

# Chapter 14

## BacMam: Versatile Gene Delivery Technology for GPCR Assays

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### Summary

BacMam viruses are modified baculoviruses that contain mammalian expression cassettes for viral gene delivery and transient expression in mammalian cells. They are easily, inexpensively, and rapidly generated and provide a versatile solution for G protein-coupled receptor (GPCR) cell-based assay development. Using BacMam technology, target gene expression levels are easily controlled and simultaneous delivery of multiple genes is possible, for example, coexpression of a receptor and a G protein or a reporter gene. BacMam viruses are compatible with the GPCR cell-based assay formats typically used in high-throughput screening and provide an unparalleled level of experimental flexibility that is simply not possible when using stable recombinant cell lines.

**Key words:** BacMam, Modified baculovirus, Transient expression, Viral transduction, G protein-coupled receptors, GPCR, Cell-based assay.

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### 1. Introduction

High-throughput screening (HTS) has become the primary approach for new lead discovery in the pharmaceutical industry and currently more than half of all screens are performed using cell-based assays (1). Increasing screening throughput of compound collections of in excess of 1 million discrete chemical entities and a trend toward HT G protein-coupled receptor functional rather than binding assays necessitates a steady supply of high-quality, assay ready cells. In addition to the cells and assays used for the primary HTS target, there is also a requirement of cell-based assays for the key selectivity target(s) as well as rodent orthologues needed for down stream lead optimization and SAR

screening. We have implemented BacMam virus gene delivery as means of rapidly generating robust and reproducible cell-based assays to support our GPCR screening activities.

We have recently reviewed our experiences with the technology and have documented the advantages we perceive in using BacMam-based transient gene delivery in lieu of stable cell lines to support cell-based drug discovery assays (2–4). Data obtained with BacMam-expressed receptors are consistent with data obtained with stable cell lines (5), but there are inherent advantages of BacMam as assays can be developed faster using transients than using stable cell lines. In addition, receptor expression levels can be easily controlled by titrating the amount of virus added to the host cells. BacMam has successfully been used to generate cell-based assays of GPCRs from each of the three main families of receptors and receptors, which are either  $G_i$ ,  $G_q$ , or  $G_s$  coupled (5). Recently, BacMam-expressed receptors have been used to support cell-based assays for several of GSK's GPCR lead optimization programs; notably fluorescent imaging plate reader (FLIPR) assays for characterization of Urotensin II receptor antagonists (6), CCR5 receptor antagonists (7), NK3 receptor antagonists (8), GPR40 receptor antagonists (9), as well as underpinning cAMP accumulation and GTP $\gamma$ S binding assays for an H3 antagonist program (10). Several reports have also reported success with BacMam-transduced cells in support of high-content  $\beta$ -arrestin-green fluorescent protein translocation GPCR assays (11–13).

Details of the BacMam transfer vectors and methods for virus production have been described previously (14, 15). It is not our intent in this chapter to describe functional assay methodology but rather to present methods for how one would go about using a BacMam virus encoding a GPCR of interest to transduce cells for use in a GPCR cell-based assay described elsewhere in this volume. While the procedures we describe will be exemplified with FLIPR assays, the procedures used to transduce the host cells and optimize the transduction conditions will be readily applicable to other assay formats.

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## 2. Materials

### 2.1. Biological Reagents, Buffers, and Kits

1. GPCR BacMam and G protein chimera BacMam (titer  $\sim 1 \times 10^8$ ) are generated as described (14, 15) and stored at 4°C in the dark.
2. U-2 OS (ATCC, Rockville, MD), Grip Tite 293 MSR (Invitrogen, Carlsbad, CA), HEK-293 (ATCC), CHOK1 (ATCC) cells, or other transducible mammalian host cells (16).

3. Dulbecco's modified Eagle's medium (DMEM)/F12 cell medium supplemented with 10% gamma-irradiated and heat-inactivated fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS).
4. Trypsin (JRH biosciences).
5. Target-specific ligands: ADP, histamine, acetylcholine (Sigma, St. Louis, MO).
6. FLIPR ligand dilution buffer: Hank's Buffered Salt Solution (HBSS) with calcium, magnesium, and 20 mM HEPES (pH 7.4; Invitrogen) and supplemented with 0.1% Bovine serum albumin (BSA; Sigma).
7. Probenecid stock: 285 mg probenecid in 1 mL 1 M NaOH (Sigma). Stock must be prepared fresh daily. Do not store.
8. FLIPR assay buffer: HBSS containing 2.5 mM probenecid (Sigma).
9. Brilliant Black-Fluo-4 kit (Molecular Devices Corporation, Sunnyvale, CA).
10. 2 mM Fluo-4 stock ( $1000\times$ ): 1 mg Fluo-4 (Molecular Devices Corporation) in 450  $\mu$ L 20% Pluronic Acid (Sigma).
11. 50 mM Brilliant Black Stock ( $100\times$ ): 2 g Brilliant Black in 46 mL distilled H<sub>2</sub>O.
12. Brilliant Black/Fluo-4 loading buffer: FLIPR assay buffer containing 500  $\mu$ M Brilliant Black and 2  $\mu$ M Fluo-4.

## 2.2. Plasticware

1. Sterile 15 mL (Corning, Lowell, MA) and 50 mL (BD Falcon, Franklin Lakes, NJ) conical tubes.
2. T75 and T150 tissue culture-treated cell culture flasks (Corning).
3. Sterile reagent troughs (Corning).
4. 384-well black, clear bottom TC-treated plates (Greiner, Monroe, NC, USA) and 384-well Black, clear bottom poly-D-lysine-coated plates (Greiner).
5. 384-well white polypropylene plates (Greiner).

## 2.3. Equipment

1. 37°C water bath.
2. Hemocytometer or other cell counting/viability instrumentation, such as CEDEX cell counter (Innovatis, Malvern, PA) or Vi-cell (Beckman coulter, Fullerton, CA).
3. Centrifuge and rotor for 15 and 50 mL tubes.
4. 8- and 12-channel (50/850  $\mu$ L) multi-channel pipettor (Matrix Technologies, Hudson, NH).
5. Humidified tissue culture incubator.
6. Flexstation, FLIPR, or FLIPR Tetra instrument (Molecular Devices Corporation).

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### 3. Methods

#### **3.1. BacMam-Transduced Cellular Systems for Robust and Reliable Functional Assays**

In both basic research and drug discovery, the ability to quickly develop specific, high-quality assays is a competitive advantage. For drug discovery efforts utilizing cellular systems, such as GPCR functional assay screening, BacMam reagents may be utilized to quickly compare multiple assay formats as well as a range of potential host systems during the initial phase of assay development. An early critical step in assay development, whether utilizing endogenous receptor, stable cell line, or transient system such as BacMam, is the characterization and choice of cellular host systems. Identification of a host system with no (or low) endogenous functional activity of target specific ligand is essential for development of a specific engineered cellular system. Besides allowing quick identification and reliable transient expression of GPCR targets for identification and characterization of agonists and antagonists, BacMam allows for changes in target expression levels which enable characterization of partial agonists/antagonists (2, 7).

For drug discovery work, the development of streamlined assay protocols with low false-positive and false-negative hit statistics, and excellent correlation of well-to-well, plate-to-plate, and day-to-day experimental results leads to high-quality data and lower costs because of fewer failed plates. During assay development, we measure efficacy (background, signal, and signal-to-background ratio), agonist and antagonist potency ( $pXC_{50}$ ), and  $Z'$  (17) from low and high signal control wells. Through multiple iterations assays are optimized for HTS and compound profiling. The methods below describe how to define and optimize a target-specific system utilizing a GPCR target BacMam to produce a robust and reliable assay.

#### **3.2. Preparation of BacMam Transductions**

The optimization of the BacMam transduction concentration, transduction time, and host cell line are important steps for the use of BacMam technology for GPCR assays. Transduction of mammalian cells can be performed in tissue culture flasks as described previously (15) or within the assay plate. To prepare for a FLIPR intracellular calcium assay, we recommend BacMam transduction directly in the assay plate. Below protocol will outline the process for initial optimization of BacMam concentration for a  $G_q$  protein-coupled receptor, assuming testing of duplicate 384-well plates in a traditional FLIPR assay for two BacMams per assay plate.

Host cells (i.e., HEK-293, CHO, U-2 OS) are maintained in Corning T150 culture flasks. Cells are cultured in DMEM/F12 medium and are split twice a week at 1:5 or 1:10 as needed depending on the host cells.

### 3.2.1. Basic BacMam Transduction Protocol

1. Recover host cells (HEK-293, CHOK1, or U-2 OS) from tissue culture flasks via trypsin treatment and add cells to conical centrifuge tubes (*see Note 1*).
2. Centrifuge cells (e.g.,  $100 \times g$ ) to remove medium and resuspend cells in growth medium to a density of approximately  $1 \times 10^6$  cells/mL.
3. Determine cell density and viability by Trypan blue exclusion via hemocytometer (or other cell counting instrument such as CEDEX or Vi-Cell).
4. Resuspend cells to a final density of  $3 \times 10^5$  viable cells/mL (*see Note 2*).
5. Combine cells and GPCR BacMam into four 15 mL conical tubes as described in **Table 1**.

**Table 1**  
**Single transduction BacMam titration table**  
**to optimize target BacMam concentration**

[BacMam] (%)	Host cell volume (mL)	BacMam volume ( $\mu$ L)
0	10	0
1	10	100
3	10	300
6	10	600

To test a range of volume-to-volume ratio of target BacMam to cells in duplicate plates, mix 10 mL of host cells ( $3 \times 10^5$  cells/mL) with BacMam volumes specified in the third column in 15 mL conical tubes. In secondary tests, one can refine or expand the range of concentrations tested, depending on the host cells and results from the initial experiment.

6. Gently mix cells and BacMam mixture by hand.
7. Transfer cells and BacMam to sterile reagent trough.
8. Using multichannel pipette, dispense 50  $\mu$ L ( $1.5 \times 10^4$  cells per well) of cell and BacMam mixture to 384-well plates as shown in **Fig. 1**.
9. Incubate cell transduction plates 18–24 h in humidified 37°C tissue culture incubator (*see Note 3*).

### 3.2.2. GPCR Functional Assay and Data Analysis

1. Load cells with appropriate FLIPR dyes as Brilliant Black-Fluo-4 Kit protocols:
  - a. Aspirate medium from wells.
  - b. Add 20  $\mu$ L loading dye.
  - c. Incubate cells at 37°C, 5% CO<sub>2</sub> for 1 h.

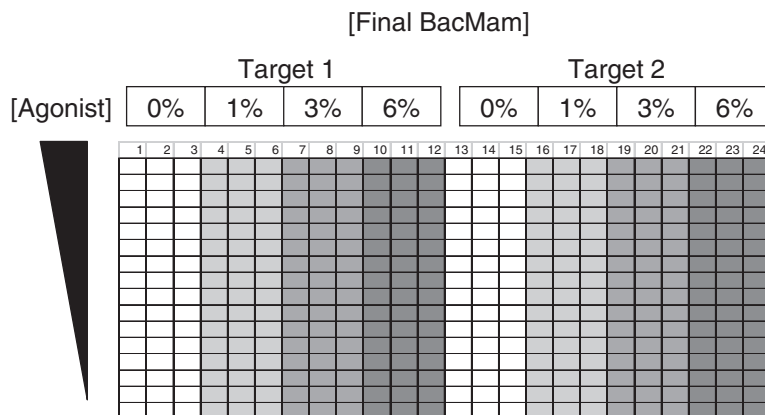


Fig. 1. A plate map of cells transduced with a range of BacMam concentrations. Agonist dilutions are prepared in 384-well polypropylene plates with high concentration in row A, 1:3 dilutions through row O, and no agonist control in row P. The plate can accommodate two targets in this format.

2. Prepare threefold concentration of serially diluted ligand in FLIPR ligand dilution buffer (1:2 or 1:3 dilutions with 12 points) in 384-well polypropylene plate (*see Note 4*).
3. Dose cells with 10  $\mu$ L per well target-specific ligand as shown in **Fig. 1** Platemap.
4. Read on FLIPR instrument as per standard protocols.
5. Graph data (for FLIPR max-min) and calculate pEC<sub>50</sub> as exemplified in **Fig. 2** in which agonist dose-response curves were obtained on FLIPR from U-2 OS cells transduced with varying concentrations of a BacMam encoding the rat P2Y1 receptor.

### 3.2.3. Interpretation of Results

From the results presented in **Fig. 2**, at the concentration tested there was no response to ADP agonist in untransduced U-2 OS cells and the potency of ADP was similar regardless of BacMam concentration. However, the most efficacious response was observed upon transduction with 1% BacMam (*see Note 5*).

### 3.3. Optimizing Cotransduction Conditions: Receptor and G protein

There are times when more than one BacMam must be introduced into host cells in order to create a biological system that is functionally responsive (*see Note 6*). For example, G<sub>i</sub>-coupled receptors can be evaluated in FLIPR assays through coexpression of chimeric G proteins. Also, new cell-based assay methods for measuring intracellular calcium levels or cAMP concentrations for G<sub>i</sub>- and G<sub>s</sub>-coupled receptors expressed in suspension cells are becoming more common place. These assay formats are amenable to BacMam

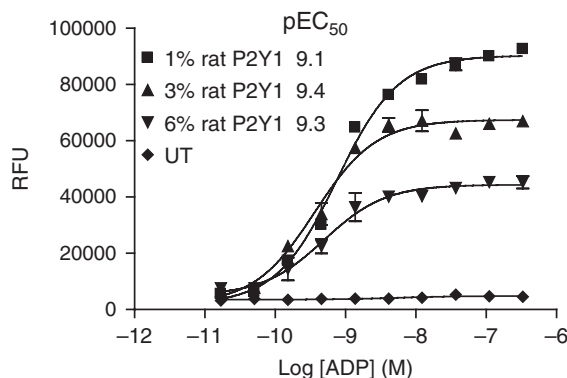


Fig. 2. Initial titration of rat P2Y1 BacMam in U-2 OS cells. U-2 OS cells ( $3 \times 10^5$  cells/mL) were transduced with 0%, 1%, 3%, and 6% rat P2Y1 BacMam for 20 h at 37°C (UT, untransduced U-2 OS cells). Transduced cells dosed with ADP agonist were read on FlexStation using Calcium 3 kit (MDC) protocol. In this experiment, the most robust results were obtained with U-2 OS cells transduced with 1% BacMam. This assay was further optimized to reduce the final BacMam concentration to lower than 1% (data not shown).

delivery as well. For example, aequorin protein may be coexpressed with GPCR target via dual transduction BacMam approach (see **Note 7**).

### 3.3.1. Dual Transduction Protocol: Target and Chimeric G Protein BacMams

1. Follow Steps 1–5 in **Section 3.2.1**.
2. Mix cells, target-specific BacMam, and chimeric G protein BacMam as described in **Table 2** (see **Note 8**).
3. Proceed with remaining steps in **Sections 3.2.1** and **3.2.2** (see **Note 9**).

### 3.3.2. Interpretation of Dual Transduction (GPCR + G Protein Chimera) Experiments

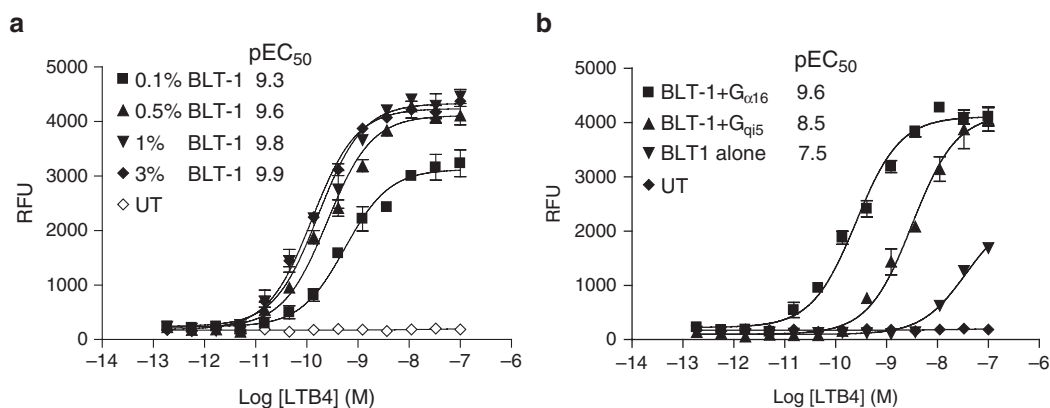
As depicted in **Fig. 3a**, changes in the target BacMam concentration will result in a range of potencies and efficacies. Since the lowest concentration of BLT-1 BacMam with the highest efficacy was 0.5% (in the presence of 0.5%  $G_{\alpha 16}$ ) in U-2 OS, this concentration was utilized for future optimization studies. BLT1 BacMam was also cotransduced with a range of chimeric G protein  $G_{qi5}$  concentrations (data not shown) in the same experiment. In **Fig. 3b**, data for 0.5% BLT1 BacMam alone versus cotransduction with 0.5%  $G_{qi5}$  or  $G_{\alpha 16}$  are plotted. Note that BLT1 alone had low efficacy and potency, while coupling with chimeric G proteins resulted in a marked increase in efficacy in response to LTB4 treatment. Dual transduction of chimeric G protein  $G_{\alpha 16}$  with BLT1 resulted in the highest potency response to ligand and therefore was the reagent selected for further assay optimization steps.

**Table 2**

**Dual transduction BacMam titration table to optimize target BacMam concentration and choose optimal chimeric G protein BacMam**

Target		G protein chimera		Host cells
[BacMam] (%)	Volume ( $\mu$ L)	BacMam [%]	Volume ( $\mu$ L)	Volume (mL)
0	0	0.50	50	10
0.10	10	0.50	50	10
0.50	50	0.50	50	10
1	100	0.50	50	10
3	300	0.50	50	10
6	600	0.50	50	10

To identify the best coupling agent for the target, use the table above to set up dual transductions with multiple chimeric G proteins in parallel. For initial testing of dual transductions, vary the GPCR target BacMam concentration while keeping the chimeric G protein BacMam concentrations constant. Once the optimal target concentration is determined, the G protein BacMam concentration is optimized by fixing the target BacMam concentration at the optimal level and varying the G protein concentration.



**Fig. 3. (A)** Initial optimization of dual transduction conditions. A range of BLT-1 BacMam concentrations (0%, 0.1%, 0.5%, 1%, and 3%) were transduced in U-2 OS cells ( $3 \times 10^5$  cells/mL) in the presence of 0.5% chimeric G protein  $G_{\alpha 16}$  BacMam (UT, untransduced U-2 OS cells). Cells were incubated 20 h at 37°C, loaded with dye, dosed with LTB4 agonist, and then read on a FLIPR Tetra using Fluo-4 Brilliant Black no-wash FLIPR protocol. Results demonstrated that 0.5% BLT-1 BacMam was the lowest concentration required for optimal efficacy and potency. Further assay development was continued with 0.5% BLT-1 BacMam-transduced cells. **(B)** Determination of best chimeric G protein BacMam for optimal target performance. Dual transductions were prepared as described in **Table 2**. Duplicate plates were prepared by mixing U-2 OS cells ( $3 \times 10^5$  cells/mL) with 0.5% BLT-1 BacMam alone or in combination with 0.5%  $G_{\alpha 16}$  or 0.5%  $G_{\alpha i5}$  BacMam (UT, untransduced U-2 OS cells). As per outlined protocol, cells were incubated for 20 h at 37°C. Following dye load and treatment with LTB4 agonist, as per Fluo-4 Brilliant Black no-wash FLIPR protocol, plates were read on FLIPR Tetra. Results demonstrated that U-2 OS cells cotransduced with BLT-1 and  $G_{\alpha 16}$  BacMams had similar efficacy but greater potency than cells cotransduced with BLT1 and  $G_{\alpha i5}$  BacMams.



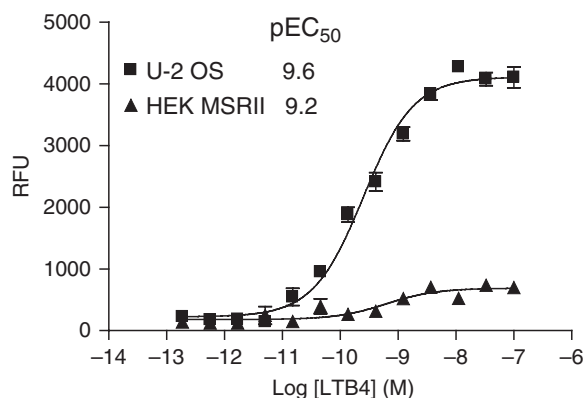
### 3.4. Optimizing Transduction Conditions: Varying Host Cells

#### 3.4.1. Characterizing BacMam Expression in a Range of Host Cells

The use of untransduced cells (no BacMam control) is an important control to determine the endogenous response to the test ligand in the host cells. If there is a weak response in the target-transduced host cells or potent and efficacious response to the ligand in the host cells in the absence of transduced target, proceed with BacMam transductions in a larger host cell panel.

1. Trypsinize different host cells, such as U-2 OS cells, CHOK1 cells, or HEK-293 cells (*see Note 10*).
2. Proceed with steps outlined in 3.2.1 and 3.2.2 (*see Notes 2 and 11*).

In **Fig. 4**, U-2 OS and HEK MSRII cells were cotransduced with 0.5% BLT1 and 0.5%  $G_{\alpha 16}$  BacMams. While the LTB4 agonist potencies were quite similar, U-2 OS cells had a more efficacious response to the ligand, which is highly desirable for development of a robust and reliable cellular assay.



**Fig. 4.** Testing host cell lines for optimal target expression. U-2 OS and HEK MSRII (HEK-293 cells engineered to express macrophage scavenger receptor) cells ( $3 \times 10^5$  cells/mL) were cotransduced with 0.5% BLT1 and 0.5%  $G_{\alpha 16}$  BacMams for 18 h at 37°C. As per Fluo-4 Brilliant Black no-wash FLIPR protocol, cells were loaded with dye, dosed with LTB4 agonist and plates were read on FLIPR Tetra. Results demonstrated that LTB4 had higher potency and efficacy in U-2 OS cells transduced with BLT1 and  $G_{\alpha 16}$  compared to HEK MSRII cells cotransduced with the same BacMams.

### 3.5. Transduction of Frozen Host Cells

Cryopreserved host cells are an excellent alternative to freshly maintained cells and may be substituted for freshly maintained cells without alterations to the base transduction protocol. In most cases, frozen cells utilized as transduction hosts have similar performance to freshly maintained cells in FLIPR assays (**Fig. 5**) (*see Note 12*).

1. Prewarm medium to 37°C.
2. Remove cryopreserved cells from liquid nitrogen.

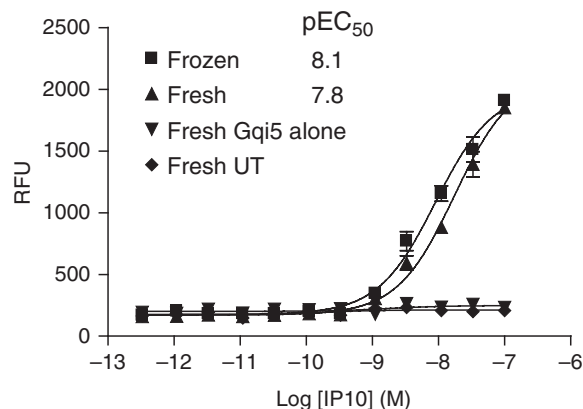


Fig. 5. Functional performance of freshly maintained versus frozen stocks of host cells. Freshly maintained and thawed frozen U-2 OS cells ( $3 \times 10^5$  cells/mL) were cotransduced with 1% mCXCR3 and 0.5% G<sub>qi5</sub> G protein chimera BacMam (UT, untransduced cells). Following 18 h transduction at 37°C, cells were loaded with dye as per standard Fluo-4 Brilliant Black no-wash FLIPR protocol and dosed with IP10 ligand. Similar IP10 efficacy and potency were observed from transductions prepared with freshly maintained and frozen U-2 OS cells.

3. Thaw frozen cell aliquots rapidly with agitation in 37°C water bath.
4. Continue with Step 2 of the BacMam transduction procedure in **Sections 3.2.1** and **3.2.2** (*see Note 13*).

#### 4. Notes

1. U-2 OS cells are a good starting place for BacMam-based GPCR assays as these cells are highly susceptible to BacMam transduction and lack many of the endogenous receptors present on HEK-293 and other mammalian host cells typically used for cell-based GPCR assays (18).
2. Optimal host cell concentration will vary, depending on the characteristics of the host cell and the assay readout. Cell density may be optimized once functional response is confirmed. For 384-well transductions for FLIPR assays,  $1.0 \times 10^4$  cells per well is often optimal transduction cell density for U-2 OS cells while higher cell densities ( $1.5 \times 10^4$  to  $2.0 \times 10^4$  cells per well) are generally best for HEK-293-derived and CHO-derived host cells for 24 h end-point read.
3. For many GPCR targets, 24 h transduction time results in optimal expression and excellent functional response. As with transient transfections, incubation time required for optimal

functional response is target specific. If signal window is low, test functional response following 48 and 72 h transduction time. BacMam concentration, transduction time, cell density, cell host line, and target GPCR are codependent variables, so changes in transduction time will often require re-optimization of some or all of the other experimental variables.

4. For each cell line, a specific ligand for an endogenous receptor may be included as an additional control (e.g., histamine for U-2 OS cells, muscarine or acetylcholine for HEK-293 cells and ADP for CHO cells).
5. At completion of the initial reagent validation experiment, there are several kinds of follow-up experiments that can be initiated to improve the assay in terms of reproducibility and agonist-induced efficacy and potency. For example, in **Fig. 2**, 1% BacMam results in the most efficacious response. We repeated the basic transduction protocol for this target, with further refinements to transduction levels (0.1%, 0.5%, 1%, 2%) and measured not only efficacy and potency via dose response, but also additional wells for  $Z'$  controls (16 no ligand wells and 16 EC<sub>100</sub> wells for agonist assays or DMSO first addition with 16 buffer and 16 EC<sub>80</sub> second addition for antagonist assays (9)). From this experiment, the final optimized BacMam concentration was 0.1%, which was the concentration with the greatest efficacy, most potent response, and lowest variation as determined by  $Z'$  calculation.
6. Multiple BacMams may be introduced to cells simultaneously. We have introduced as many as five BacMams into host cells concomitantly. It must be noted, however, that individual host cells will have some transduction limit. Also, extreme overexpression of some targets may have deleterious effects on cell health or functional performance.
7. For suspension assay formats, BacMam transductions may be performed in culture flasks or cell stacks. Following the 24–48 h transduction time, cells are harvested and used directly as per the aequorin or cAMP detection protocols.
8. G<sub>α16</sub> and G<sub>qi5</sub> are commonly used G protein chimeras. Refer to **Table 2** to set up separate transductions in parallel with the target in combination with each chimeric G protein. Other G protein chimeras or accessory proteins may also be cotransduced as additional variables.
9. If background is observed because of G protein coupling to endogenous GPCRs, optimize the G protein BacMam concentration by fixing the target BacMam concentration at the lowest dose that results in highest efficacy and potency and cotransduce a range of G protein BacMam concentrations as described in **Table 2** for the optimization of target BacMam concentration.

10. When using HEK-293 cells in FLIPR assays, utilize poly-D-lysine-coated assay plates to improve well-to-well reproducibility. Alternatively, HEK-293 cells that are engineered to express macrophage scavenger receptor II (MSR<sup>II</sup>) (19), such as the commercially available GripTite cells (Invitrogen), retain the high transduction efficiency of HEK-293 cells, yet display superior adherent properties. For these reasons, HEK-293 cells expressing MSR<sup>II</sup> are an excellent choice host cell for BacMam-transduced FLIPR assays and other adherent cell assays requiring wash steps.
11. HEK-293 and U-2 OS cells are excellent choices for first host cell panel, due to their excellent transduction efficiencies. A secondary panel of transducible host cells might include Saos-2, SH-SY5Y, CHOKI, BHK, Cos-1 and CV-1 cells, for example.
12. Cells to be used for FLIPR, aequorin, or cAMP measurements may also be transduced in flasks or cell stacks, harvested, and used directly or frozen for later use. If the transduced frozen cells are to be utilized for FLIPR assays, test effect of transduction time (24, 48, and 72 h) on freshly transduced cells prior to freezing cells. If there is a decline in efficacy or potency at 72 h, for example, keep the total time (transduction time plus the time cells are in assay plate) to 48 h, or where performance is optimal for transduction of fresh cells. For suspension assays formats such as cAMP measurement or aequorin, cells that are transduced, harvested, frozen, and then thawed for assay may require a thaw recovery time for best assay performance. For post-thaw recovery, use growth medium with gentle stirring and test various recovery times (15 min to several hours) and temperatures (37°C, room temperature, and 4°C).
13. The use of untransduced cells (no BacMam control) is important to determine the endogenous responses to ligand. If the G protein chimera is cotransduced with the target, then the G protein chimera transduced cells without the target is used as an additional control.

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