentrations of EGF for 8 minutes. 6 x lysis buffer containing 12 nM Tb-anti-ERK2 [pThr185/pTyr187] was added to the cells and incubated at room temperature for 2 hours. TR-FRET emission ratios were determined on a BMG PHERAstar fluorescence plate reader (excitation at 340 nm, emission 520 nm and 490 nm)

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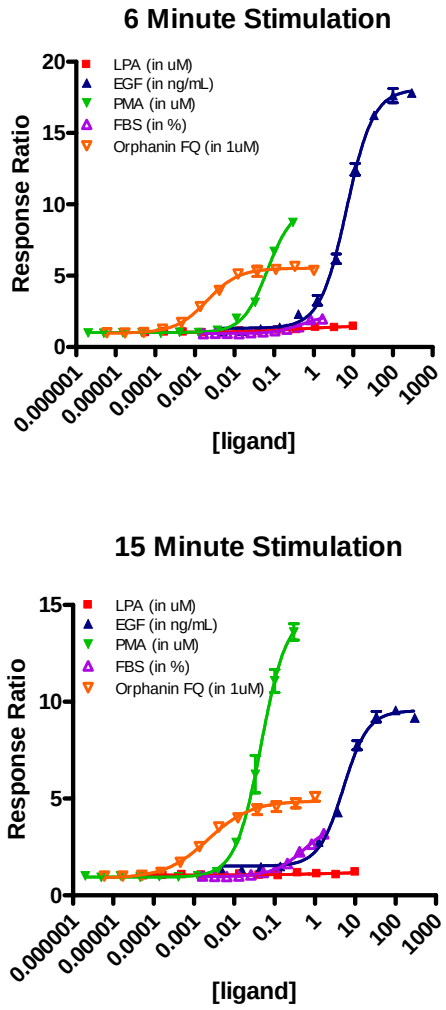
Figure 4e GeneBLAzer® OPRL-Gqi5-NFAT-*bla* FreeStyle™ 293F



Measurement of ERK2 Phosphorylation using the BacMam ErK2 [pThr185/pTyr187] Cellular Assay. GeneBLAzer® OPRL-Gqi5-NFAT-*bla* FreeStyle™ 293F (Invitrogen, K1714) cells were harvested, resuspended in assay medium and mixed with BacMam ERK2 Reagent to achieve 5% virus (v/v) concentrations. The mix was applied to designated wells of a 384-well plate, yielding a cell density of ~20,000 cells per well. Following a 24 hour incubation, cells were treated for 6 and 15 minutes with a serial dilution of the indicated ligand. Cells were lysed by adding fully-supplemented 6X lysis buffer containing Tb-anti-ERK2 [pThr185/pTyr187] Antibody and equilibrated for 3 hours at room temperature before TR-FRET was measured on a BMG Labtech Pherastar plate reader.

General References:

- Carlson, C. B.; Mashock, M.J.; Bi, K., BacMam-enabled LanthaScreen cellular assays for PI3K/Akt pathway compound profiling in disease-relevant cell backgrounds. *J Biomol Screen* **2010**, *15*, 327-334.
- Robers, M. B.; Horton, R. A.; Bercher, M. R.; Vogel, K. W.; Machleidt, T., High-throughput cellular assays for regulated post-translational modifications. *Anal. Biochem*. **2008**, *372*, 189–197.
- Huwiler, K.G.; Machleidt, T.; Chase, L.; Hanson, B.; Robers, M. B., Characterization of serotonin 5-hydroxytryptamine-1A receptor activation using a phosphor-extracellular-signal regulated kinase 2 sensor. *Anal Biocheml*. **2009**, *393*, 95-104.
- Kost, T. A.; Condreay, J. P.; Ames, R. S.; Rees, S.; Romanos, M. A., Implementation of BacMam virus gene delivery technology in a drug discovery setting. *Drug Disc Today* **2007**, *12*, 396-403.