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BacMam ERK2 [pThr185/pTyr187] Cellular Assay User Guide

Catalog no. A12888 **Shipping: Varies** **Storage: Varies**

Protocol part no. A12888.pps Revision date: 10 January 2011

Table of Contents

Kit Contents and Handling

Component	SKU#	Amount	Storage	Handling
BacMam ERK2 Reagent	A12889	2 × 25 mL	4°C	• Do not freeze • Avoid extended exposure to ambient room light • Use sterile technique • Aliquot into sterile containers to minimize handling, if necessary
LanthaScreen® Tb-anti-ERK2 [pThr185/pTyr187] Antibody	PV5833*	10 µg	–20°C	Aliquot if necessary to avoid multiple freeze/thaw cycles
6X LanthaScreen® Cellular Assay Lysis Buffer	A12891	6 mL	4°C	On the day of assay, supplement with inhibitor cocktails (not provided; see page 3) and antibody
BacMam Enhancer Solution (1000X)	PV5835	150 µL	–20°C	Aliquot if necessary to avoid multiple freeze/thaw cycles

*To order additional antibody, use catalog no. PV5269 (25 µg amount).

Overview

BacMam Cellular Assays use the BacMam gene delivery system in conjunction with LanthaScreen® Cellular Assays to measure post-translational modifications of a target substrate, as described below. The combination of the two technologies provides a fast, convenient, and robust method for interrogating specific signal transduction events in a cell background of choice.

LanthaScreen® Cellular Assay

LanthaScreen® Cellular Assays are high-throughput screening (HTS) compatible immunoassays used to interrogate target-specific post-translational modifications in a cell-based format. Target proteins are expressed as fusions with green fluorescent protein (GFP) in living cells, and modification-specific antibodies labeled with terbium (Tb) are used to detect stimulus-induced post-translational modifications in a time-resolved fluorescence resonance energy transfer (TR-FRET) format.

The use of GFP as a FRET acceptor circumvents the need to use complex antigen-capturing reagents, thereby providing a high-throughput alternative to commonly used analytical methods such as Western blot and ELISA.

For more information, visit www.invitrogen.com/lanthascreen.

BacMam Technology

BacMam technology uses a modified baculovirus to efficiently deliver and express genes (in this case, GFP-substrate fusion gene) in mammalian cells. The virus is non-replicating in mammalian cells, rendering them safe as research reagents.

This technology has several advantages over traditional transient methods for heterologous gene expression:

- High transduction efficiency across a broad range of cell types, including primary and stem cells
- Little-to-no observable cytopathic effects
- Reproducible and titratable target gene expression
- Compatibility with simultaneous delivery of multiple genes.

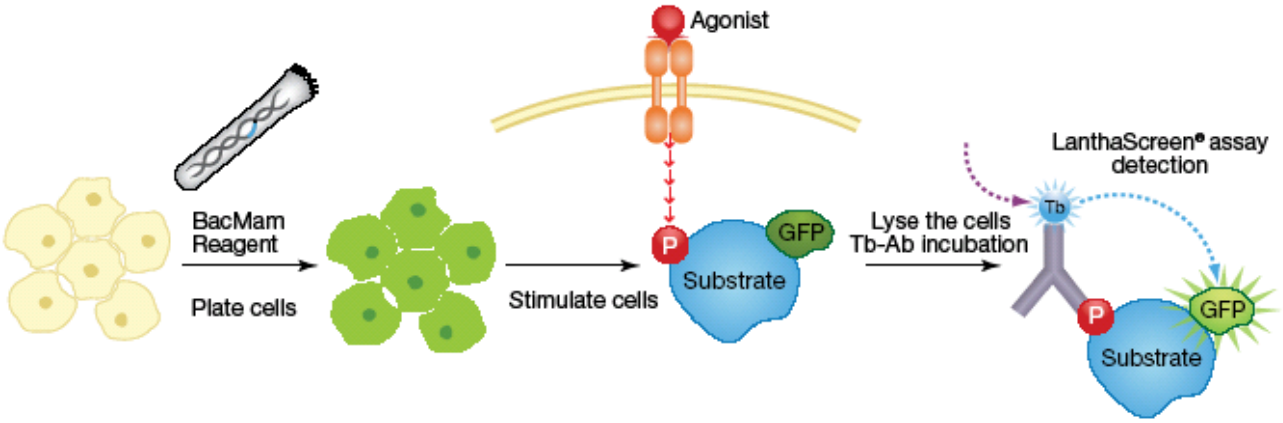

Figure 1. Illustration of Representative Assay Workflow. Cells are treated with BacMam reagent encoding a GFP-fusion protein and plated in 384-well format; 24 hours post-transduction, the cells are stimulated to induce post-translational modification of the GFP-substrate (e.g., phosphorylation as shown). Cells are then lysed in the presence of a terbium-anti-modification-specific antibody prior to the LanthaScreen® assay readout.

See Kost, TA et. al *Drug Disc. Today* **2007**, *12*, 396-403 for more information on BacMam gene expression in cells. For more information on BacMam, visit www.invitrogen.com/bacmam.

Intended Use

For research use only. Not intended for human or animal diagnostic or therapeutic uses.

Before Starting

Materials Required but Not Provided

Materials	Recommended Source	Part #
Positive Control Growth Factor **We recommend:** EGF (if using U-2 OS cells)	Invitrogen	PHG0314
Positive Control GPCR agonist **We recommend:** 5-HT (if using CHO cells expressing 5-HT1A)	Sigma	H9523
Cell line of interest	various	various
DMSO	Fluka	41647
Protease Inhibitor Cocktail	Sigma	P8340
Phosphatase Inhibitor Cocktail	Sigma	P0044
White tissue culture-treated, 384-well assay plates	Corning	3570
Fluorescence plate reader with top-read and TR-FRET capability	Visit www.invitrogen.com/instrumentsetup	
Optional: Clear-bottom, tissues culture treated, 384-well assay plates for visualization of GFP-fusion	Corning	3712

CHO Cell Culture Reagents (optional)

Media/Reagents	Recommended Source	Part #
GeneBLAzer® 5HT1A Gα15-NFAT-*bla* CHO-K1 Cells (CHO cells expressing 5-HT1A Receptor)	Invitrogen	K1712
Dulbecco's Modified Eagle Medium (D-MEM) (1X), liquid (with GlutaMAX™-I)	Invitrogen	10569-010
Opti-MEM® I Reduced-Serum Medium (with HEPES and L-glutamine, without Phenol Red)	Invitrogen	11058-021
Fetal Bovine Serum (dialyzed)	Invitrogen	26400-036
Nonessential amino acids (NEAA)	Invitrogen	11140-050
Sodium Pyruvate	Invitrogen	11360-070
Penicillin/Streptomycin (antibiotic)	Invitrogen	15140-122
Dulbecco's Phosphate-buffered saline (D-PBS) without Ca^{2+} and Mg^{2+}	Invitrogen	14190-136
Dulbecco's Phosphate-buffered saline (D-PBS) with Ca^{2+} and Mg^{2+}	Invitrogen	14040-133
HEPES Buffer Solution (1 M)	Invitrogen	15630-080
Trypsin/EDTA	Invitrogen	25300-062

U-2 OS Cell Culture Reagents (optional)

Media/Reagents	Recommended Source	Part #
U-2 OS cells	ATCC®	HTB-96™
Opti-MEM® I Reduced-Serum Medium (with HEPES and L-glutamine, without Phenol Red)	Invitrogen	11058-021
McCoy's 5A Medium (modified) (1X), liquid	Invitrogen	16600-108
Fetal Bovine Serum (dialyzed)	Invitrogen	26400-036
Nonessential amino acids (NEAA)	Invitrogen	11140-050
Sodium Pyruvate	Invitrogen	11360-070
Penicillin/Streptomycin (antibiotic)	Invitrogen	15140-122
Dulbecco's Phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+}	Invitrogen	14190-136
HEPES Buffer Solution (1 M)	Invitrogen	15630-080
Trypsin/EDTA	Invitrogen	25300-062

Guidelines for Working with BacMam Reagent

- For first time users of BacMam reagent, we recommend including a control cell line that can be transduced exceptionally well, such as U-2 OS (ATCC® catalog number HTB-96™).
- Most cell types can be transduced efficiently using the protocol described for U-2 OS cells. However, some challenging cell types require the alternative protocol described for CHO cells.
- BacMam Enhancer Solution is not required for this BacMam reagent if using U-2 OS cells.
- We recommend the following steps for optimizing the transduction conditions for your cell background of interest in a small scale in order to minimize reagent consumption:
    1. Prepare a virus titration to determine the optimal percentage of virus to use.
    2. For untested cell backgrounds, perform a transduction in the presence and absence of BacMam Enhancer Solution included in the kit and then analyze the expression of GFP-ERK2.

Titration of BacMam ERK2 Reagent

We recommend performing a titration of the BacMam ERK2 Reagent to determine the optimal percentage of virus for the transduction in your cell background of interest. Select the lowest percentage of BacMam reagent that yields the largest assay window (response ratio). See the examples below.

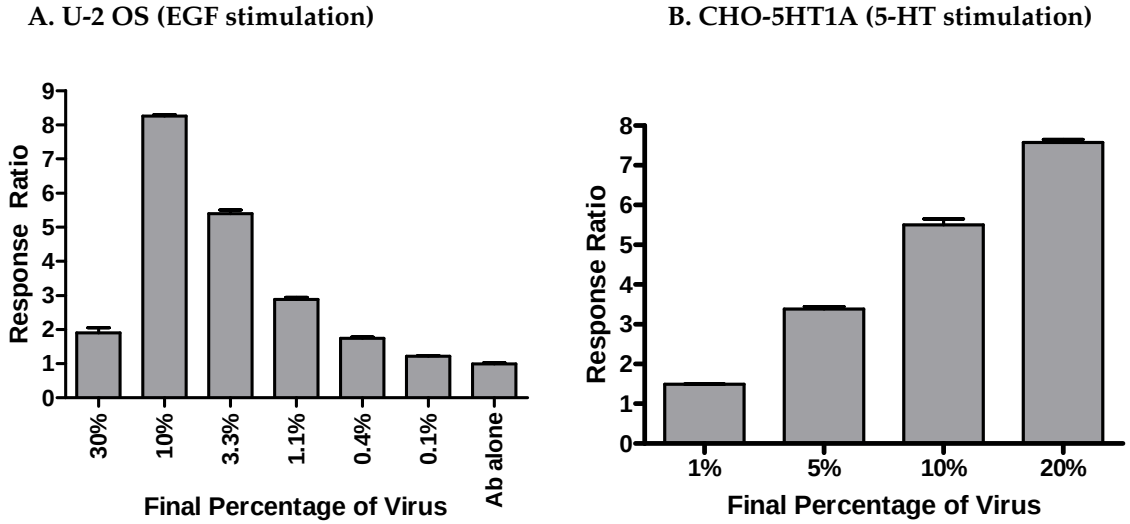

Figure 2. Detection of ERK2 phosphorylation at Thr185/Tyr187 in (**A**) U-2 OS cells transduced with various concentrations of BacMam ERK2 Reagent and stimulated with 100 ng/mL EGF; (**B**) CHO-5HT1A cells transduced with various concentrations of BacMam ERK2 and stimulated with 0.3 µM 5-HT.

Guidelines for Optimizing LanthaScreen® Assays

First-time LanthaScreen® users

LanthaScreen® assays require the detection of terbium TR-FRET. For more information about your specific instrument and to purchase filters, visit www.invitrogen.com/instrumentsetup. For a protocol describing how to test whether your instrument is able to detect a terbium/GFP TR-FRET signal, please contact Drug Discovery Technical Support at drugdiscoverytech@invitrogen.com or 760-603-7200 (extension 40266).

Important assay parameters for optimization

- Confluence of cells at harvest for assay set-up may impact results, such as the assay window. In general, cells should be harvested at the maximum density at which they are still healthy.
- Cell plating density (i.e., cell number per well in the assay plate)
- Ligand stimulation time
- Assay equilibration time

Validation packet

Visit www.invitrogen.com and search for A12888 to download the validation packet for the BacMam ERK2 Cellular Assay. The validation packet is located under the "How to Use" tab on the product page, and contains more information about ERK2 applications.

Quick Reference Protocols for CHO Cells

The following agonist and antagonist GPCR-mediated ERK2 phosphorylation protocols have been developed for CHO cells over-expressing the serotonin receptor HTR1A. Conditions such as plating density, stimulant, or stimulation time may need to be optimized for CHO cell lines expressing different GPCRs. Phosphorylation of ERK2 at [Thr185/Tyr187] can also be induced in CHO cells with either 10% serum or 100 nM PMA.

GPCR Agonist Assay—CHO Cells

		Cell-Free Control Wells	Unstimulated Control Wells	Stimulated Control Wells	Test Compound Wells
BacMam Transduction	**Step 1** Grow and Transduce Cells	• Grow cells in Growth Medium* to 90–100% confluence (~1–2 × 10^5 cells/cm^2). • Perform five 3-fold serial dilutions of BacMam reagent in PBS with Ca^{2+} and Mg^{2+}. • Remove media from cells and add the serial diluted BacMam virus. • Incubate at room temperature protected from light for 3–4 hrs.			
	Step 2 Add Enhancer and Incubate Cells	Remove virus and add Growth Medium* plus 1X BacMam Enhancer Solution. Incubate the plate at 37°C/5% CO_2 for 20–24 hours.			
LanthaScreen® Assay	**Step 3** Harvest Cells	Harvest cells, wash once with Assay Medium** and resuspend in Assay Medium at 0.75 × 10^6 cells/mL (determine the optimal cell density for your cell line).			
	Step 4 Plate Cells	20 µL/well Assay Media only	20 µL transduced cells/well (about 15,000 cells/well), quick spin of the plate		
	Step 5 Serum-starve and Incubate Cells	Incubate the plate at 37°C/5% CO_2 for 4 hours			
	Step 6 Prepare Complete 6X Lysis Buffer	To 1 mL 6X Lysis Buffer, add 30 µL of 100x protease inhibitor, 30 µL 100x phosphatase inhibitor , and Tb-anti-ERK2 [pThr185/pTyr187] Antibody to 12 nM .			
	Step 7 Add Agonist	10 µL/well of 0.3% DMSO in Assay Media		10 µL/well of 3X 5-HT (900 nM) in Assay Media	10 µL/well of 3X Test Compound in Assay Media
	Step 8 Stimulate Cells	Incubate the plate at 37°C/5% CO_2 for 6–8 minutes			
	Step8 Add Lysis Buffer (including Tb-Ab)	Add 6 µL/ well of Complete 6X Lysis Buffer to each well			
	Step 9 Cell Lysis/Assay Equilibration	Incubate plate for ~3 hours at room temperature in the dark			
	Step 10 Read Plate and Analyze Data	See **LanthaScreen® Detection**, page 14—Excitation filter: 337 nm (30 nm bandwidth); Emission filters: 490 nm (10 nm bandwidth) and 520 nm (25 nm bandwidth)			

* **Growth Media:** D-MEM Media (Invitrogen 10569) with 10% dFBS, 10 mM HEPES, 0.1 mM NEAA, and 100 U/mL Penicillin/100 µg/mL Streptomycin

** **Assay Media:** Opti-MEM® I (Invitrogen 11058) with 0.1% cdFBS (or dialyzed FBS), 0.1 mM NEAA, 1 mM Sodium Pyruvate, and 100 U/mL Penicillin/100 µg/mL Streptomycin

GPCR Antagonist Assay—CHO Cells

		Cell-Free Control Wells	Unstimulated Control Wells	Stimulated Control Wells	Control Compound wells	Test Compound Wells
BacMam Transduction	**Step 1** Grow and Transduce Cells	• Grow cells in Growth Medium* to 90–100% confluence (~1–2 × 10^5 cells/cm^2). • Perform five 3-fold serial dilutions of BacMam reagent in PBS with Ca^{2+} and Mg^{2+}. • Remove media from cells and add the serial diluted BacMam virus. • Incubate at room temperature protected from light for 3–4 hrs.				
	Step 2 Add Enhancer and Incubate Cells	Remove virus and add Growth Medium* plus 1X BacMam Enhancer Solution. Incubate the plate at 37°C/5% CO_2 for 20–24 hours.				
LanthaScreen® Assay	**Step 3** Harvest Cells	Harvest cells, wash once with Assay Medium** and resuspend in Assay Medium at 0.75 × 10^6 cells/mL (You need to determine the optimal cell density for your cell line).				
	Step 4 Plate Cells	20 µL/well Assay Media only	20 µL transduced cells /well (about 15,000 cells/well), quick spin of the plate			
	Step 5 Serum-starve and Incubate Cells	Incubate the plate at 37°C/5% CO_2 for 4 hours				
	Step 6 Prepare Complete Lysis Buffer	To 1 mL 6X Lysis Buffer, add 30 µL of 100x protease inhibitor, 30 µL 100x phosphatase inhibitor , and Tb-anti-ERK2 [pThr185/pTyr187] Antibody to 12 nM .				
	Step 7 Add Antagonist	5 µL/well of 0.5% DMSO in Assay Medium			5 µL/well of 5X control antagonist in Assay Medium	5 µL/well of 5X test compound in Assay Medium
	Step 8 Incubate	Incubate the plate at 37°C/5% CO_2 for 30–45 minutes				
	Step 9 Add Agonist	5 µL/well Assay Medium		5 µL/well of 6X agonist (for 5-HT, use 180 nM) in Assay Medium		
	Step10 Stimulate Cells	Incubate the plate at 37°C/5% CO_2 for 6–8 minutes				
	Step 11 Add Lysis Buffer (including Tb-Ab)	Add 6 µL/ well of Complete 6X Lysis Buffer to each well				
	Step 12 Cell Lysis/Assay Equilibration	Incubate plate for ~3 hours at room temperature in the dark				
	Step 13 Read Plate and Analyze Data	See **LanthaScreen® Detection**, page 14—Excitation filter: 337 nm (30 nm bandwidth); Emission filters: 490 nm (10 nm bandwidth) and 520 nm (25 nm bandwidth)				

* **Growth Media:** D-MEM Media (Invitrogen 10569) with 10% dFBS, 10 mM HEPES, 0.1 mM NEAA, and 100 U/mL Penicillin/100 µg/mL Streptomycin

** **Assay Media:** Opti-MEM® I (Invitrogen 11058) with 0.1% cdFBS (or dialyzed FBS), 0.1 mM NEAA, 1 mM Sodium Pyruvate, and 100 U/mL Penicillin/ 100 µg/mL Streptomycin

Quick Reference Protocols for U-2 OS Cells

In the following agonist and antagonist ERK2 phosphorylation protocols have been developed for U-2 OS cells. They may be applied to many other cell types such as HEK293, HeLa, and A549. The cell harvesting and plating densities, growth medium, and assay medium must be optimized for your particular cell line(s).

The cells are incubated with virus at the time of plating onto the assay plate. These protocols include a 20–24 hour incubation period.

Agonist Assay—U-2 OS Cells

<table>
<tr><th></th><th></th><th>Cell-Free Control Wells</th><th>Unstimulated Control Wells</th><th>Stimulated Control Wells</th><th>Test Compound Wells</th></tr>
<tr><td rowspan="3">BacMam Transduction</td><td>Step 1
Grow, Harvest Cells and Transduce</td><td colspan="4">• Grow cells in Growth Medium* to 90-100% confluence (~0.8–1.2 × 10^5 cells/cm^2).
• Harvest and wash cells and resuspend in Assay Medium** at 8.4 × 10^5 cells/mL.
• Perform five 3-fold serial dilutions of BacMam reagent in Assay Medium.
• Add 0.4 mL undiluted or serial diluted BacMam reagent to 1 mL cells to generate a virus titration range of 0.1% to 30% (v/v) final concentration.</td></tr>
<tr><td>Step 2
Plate Cells/virus mixture</td><td>Add 20 µL/well Assay Media only</td><td colspan="3">Add 20 µL cells and BacMam mixture per well (~12,000 cells/well), and quick spin the plate</td></tr>
<tr><td>Step 3
Incubate Cells</td><td colspan="4">Incubate the plate at 37°C/5% CO_2 for 20–24 hours</td></tr>
<tr><td rowspan="6">LanthaScreen® Assay</td><td>Step 4
Prepare Complete 6X Lysis Buffer</td><td colspan="4">To 1 mL 6X Lysis Buffer, add 30 µL of 100x protease inhibitor, 30 µL 100x phosphatase inhibitor , and Tb-anti-ERK2 [pThr185/pTyr187] Antibody to 12 nM</td></tr>
<tr><td>Step 5
Add media or ligand</td><td colspan="2">Add 10 µL/well of Assay Media with 0.3% DMSO</td><td>Add 10 µL/well of 3X Agonist in Assay Media with 0.3% DMSO (for EGF, use ~600 ng/mL)</td><td>Add 10 µL/well of 3X Test Compound in Assay Media (0.3% DMSO)</td></tr>
<tr><td>Step 6
Stimulate Cells</td><td colspan="4">Incubate the plate at 37°C/5% CO_2 for 6–8 minutes</td></tr>
<tr><td>Step 7
Add Lysis Buffer (including Tb-Ab)</td><td colspan="4">Add 6 µL/well of Complete 6X Lysis Buffer to each well</td></tr>
<tr><td>Step 8
Cell Lysis/Assay Equilibration</td><td colspan="4">Incubate plate for ~3 hours at room temperature in the dark</td></tr>
<tr><td>Step 9
Read Plate and Analyze Data</td><td colspan="4">See LanthaScreen® Detection, page 14—Excitation filter: 337 nm (30 nm bandwidth); Emission filters: 490 nm (10 nm bandwidth) and 520 nm (25 nm bandwidth)</td></tr>
</table>

* **Growth Media for U-2 OS Cells:** McCoy's 5A Media with 10% dFBS, 10 mM HEPES, 0.1 mM NEAA, 1 mM Sodium Pyruvate, and 100 U/mL Penicillin/ 100 µg/mL Streptomycin

** **Assay Media:** Opti-MEM® I (Invitrogen 11058) with 0.1% cdFBS (or dialyzed FBS), 0.1 mM NEAA, 1 mM Sodium Pyruvate, and 100 U/mL Penicillin/ 100 µg/mL Streptomycin

Antagonist Assay—U-2 OS Cells

		Cell-Free Control Wells	Unstimulated Control Wells	Stimulated Control Wells	Control Compound wells	Test Compound Wells
BacMam Transduction	**Step 1** Grow, Harvest Cells and Transduce	• Grow cells in Growth Medium* to 70–90% confluency (~0.8–1.2 × 10^5 cells/cm^2). • Harvest, wash cells and resuspend in Assay Medium** at 8.4 × 10^5 cells/mL. • Perform five- 3 fold serial dilutions of BacMam reagent in Assay Medium. • Add 0.4 mL undiluted or serial diluted BacMam reagent to 1 mL cells to generate a virus titration range of 0.1% to 30% (v/v) final concentration.				
	Step 2 Plate Cells/virus mixture	Add 20 µL/well Assay Media only	Add 20 µL cells and BacMam mixture per well (~12,000 cells/well), and quick spin the plate			
	Step 3 Incubate Cells	Incubate the plate at 37°C/5% CO_2 for 20–24 hours				
LanthaScreen® Assay	**Step 4** Add media, control, or test compounds	Add 5 µL/well of 0.5% DMSO in Assay Media			Add 5 µL/well of 5X Iressa (2.5 µM) in Assay Media	Add 5 µL/well of 5X Test Compound in Assay Media
	Step 5 Compound pretreatment	Incubate the plate at 37°C/5% CO_2 for 30–60 minutes				
	Step 6 Prepare Complete 6X Lysis Buffer	To 1 mL 6X Lysis Buffer, add 30 µL of 100x protease inhibitor, 30 µL 100x phosphatase inhibitor , and Tb-anti-ERK2 [pThr185/pTyr187] Antibody to 12 nM				
	Step 7 Add media or ligand	Add 5 µL/well Assay Media	Add 5 µL/well of 6X Agonist (for EGF, 600 ng/mL) in Assay Media			
	Step 8 Stimulate Cells	Incubate the plate at 37°C/5% CO_2 for 6–8 minutes				
	Step 9 Add Lysis Buffer (including Tb-Ab)	Add 6 µL/well of Complete 6X Lysis Buffer to each well				
	Step 10 Cell Lysis/Assay Equilibration	Incubate plate for ~3 hours at room temperature in the dark				
	Step 11 Read Plate and Analyze Data	See **LanthaScreen® Detection**, page 14—Excitation filter: 337 nm (30 nm bandwidth); Emission filters: 490 nm (10 nm bandwidth) and 520 nm (25 nm bandwidth)				

* **Growth Media for U-2 OS Cells:** McCoy's 5A Media with 10% dFBS, 10 mM HEPES, 0.1 mM NEAA, 1 mM Sodium Pyruvate, and 100 U/mL Penicillin/ 100 µg/mL Streptomycin

** **Assay Media:** Opti-MEM® I (Invitrogen 11058) with 0.1% cdFBS (or dialyzed FBS), 0.1 mM NEAA, 1 mM Sodium Pyruvate, and 100 U/mL Penicillin/ 100 µg/mL Streptomycin

Detailed Protocol for GPCR-mediated ERK2 phosphorylation in CHO Cells

The following detailed protocol provides separate workflows for agonist and antagonist assays. Cells are allowed to adhere to the tissue-culture flasks before transduction with BacMam reagent. For easy-to-transduce cells, an alternative detailed transduction protocol is provided starting on page 14.

Day 1. BacMam Transduction

1. Begin with cells grown to near-complete confluence under normal growth conditions (e.g., CHO cells should be grown to 90–100% confluence, about 1–2 × 10^5cells/cm^2). **Confluence of cells may impact results, such as the assay window (i.e., less than 90% confluence may result in a significantly lower assay window).**
2. Perform five 3-fold serial dilutions of BacMam reagent in D-PBS with Ca^{2+} and Mg^{2+}.
3. Remove media from cells and add the serial diluted BacMam virus. A typical final concentration of BacMam reagent is 0.12–30% (v/v).
4. Incubate at room temperature for 3–4 hours in the dark.
5. Remove the BacMam reagent from the cells. Add growth medium containing 1X BacMam Enhancer Solution.
6. Incubate the cells in a 37°C/5% CO_2 incubator for 20–24 hours.

Day 2. LanthaScreen® Cellular Assay

7. Harvest the cells and wash once with assay medium, then resuspend the cells in assay medium at 0.75 × 10^6 cells/mL. You will need to determine the optimal cell density for your cell line of interest.
8. Plate 20 µL/well cells in assay medium onto a 384-well assay plate (about 15,000 cells/well). Plate 20 µL/well assay medium in cell-free control wells.
9. **Optional:** Transfer some cells to the wells of a clear-bottom 384-well plate for image analysis of GFP expression.
10. Incubate the cells in a 37°C/5% CO_2 incubator for ~4 hours for serum-starvation. Depending on the cell type, you may need to incubate longer.
11. **Optional:** Analyze GFP expression levels in the clear-bottom 384-well plate by fluorescence microscopy using standard FITC filter sets.
12. During incubation, prepare Complete 6X Lysis Buffer by adding both protease inhibitor and phosphatase inhibitor cocktails to the provided 6X Lysis Buffer, at a 1:33 dilution of 100X stock (e.g., 30 µL of 100X stock inhibitors per 1,000 µL of 6X Lysis Buffer) and add LanthaScreen® Tb-anti-ERK2 [pThr185/pTyr187] Antibody to 12 nM Mix gently by inversion. Store on ice until use.
13. Proceed with the following agonist or antagonist assay setup.

    Agonist Assay Setup:

    a. Prepare a stock solution of 0.3% DMSO in assay medium. **Note:** If you are using a solvent other than DMSO for the agonist, change the solvent used in the control wells accordingly. Be careful to keep the amount of solvent consistent in all wells.

    b. Prepare 3X control agonist in assay medium with 0.3% DMSO (e.g., if treating CHO-5HT1A cells with 5-HT, prepare 900 nM 5-HT in assay medium for a 3X concentration). Run a dose response curve to determine the EC_{100} for your control agonist solution.

    c. Prepare 3X test compound in assay medium (if the test compound is dissolved in DMSO, make sure the DMSO concentration for the 3X solution is 0.3%).

d. Add 10 µL of assay medium with 0.3% DMSO to each unstimulated control and cell-free control well.

e. Add 10 µL of the 3X control agonist (5-HT) in assay medium to each stimulated control well.

f. Add 10 µL of the 3X test compound in assay medium to each test compound well.

g. Proceed to the next numbered step.

Antagonist Assay Setup:

a. Prepare a stock solution of 0.5% DMSO in assay medium. **Note:** If you are using a solvent other than DMSO for the agonist, change the solvent used in the control wells accordingly. Be careful to keep the amount of solvent consistent in all wells.

b. Prepare a 5X control antagonist in assay medium. Run a dose response curve to determine the IC_{100} for your control inhibitor solution.

c. Prepare a 5X test compound in assay medium (if the test compound is dissolved in DMSO, make sure the DMSO concentration for the 5X solution is 0.5%).

d. Add 5 µL of the assay medium with 0.5% DMSO to each unstimulated, stimulated, and cell-free control well.

e. Add 5 µL of the 5X control antagonist in assay medium to each control compound well.

f. Add 5 µL of the 5X test compound in assay medium to each test compound well.

g. Incubate the plate in a humidified 37°C/5% CO_2 incubator for 30–60 minutes.

a. Prepare a 6X stock of agonist in assay medium (e.g., if using CHO-5HT1A cells, prepare 180 nM 5-HT in assay medium for a 6X concentration). Run a dose response curve to determine the EC_{80} for your agonist solution.

h. Add 5 µL of the assay medium to each unstimulated and cell-free control well.

i. Add 5 µL of 6X stock of agonist (5-HT) to each stimulated, control, and test compound well

14. Cover the plate and stimulate the cells by incubating in a humidified 37°C/5% CO_2 incubator for 6–8 minutes.

Tip: The stimulation time must be optimized for each cell type and agonist. "Stimulation time" refers to the time from agonist addition to the addition of Complete 6X Lysis Buffer.

15. After stimulation, immediately add 6 µL of Complete 6X Lysis Buffer to each well. Cover the plate.

16. Incubate the covered plate at room temperature in the dark for 3 hours or other desired equilibration time. The equilibration time can be optimized for your cell line of interest.

Note: Assay plates may be stored at 4°C overnight prior to reading. Let the plate warm to room temperature prior to reading.

17. Proceed to **LanthaScreen® Detection**, page 14.

Detailed Protocol for ERK2 Phosphorylation in U-2 OS cells

The following detailed protocol provides separate workflows for agonist and antagonist assays. Cells are incubated with virus at the time of plating onto the assay plate. This protocol includes a 20–24-hour incubation.

Day 1. BacMam Transduction

1. Begin with cells grown to near-complete confluence under normal growth conditions (e.g., U-2 OS cells should be grown to 100% confluence). Confluence of cells may impact results, such as the assay window.

   Note: For many cell types, such as U-2 OS, a cell seeding density of ~30,000 cells/cm^2 for 3 days with a harvest density of ~0.8–1.2 × 10^5 cells/cm^2 is optimal. In general, cells should be transduced at the maximum density at which the cells are still healthy.

2. Harvest and wash the cells and resuspend in low-serum assay medium, using the appropriate conditions for your particular cell line (for U-2 OS cells, resuspend at 7 × 10^5 cells/mL).

   Note: For the BacMam ERK2 assay, it is important to wash away any residual serum following harvest to ensure optimal starve.

3. Add BacMam ERK2 Reagent to the cells. A typical final concentration of BacMam reagent is 1–30% (v/v). Mix gently by inversion.

   Note: We recommend testing a range of v/v dilutions of BacMam reagent. For U-2 OS cells, we recommend preparing five three-fold serial dilutions of the BacMam reagent in assay medium. Then mix 1 mL cells at 8.4 × 10^5 cells/mL with 0.4 mL of each BacMam reagent dilution. This should yield a final cell concentration of 6 × 10^5 cells/mL and final reagent concentrations of ~30%, 10%, 3%, 1%, 0.3%, and 0.1% (v/v).

4. Transfer 20 µL/well of cells/BacMam reagent mixture to a white 384-well assay plate. The number of cells per well may need to be optimized (e.g., seed U-2 OS cells at ~12,000 cells/well).

5. **Optional:** Transfer 20 µL/well of cells to at least one well of a clear-bottom 384-well plate for image analysis of GFP expression.

6. Incubate plates for 20–24 hours in a humidified incubator at 37°C/5% CO_2.

Day 2. LanthaScreen® Cellular Assay

7. **Optional:** Twenty-four hours post-transduction, analyze GFP expression levels in the clear-bottom 384-well plate by fluorescence microscopy using standard FITC filter sets.

8. During incubation, prepare Complete 6X Lysis Buffer by adding both protease inhibitor and phosphatase inhibitor cocktails to the provided 6X Lysis Buffer, at a 1:33 dilution of 100X stock (e.g., 30 µL of each 100X stock inhibitor per 1,000 µL of 6X Lysis Buffer) and add LanthaScreen® Tb-anti-ERK2 [pThr185/pTyr187] Antibody to 12 nM Mix gently by inversion. Store on ice until use.

9. Proceed with the following agonist or antagonist assay setup.

   Agonist Assay Setup:

   a. Prepare a stock solution of 0.3% DMSO in assay medium. **Note:** If you are using a solvent other than DMSO for the agonist, change the solvent used in the control wells accordingly. Be careful to keep the amount of solvent consistent in all wells.

   b. Prepare 3X control agonist in assay medium with 0.3% DMSO (e.g., if treating U-2 OS cells with EGF, prepare 600 ng/mL in Assay Media for a 3X concentration). Run a dose response curve to determine the EC_{100} for your control agonist solution.

   c. Prepare 3X test compound in assay medium (if the test compound is dissolved in DMSO, make sure the DMSO concentration for the 3X solution is 0.3%).

d. Add 10 µL of assay medium with 0.3% DMSO to each unstimulated control and cell-free control well.

e. Add 10 µL of the 3X control agonist (EGF) in assay medium to each stimulated control well.

f. Add 10 µL of the 3X test compound in assay medium to each test compound well.

g. Proceed to the next numbered step.

Antagonist Assay Setup:

a. Prepare a stock solution of 0.5% DMSO in assay medium. **Note:** If you are using a solvent other than DMSO for the agonist, change the solvent used in the control wells accordingly. Be careful to keep the amount of solvent consistent in all wells.

b. Prepare a 5X control compound Iressa in assay medium (e.g., prepare a 10 mM stock solution of Iressa in DMSO, then dilute to 2.5 µM in Assay Media for a 5X concentration). Run a dose response curve to determine the IC_{100} for your control inhibitor solution.

c. Prepare a 5X test compound in assay medium (if the test compound is dissolved in DMSO, make sure the DMSO concentration for the 5X solution is 0.5%).

d. Add 5 µL of the assay medium with 0.5% DMSO to each unstimulated, stimulated and cell-free control well.

e. Add 5 µL of the 5X control inhibitor Iressa in assay medium to each control compound well.

f. Add 5 µL of the 5X test compound in assay medium to each test compound well.

g. Incubate the plate in a humidified 37°C/5% CO_2 incubator for 30–60 minutes.

h. Prepare a 6X stock of agonist (EGF) in assay medium (e.g., if using U-2 OS cells, prepare 600 ng/mL in Assay Media for a 6X concentration). Run a dose response curve to determine the EC_{80} for your agonist solution.

i. Add 5 µL of the assay medium to each unstimulated and cell-free control well.

j. Add 5 µL of 6X stock of agonist (EGF) to each stimulated, control and test compound well

10. Cover the plate and stimulate the cells by incubating in a humidified 37°C/5% CO_2 incubator for 6 minutes.

Tip: The stimulation time must be optimized for each cell type and agonist. "Stimulation time" refers to the time from agonist addition to the addition of Complete 6X Lysis Buffer.

11. After stimulation, immediately add 6 µL of Complete 6X Lysis Buffer to each well. Cover the plate.

12. Incubate the covered plate at room temperature in the dark for 3 hours or other desired equilibration time. The equilibration time can be optimized for your cell line of interest.

Note: Assay plates may be stored at 4°C overnight prior to reading. Let the plate warm to room temperature prior to reading.

13. Proceed to **LanthaScreen® Detection**.

LanthaScreen® Detection

Instruments and Filters

Detection can be performed on a variety of plate readers, including the PE Envision. The data presented on page 4 were generated using a BMG PHERAstar plate reader using the LanthaScreen® filter block available from BMG.

Visit www.invitrogen.com/instrumentsetup or contact Invitrogen Discovery Sciences technical support (drugdiscoverytech@invitrogen.com or 760-603-7200 (extension 40266)) for more information on performing LanthaScreen® Cellular Assays on your particular instrument.

Note: We do not recommend using monochromator-based instruments without adjustable bandwidth, as the sensitivity of these instruments is not sufficient to adequately detect the TR-FRET signal.

Reading the Assay Plate and Data Analysis

All measurements should be taken at room temperature from the top of the wells.

1. Let the assay plate warm to room temperature before reading, if necessary.
2. Set the fluorescence plate reader to top/time-resolved read mode.
3. Allow the lamp in the plate reader to warm up for at least 10 minutes before making measurements.
4. Use the filter selections described below. To obtain an assay window, filter bandwidths are critical and cannot be approximated.

	Settings to Measure Donor (Terbium) Signal	Settings to Measure Acceptor (TR-FRET to GFP) Signal
Excitation filter:	337 nm (30 nm bandwidth)	
Emission filter:	490 nm (10 nm bandwidth)	520 nm (25 nm bandwidth)
Dichroic Mirror:	Variable	
Delay Time:	100 µs	
Integration Time:	200 µs	

5. Calculate the acceptor/donor Emission Ratio (TR-FRET Ratio, 520 nm/490 nm) for each well, by dividing the acceptor emission values by the donor emission values.
6. **Optional:** Convert the data to a response ratio by dividing each emission ratio value by the value from unstimulated cells (cells not receiving agonist).

Appendix

Alternative Transduction Protocol (Tested for NIH3T3 and HCT116 cells)

In this protocol, cells are incubated with virus at the time of adhering to the tissue-culture plate, usually 24 hours prior to re-plating onto the assay plate.

Day 1

1. Begin with cell cultures grown to complete confluence under normal growth conditions. Confluence of cells may impact results, such as the assay window.
2. Trypsinize to harvest adherent cells as recommended by the cell-line manufacturer.

3. Resuspend cells in growth medium. Seed cells such that the monolayers will be approximately 50–80% confluent once attached and spread. Avoid plating the cells such that 80% confluence is exceeded 24 hours post-transduction.

Note: For first time users, it may be useful to work with 6-well plates to minimize reagent consumption during the transduction optimization process.

Tip: For most cell lines (with a doubling time of approximately 24 hours), a seeding density of 2–4 × 10^4 cells/cm^2 is optimal for BacMam transduction. It may be desirable to optimize the cell density for specific cell backgrounds.

4. Immediately after seeding the cells, add the desired amount of BacMam reagent to the cells. For initial optimization, we recommend testing 30%, 10%, 3%, and 1% v/v dilutions of BacMam reagent.

5. Add BacMam Enhancer Solution at a 1X final concentration if required. **Note:** For untested cell backgrounds, we recommend performing the transduction in the presence and absence of Enhancer Solution and then analyzing the expression of the BacMam target.

6. Place cells in a humidified 37°C/5% CO_2 incubator for 16–24 hours to allow for the transduction and expression of the GFP fusion protein.

Day 2

7. **Optional:** Analyze GFP expression levels by fluorescence microscopy using standard FITC filter sets.

8. Harvest the transduced cells and be careful not to over-trypsinize the cells as this can result in poor viability and a decreased assay window.

9. Resuspend the harvested cells in growth medium with serum to inactivate the trypsin. Centrifuge the cells at 200 × g for 5 minutes. Aspirate the growth medium and resuspend the cell pellet in assay medium (usually low serum).

10. Centrifuge the cells at 200 × g for 5 minutes. Aspirate the assay medium and resuspend the cell pellet in assay medium at the desired density.

Tip: The number of cells per well will affect the assay window and can be optimized. We recommend starting with 10,000–20,000 cells per well seeded in 20 µL of assay medium. Therefore, resuspend cells to 0.5–1 × 10^6 cells/mL.

11. Plate 20 µL of transduced cells in assay medium into white tissue culture-treated 384-well plates and incubate the plates in a 37°C/5% CO_2 incubator (or appropriate) for 16–20 hours.

Note: Serum starvation in low-serum or serum-free media is required for most cell types analyzed with this assay. We recommend starting with a 16–20 hour serum starvation and optimizing as needed.

Day 3

12. Proceed to the **LanthaScreen® Cellular Assay** on page 10 or 12.

Troubleshooting Guide

Observation	Potential Solutions
Weak/no expression of GFP-fusion in the cell line of interest in a clear-bottom 384-well plates.	Confirm that your fluorescence microscope is configured appropriately for detection of GFP/FITC.
	Perform a virus titration to find the optimal virus concentration for your cell background.
	Confirm that no contamination of the BacMam reagent has occurred.
	For first-time users, we recommend the standard transduction protocol using U-2 OS cells at 100% confluence. Overly confluent or unhealthy cells will not transduce efficiently.
	If the standard protocol works for U-2 OS cells but not for your cells, please try Alternative Transduction Protocol in the Appendix.
>50% expression of GFP-fusion is observed, but weak/no detectable stimulation of the posttranslational modification of the GFP-fusion in cell line of interest	Confirm that the fluorescence plate reader is configured appropriately for LanthaScreen® detection. Filter bandwidth requirements are exact. For more information about your specific instrument and to purchase filters, visit www.invitrogen.com/instrumentsetup. Contact Drug Discovery Technical Support for more information.
	Be sure that the cells are grown to the highest density at which they remain healthy before proceeding to the transduction step, to enhance the sensitivity of the cells to stimulation.
	For first-time users, we recommend following the standard transduction protocol using U-2 OS cells.
	Perform a stimulation time course experiment to find out the optimal stimulation time for your agonist and cell line of interest. (The stimulation time refers to the time from agonist addition to lysis).
	Image the cells in clear-bottom microtiter plates. Ensure that cells are adhered to the bottom of the plate and are not expressing very high levels of GFP. Dimly green cells are desirable. Excessive expression of the GFP-fusion may be deleterious to cell health.
> 60% expression of GFP-fusion, but very low assay window due to high background (stimulated and unstimulated cells generate high emission ratios compared to cell-free/Tb-antibody alone).	Image the cells in clear-bottom microtiter plates. Ensure that cells are adhered to the bottom of the plate and are not expressing very high levels of GFP. Dimly green cells are desirable. Excessive expression of the GFP-fusion may be deleterious to cell health.
Following transduction with the BacMam reagent, cells have a rounded/unspread phenotype and appear to be in poor health.	Under serum-starvation conditions, many cell types will appear rounded when imaged 24 or 48 hours post-transduction. This is common among cell types such as HEK293 and CHO. Under these conditions, cells are capable of modifying the GFP-fusion target, and should remain adhered despite poor cell spreading.
	Cells that appear rounded and detached from the clear-bottom microtiter plate may be unsuitable for GFP-fusion activation. Ensure that cells are not expressing excessive GFP-fusion as indicated above.
Day-to-day fluctuations in assay window are observed.	Be sure to use cells with the same growth conditions (e.g., same harvest density).

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