Catalog no. A12899 **Shipping: Varies** **Storage: Varies**

Protocol part no. A12899pps (MAN0003213) Rev. date: 12 August 2010

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Kit Contents and Handling

Component	Amount	Storage	Handling
BacMam AKT Reagent	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-AKT [pSer473] Antibody	10 µg	–20°C	Aliquot if necessary to avoid multiple freeze/thaw cycles
6X LanthaScreen® Cellular Assay Lysis Buffer	6 mL	4°C	On the day of assay, supplement with inhibitor cocktails* and antibody

*See **Materials Required but Not Provided** on page 3.

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 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, including:

- 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.

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.

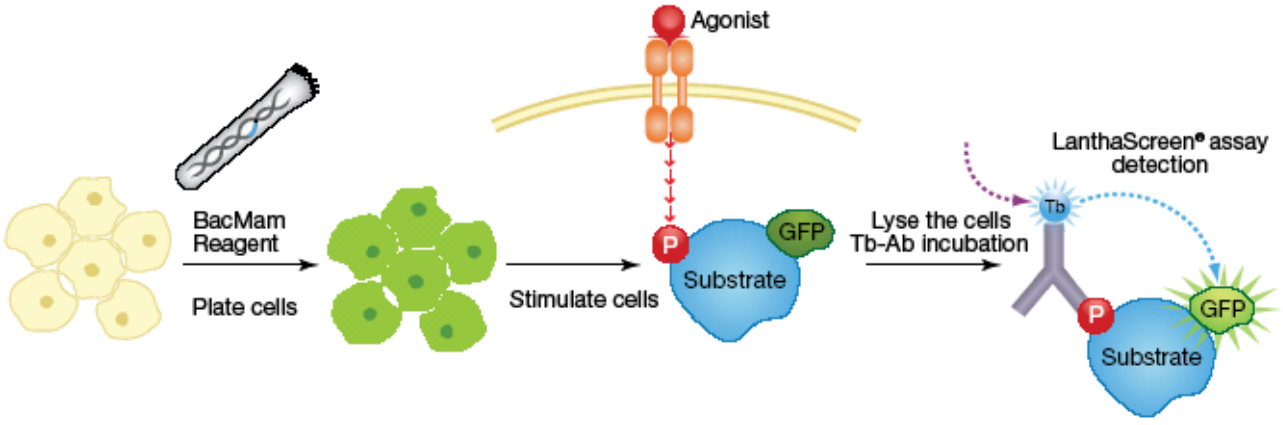

Figure 1. Illustration of Representative Assay Workflow. Cells are treated with BacMam Reagent encoding a GFP-fusion protein and plated in 384-well format; 24 hours post-transduction, the cells are stimulated to induce post-translational modification of the GFP-substrate (e.g., phosphorylation as shown).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

Workflow for the BacMam AKT [pSer473] Cellular Assay

Day 1: Cells are transduced with BacMam GFP-AKT virus and starved overnight, resulting in expression of a GFP-AKT fusion protein that can be visualized by fluorescence microscopy.

Day 2: Phosphorylation at Ser473 of AKT expressed as a GFP fusion protein is induced with a growth factor, such as PDGF. Minimal phosphorylation occurs in untreated cells. Cells are lysed in the presence of a Tb-labeled anti-AKT phospho-Ser473 (pSer473) antibody, and the protein modification event is measured on a TR-FRET-compatible plate reader. Little or no TR-FRET is observed in untreated cells, whereas PDGF-treated cell samples display high TR-FRET.

Before Starting

Materials Required but Not Provided

Materials	Recommended Source	Part #
Positive Control Agonist (growth factor) **We recommend:** PDGF-BB (prepare stock solution at 100 µg/mL in 100 mM acetic acid)	Invitrogen	PHG0045
Control Antagonist (inhibitor) **We recommend:** PI-103	EMD Biosciences	528100
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 (Excitation filter: 337 nm (30 nm bandwidth); Emission filters: 490 nm (10 nm bandwidth) and 520 nm (25 nm bandwidth))	Visit www.invitrogen.com/instrumentsetup for details	
Optional: Clear-bottom, tissues culture treated, 384-well assay plates for visualization of GFP-fusion	Corning	3712

U-2 OS Cell Culture Reagents (optional, if using U-2 OS cells)

U-2 OS cells transduce exceptionally well, and we recommend their use as a control cell line.

Media/Reagents	Recommended Source	Part #
U-2 OS cells	ATCC	HTB-96
DMEM (low glucose, without Phenol Red)	Invitrogen	11054-020
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 which transduces exceptionally well, such as U-2 OS (ATCC® number: HTB-96).
- BacMam Enhancer (catalog no. PV5835) is not required for this BacMam Reagent.
- Most cell types can be transduced efficiently using the protocol described here for U-2 OS. However, some challenging cell types, such as CHO, require alternative protocols as described in the **Appendix**.

Titration of BacMam AKT Reagent

We recommend performing a titration of the BacMam AKT 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 example below.

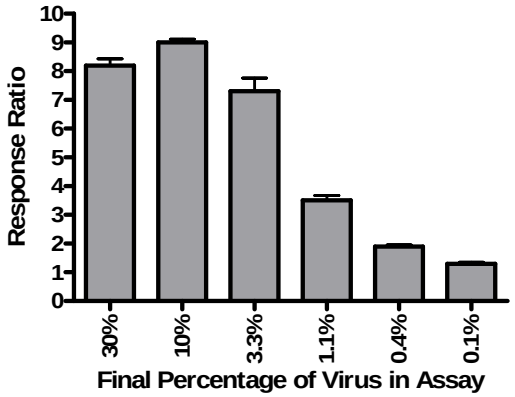

Figure 2. Detection of AKT phosphorylation at Ser473 in U-2 OS cells transduced with various concentrations of BacMam AKT Reagent and stimulated with 100 ng/mL PDGF.

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 A12899 to download the validation packet for the BacMam AKT Cellular Assay. The validation packet is located under the "How to Use" tab on the product page, and contains more information about AKT applications.

Assay Protocol

In the following protocol, cells are incubated with virus at the time of plating onto the assay plate. This protocol includes a 20–24-hour incubation period.

For difficult-to-transduce cells: Two alternative transduction protocols are provided in the **Appendix** on page 10.

The cell harvesting and plating densities, growth medium, and assay medium must be optimized for your particular cell line(s). The following protocol was developed for U-2 OS cells. It may be applied to many other cell types such as HEK293, HeLa and A549. Conditions may need to be optimized for different cell types.

Quick Reference Protocol for U-2 OS Cells– Agonist Assay

This quick reference protocol is designed for experienced users using U-2 OS cells. Conditions may need to be optimized for different cell types. For a detailed protocol, see page 7.

<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 70–90% confluence (~0.75 to 1.0×10^5 cells/cm^2).
• Harvest, wash cells and resuspend in Assay Medium** at 7 × 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 (~10,000 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
Add Media, Control, and Test Compound</td><td colspan="2">Add 10 µL/well of Assay Media with 0.3% DMSO</td><td>Add 10 µL/well of 3X Positive Control Agonist in Assay Media</td><td>Add 10 µL/well of 3X Test Compound in Assay Media</td></tr>
<tr><td>Step 5
Stimulate Cells</td><td colspan="4">Incubate the plate at 37°C/5% CO_2 for 15 minutes</td></tr>
<tr><td>Step 6
Prepare Complete 6X Lysis Buffer</td><td colspan="4">To 1 mL of 6X Lysis Buffer, add 30 µL 100X protease inhibitor, 30 µL 100X phosphatase inhibitor, and LanthaScreen® Tb-anti-AKT [pSer473] Antibody to 12 nM</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 on page 9—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

** **AKT Assay Media:** DMEM, low-glucose (Invitrogen # 11054)

Quick Reference Protocol for U-2 OS Cells– Antagonist Assay

This quick reference protocol is designed for experienced users using U-2 OS cells. Conditions may need to be optimized for different cell types. For a detailed protocol, see page 7.

		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 (~0.75 to 1.0×10^5 cells/cm^2). • Harvest, wash cells and resuspend in Assay Medium** at 7×10^5 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 (~10,000 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, and Test Compounds	Add 5 µL/well of 0.5% DMSO in Assay Media			Add 5 µL/well of 5X Control Antagonist 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 to 60 min				
	Step 6 Add Media and Agonist	Add 5 µL/well Assay Media	Add 5 µL/well of 5X Agonist (for PDGF, 200 ng/mL) in Assay Media			
	Step 7 Stimulate Cells	Incubate the plate at 37°C/5% CO_2 for 15 min				
	Step 8 Prepare Complete 6X Lysis Buffer	To 1 mL of 6X Lysis Buffer, add 30 µL 100X protease inhibitor, 30 µL 100X phosphatase inhibitor, and Tb-anti-AKT [pSer473] Antibody to 12 nM				
	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** on page 9—Excitation filter: 337 nm (30 nm bandwidth); Emission filters: 490 nm (10 nm bandwidth) and 520 nm (25 nm bandwidth)				

Detailed Protocol

For difficult-to-transduce cells, two alternative detailed transduction protocols are provided in the **Appendix** starting on page 10.

Day 1. BacMam Transduction

1. Begin with cell cultures grown to near-complete confluence under normal growth conditions (e.g., U-2 OS cells should be grown to 70–90% 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 ~0.75 × 10^5 to 1 × 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, wash cells and resuspend in assay medium, using the appropriate conditions for your particular cell line (for U-2 OS cells, resuspend at 7.0 × 10^5 cells/mL).

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

3. Add BacMam AKT 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 5 three-fold serial dilutions of the BacMam Reagent in assay media. Then mix 1 mL cells at 7.0 × 10^5 cells/mL with 0.4 mL of each BacMam Reagent dilution. This should yield a final cell concentration of 2.5 × 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 ~10,000 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. Set up the agonist or antagonist assay, as follows:

Agonist Assay Setup

a. Prepare a stock solution of 0.3% DMSO in assay medium. (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 3X stock of positive control agonist in assay medium with 0.3% DMSO. If you are using PDGF, prepare 600 ng/mL 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 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 Step 9.

Antagonist Assay Setup

a. Prepare a stock solution of 0.5% DMSO in assay medium. (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 stock of control antagonist in assay medium. If you are using PI-104, prepare a 10 mM stock solution of PI-103 in DMSO, then dilute to 5 µM in Assay Media for a 5X concentration. Run a dose response curve to determine the IC_{100} for your control antagonist solution.

c. Prepare a 5X stock of 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 to 60 min.

a. Prepare a 5X stock of agonist in assay medium. If using PDGF, prepare 200 ng/mL in assay medium for a 5X 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 5X stock of agonist in assay medium to each stimulated, control and test compound well

9. Following agonist or antagonist assay setup, cover the plate and stimulate the cells by incubating in a humidified 37°C/5% CO_2 incubator for 15 minutes. During incubation, proceed to step 10.

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.

10. 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-AKT [pSer473] Antibody to the 6X Lysis Buffer at a concentration of 12 nM. Mix gently by inversion. Store on ice until use.

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 another 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 reading the plate, as described in the next section.

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

The following alternative transduction protocols may be used with difficult-to-transduce cell lines. In short, **Protocol A** requires that the cells be allowed to adhere to the tissue culture flasks prior to the transduction with the BacMam Reagent (the longest protocol, but allows for higher transduction efficiency for difficult cell types). **Protocol B** requires that cells are incubated with virus at the time of adhering to tissue culture plate, usually 24 hours prior to re-plating onto the assay plate.

Alternative Transduction Protocol A

In this protocol, cells are allowed to adhere to the tissue-culture flasks before transduction with BacMam Reagent.

Day 1

1. Begin with cells grown to complete confluence in normal tissue-culture flasks. Confluence of cells may impact results, such as the assay window.
2. Trypsinize and harvest adherent cells as recommended by the cell line manufacturer.
3. Plate the desired number of cells in growth medium and allow them to adhere under normal growth conditions for 16–24 hours.

Tip: For many cell types (with a doubling time of approximately 24 hours), a seeding density of approximately $2–4 \times 10^4$ cells/cm^2 will result in 50–80% confluence 24 hours after seeding. This has proven optimal for transducing cell lines such as CHO. It may be necessary to optimize the cell density for specific cell backgrounds.

Day 2

4. Determine the volume of BacMam Reagent necessary to cover the adhered cells in the tissue culture flask. We recommend ~1 mL of BacMam solution (diluted as in the next step) for every 10 cm^2 of flask surface area.
5. Prepare a dilution of BacMam Reagent (v/v) in Dulbecco's Phosphate Buffered Saline (dPBS) with Ca^{2+} and Mg^{2+} (Invitrogen, 14040-133). We recommend testing a range of v/v dilutions of BacMam Reagent—30%, 10%, 3%, and 1% (v/v) as a starting point (e.g., add 1 mL of BacMam Reagent per 10 mL of dPBS for a 10% v/v dilution).
6. Gently wash the cells once with dPBS with Ca^{2+} and Mg^{2+}.
7. Remove dPBS from Step 6, and gently add the solution of the diluted BacMam Reagent from Step 5 to the cells. Incubate the cells at room temperature (20–25ºC) for 3–4 hours protected from light.
8. Aspirate the transduction solution from the cell culture dish.
9. Add an appropriate volume of complete cell culture growth medium.
10. Add Enhancer Solution (PV5835) to 1X 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.
11. Incubate cells for 16–24 hours in optimal growth conditions (e.g., a humidified 37°C/5% CO_2 incubator).

Day 3

12. **Optional:** Analyze GFP expression levels by fluorescence microscopy using standard FITC filter sets.
13. 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.

14. 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).

15. 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.

16. Plate 20 µL of transduced cells in assay medium into white tissue culture-treated 384-well plates and incubate the plates in a humidified 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 4

17. Proceed to the **LanthaScreen® Cellular Assay** on page 7.

Alternative Transduction Protocol B (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 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 7.

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-2OS cells at 70–90% 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 A or B 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.

Observation	Potential Solutions
> 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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