Chapter 5

cAMP Assay for GPCR Ligand Characterization: Application of BacMam Expression System

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Abstract

Cyclic adenosine monophosphate (cAMP) is a second messenger of many G-protein-coupled receptors. The change in cellular cAMP level has widely been used to estimate the biological activity of various GPCR-specific agents. Förster resonance energy transfer (FRET) biosensors have been around for almost 10 years and became increasingly popular for cAMP detection. Ratiometric sensitized emission assay of a FRET biosensor can easily be implemented on fluorescence plate reader platforms. For such assays a considerable amount of cells expressing the desired biosensor is needed. A method to achieve sufficient and reproducible level of cAMP biosensor protein expression with the means of BacMam transduction system is the subject of this chapter.

Key words cAMP assay, BacMam, Baculovirus, $^{T}Epac^{VV}$, FRET, Epac-camps, Adjustable protein expression

1 Introduction

From a variety of cellular cAMP assays [1], Förster resonance energy transfer (FRET)-based biosensors allow real-time detection of changes in cellular cAMP levels. Fluorescence lifetime imaging microscopy (FLIM) and detection of sensitized emission (SE) are the techniques to assay the changes in cAMP concentration using FRET-based biosensors. The SE measurement relies on the relative fluorescence of donor and acceptor fluorophores genetically fused to a cAMP binding protein moiety. The sensor proteins are freely distributed in the cell cytosol, and binding of cAMP leads to a conformational change associated with an increase in the distance between the fluorophores, which results in a decrease in acceptor fluorescence and an increase in donor fluorescence. The calculated acceptor/donor emission ratio correlates with the change in the level of intracellular cAMP. In our laboratory we have successfully used Epac2-camps biosensor [2] and have now moved on to a next

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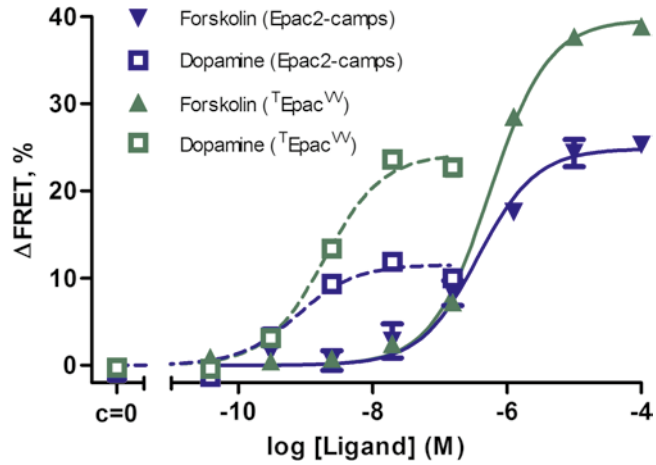

Fig. 1 Cellular responses to forskolin and dopamine measured with Epac2-camps and $^{T}Epac^{W}$ biosensors. HEK293 cells stably expressing recombinant dopamine D1 receptors were transduced with BacMam virus for 3 h and further incubated for 21 h in complete growth medium supplemented with 10 mM sodium butyrate. Cells were treated with adenylate cyclase activator forskolin or D1R agonist dopamine for 10 min at 37 °C. The calculated potencies determined with two different biosensors were in excellent agreement, micromolar for forskolin and nanomolar for dopamine. The dynamic range (FRET change window) was almost two times wider for the $^{T}Epac^{W}$ biosensor. Graph showing data from a representative experiment performed on triplicates

generation $^{T}Epac^{VV}$ biosensor [3] (Fig. 1). Both Epac2-camps and $^{T}Epac^{VV}$ reflect changes in intracellular cAMP from sub- to high micromolar concentrations, and their potencies for cAMP (μM range) are well suited for GPCR ligand screening.

The pharmacological validity of the cAMP biosensor expressed using the BacMam system (baculovirus for gene delivery to mammalian cells) was confirmed by melanocortin MC1 receptor activation with a set of known full and partial agonists [4]. In our laboratory we have also been successfully detecting activation of other GPCRs in various cell lines (e.g., recombinant dopamine receptor subtypes and endogenous β2-adrenergic receptors in HEK293 cells, recombinant melanocortin receptor subtypes in CHO-K1 cells, and recombinant LHCG receptors in COS7 cells). Assay conditions for the particular receptor of interest may require additional optimization, but the BacMam technology is compatible with a broad range of cells including primary and stem cells [5]. Using BacMam provides advantages such as adjustable protein expression levels, low cytotoxicity to host cells, safety in production and handling (Biosafety Level 1), and ease of use. Since the constant need for transfection reagents is eliminated, the relatively low cost of BacMam system is also a considerable advantage.

Klarenbeek and Jalink have recently described a protocol on how to use the $^{T}Epac^{VV}$ cAMP biosensor for microscopy on live cells [6]. Our focus here is to describe a protocol for the generation of the BacMam virus and for setting up the subsequent assay in a fluorescence microplate reader with simultaneous dual emission mode of SE (emission ~480 nm and ~530 nm, excitation ~430 nm). We will provide a detailed protocol for sufficient and adjustable expression of the biosensor proteins in most mammalian cell lines.

We describe the sequential steps required to generate, harvest, titrate, and concentrate the BacMam viruses with the cAMP biosensor gene cloned into the baculovirus genome under the control of cytomegalovirus (CMV) promoter. We will cover the expression of the biosensor protein in mammalian cells and give a protocol for a simple, ratiometric cAMP assay to determine the potencies of GPCR ligands in real time. The selection of the best cAMP sensor construct is vital, and we suggest consulting further materials for detailed information and the best choice for your application [1, 6]. Our protocol is applicable for generation of the BacMam expression system with any desired biosensor DNA construct.

2 Materials

All solutions for molecular cloning and for the assay are made with Milli-Q water unless stated otherwise. Diligently follow all waste disposal regulations when disposing waste materials and/or biological material.

2.1 Cell Culture

1. Sf9 isolated from pupal ovarian tissue of the fall armyworm *Spodoptera frugiperda* (*see* **Note 1**).
2. HEK293 cell line expressing the GPCRs of interest (*see* **Note 2**).
3. Sf9 growth medium: EX-CELL 420.
4. HEK293 growth medium: DMEM supplemented with 10 % FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin and if necessary 400 µg/mL of Geneticin (G418) to ensure stable expression of recombinant GPCR of interest, in our case dopamine D1 receptor.
5. Polylysine-coated plastic dishes.
6. Humidified CO_2 incubator.
7. Laminar flow cabinets (*see* **Notes 3** and **4**).

2.2 Plasmids and Generation of BacMam Virus

1. $^{T}Epac^{VV}$ gene in pcDNA3.1 expression vector: mTurqDelEPAC(dDEPCD)-cp173Venus(d)-Venus(d) (H74) [3].
2. Epac2-camps gene in pcDNA3.1 expression vector [2].

3. *E. coli* cells: DH5α for amplification of plasmid DNA and DH10BAC for production of bacmid DNA.
4. Baculovirus vector: pFastBac1 vector (Invitrogen Life Technologies).
5. Restriction enzymes Bst1107I (BstZ17I), Bsp68I (NruI), Eco105I (SnaBI), KspAI (HpaI).
6. Ligase.
7. Plasmid DNA purification kit.
8. Agar-LB plates: 2 % agar-LB plates with 100 μg/mL X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside), 50 μg/mL IPTG (isopropyl-beta-D-thiogalactopyranoside), 50 μg/mL kanamycin, 10 μg/mL tetracycline, 7 μg/mL gentamicin. Also 2 % agar-LB plates with 100 μg/m μg ampicillin.
9. Transfection reagent: polyethylene imide-based reagent ExGen500 (Fermentas).
10. Dulbecco's phosphate-buffered saline (DPBS) for storage of virus stocks.
11. Beckman Coulter cell counter (Z2™ Series COULTER COUNTER® Cell and Particle Counter) for determination of the virus titer.

2.3 Tangential Flow Filtration (TFF) System

1. TFF system from PALL containing the Centramate™ LV cassette holder with Omega LV Centramate™ polyethersulfone membrane T-series cassettes (0.02 m^2, nominal molecular weight cutoff: 300 kDa) and connected to a peristaltic pump (ECOLINE VC-360) that is combined with high flexibility tubing and pressure gauges to monitor feed and retentate pressures.

2.4 cAMP Biosensor Protein Expression

1. Recombinant baculovirus.
2. 70–80 % confluent mammalian cells on petri dishes.
3. Serum-free RPMI for transduction with the recombinant baculovirus.
4. Sodium butyrate at final concentration of 5–10 mM for enhancing of protein expression after the transduction with the recombinant baculovirus.

2.5 Fluorescence Microplate Reader for the cAMP Assay

1. Fluorescence from $^{T}Epac^{VV}$ biosensor (or Epac2-camps biosensor) is measured using a microplate reader (in our case PHERAstar, BMG LABTECH GmbH).
2. Excitation filter 427(20) nm.
3. Simultaneous dual emission filters 480(20) nm and 530(20) nm.
4. The GAIN is adjusted and in our case set to 1,000 for both emission channels.
5. The focal height is adjusted and in our case set to 4.2 mm.

3 Methods

3.1 Plasmids and Generation of BacMam Virus

1. Clone the $^{T}Epac^{VV}$ construct under the control of the cytomegalovirus (CMV) promoter into the pFastBac1 vector using the restriction enzymes Bst1107I (BstZ17I) and Bsp68I (NruI) for pcDNA3.1(+) and Eco105I (SnaBI) and KspAI (HpaI) for pFastBac1, respectively (*see* **Note 5**).
2. Isolate the desired donor and recipient fragments using agarose-gel electrophoresis, ligate the fragments, transform the ligation mixture into *E. coli* DH5α cells, and plate the cells on 2 % agar-LB plates containing 100 μg/mL ampicillin.
3. Amplify the selected bacterial colony (*see* **Note 6**).
4. Purify the pFastBacMam plasmid using any DNA purification kit. In brief: lyse the bacterial pellet, neutralize the lysate, wash the DNA with ice-cold isopropanol and 70 % ethanol (some kits provide DNA binding columns for easy washing procedure), remove the residual alcohol, and resuspend the plasmid DNA in Milli-Q water.
5. Transform pFastBacMam plasmid into *E. coli* DH10BAC competent cells and plate them on agar-LB plates supplemented with 100 μg/mL X-gal, 50 μg/mL IPTG, 50 μg/mL kanamycin, 10 μg/mL tetracycline, and 7 μg/mL gentamicin.
6. Amplify the selected white bacterial colony (*see* **Note 7**).
7. Purify the recombinant bacmid DNA using the same lysis, neutralization, and alcohol solutions as in plasmid purification, but do not use DNA binding columns, because bacmid DNA is a large molecule (~140 kbp) and will clog the column.
8. Transfect the purified bacmid DNA into Sf9 insect cells using the ExGen500 reagent (*see* **Note 8**) to prepare BacMam virus stocks according to the Invitrogen Life Technologies Bac-to-Bac expression system manual [7].
9. Incubate the cells for 5 days and transfer the supernatant fraction that contains recombinant baculoviruses (P0) to 10 mL Sf9 cell suspension at a density of $1–1.5 \times 10^6$ cells/mL. Incubate the cells for 3–5 days.
10. Harvest the P1 virus, determine the titer of the stock, and store it at +4 °C as an initial transfection virus stock (*see* **Note 9**).
11. Amplify P1 virus by infecting the desired volume of Sf9 cells at the density of $1–1.5 \times 10^6$ cells/mL at multiplicity of infection (MOI) 0.01–0.1, or if the titer of the virus stock is unknown, just use up to 1 % of the total volume.

12. Harvest P2 virus after 3–5 days and determine the titer. Concentrate P2 viral stocks ten times (no lower than 10^8 pfu/mL) and store the aliquots in DPBS at +4 °C until the day of the experiment (*see* **Notes 10** and **11**).

3.2 Collection, Concentration, and Storage of BacMam Virus

1. Harvest the Sf9 cell suspension after the cell viability has dropped below 50 % (*see* **Note 12**).
2. Centrifuge the cells for 10 min at 1,000 × *g* and transfer the supernatant to sterilized (e.g., autoclaved, UV light, or plasma cleaner treated) centrifuge tubes or into a TFF feed vessel.
3. If you concentrate by centrifugation, use at least 40,000 × *g* for 40 min. Resuspend the pellet in DPBS. Aliquots can be stored protected from light at +4 °C for about 2 months (*see* **Note 13**).
4. If you concentrate by tangential flow filtration (TFF), use a polyethersulfone membrane cassette (nominal molecular weight cutoff: 300 kDa) (*see* **Note 14**). Perform the TFF concentration in a cold room (+4 °C) to ensure an inert environment for the samples (*see* **Note 15**).
5. Set the transmembrane feed pressure to 1.5–3 bar and the retentate pressure to 0.2 bar.
6. Pour the baculovirus-containing supernatant into the feed vessel to start the TFF procedure. Concentrate the initial volume of baculovirus suspension about ten times (*see* **Note 16**) by controlling/monitoring the volume of permeate solution (*see* **Note 17**).
7. Perform the diafiltration in order to exchange the cell culture medium for the storage buffer (DPBS)—for this add DPBS (equal volume of the circulating retentate solution) to the feed vessel and concentrate again to the desired volume. Repeat this procedure five times to ensure 99.7 % of buffer exchange.
8. Pour your concentrated baculovirus solution (retentate) in a sterile graduated vessel (usually, 50 mL tube).
9. In order to recover most of the virus from the TFF membrane, rinse it with additional 5–10 mL of DPBS, collect the retentate, and combine with the previously collected portion of budded baculovirus solution (*see* **Note 18**).
10. Store aliquots at –90 °C until used for cAMP biosensor expression.
11. Before each usage of the TFF system, it should be sanitized according to the manufacturer's instructions. This procedure takes about 45–60 min and is performed in the cold room. Briefly, perform the sanitization by recirculating 1 L of 1 M NaOH for 30 min through the whole TFF system. Then rinse the system with 1 L of Milli-Q water and thereafter check the pH of permeate and retentate solutions.

12. After the usage of TFF system, rinse it with 1 L of 1 M NaOH for 30 min followed by further rinsing with 1 L of 0.2 M NaOH for another 30 min.
13. Store the membranes of TFF system in 0.2 M NaOH at +4 °C.

3.3 Determination of Virus Titer with Cell Size-Based Assay

In our laboratory viral titers are determined using a cell size-based assay [8] with modifications to simplify it for routine use.

1. Seed Sf9 cells on 24-well cell culture plates at 0.2×10^6 cells/well in 250 µL of EX-CELL 420 cell culture medium and allow for the cells to adhere for 30–60 min.
2. Add 250 µL of dilution samples to wells (threefold serial dilution of harvested virus supernatant or of concentrated virus in EX-CELL 420 medium).
3. Incubate the cells in the presence of virus for 24 h and thereafter determine the average cell diameter using a Beckman Coulter cell counter (*see* **Note 19**).
4. Plot the average cell diameter versus log (virus dilution) on graph and calculate the virus concentration in infectious units per mL (IU/mL) from a sigmoidal dose-response curve using Eq. 1:

$$\text{Virus concentration}\left(\text{IU}/\text{ml}\right) = \frac{\frac{1}{\text{ED}_{50}} \times 50\,\%\ \text{of infected cells}}{V}, \qquad (1)$$

where V is the sample volume in wells in milliliters (here 0.5 mL), ED_{50} is 50 % effective virus dilution corresponding to the dilution at which the average cell diameter has changed 50 %, and 50 % of infected cells is 50 % of the cells in wells at the time of infection (here 0.1×10^6 cells) given that the number of cells is roughly equal to the number of infective viral particles and the proportion of secondary infection is minimal [9].

To present virus titers in commonly used plaque-forming units (pfu/mL), the titers in IU/mL, calculated according to Eq. 1, are transformed using a linear in-lab correlation Eq. 2 obtained from plotting titers of different virus stocks as IU/mL versus pfu/mL, derived experimentally in cell size-based assay and plaque assay, respectively. Plaque assay was performed as described in Invitrogen Life Technologies Bac-to-Bac expression system manual [7].

$$\log Y = 0.92 \times \log X + 0.67, \qquad (2)$$

where Y is the desired virus titer in pfu/mL and X is the experimentally derived titer in IU/mL calculated according to Eq. 1. The titers (pfu/mL) obtained by Eq. 2 are used for determination of MOI of BacMam baculoviruses (*see* **Note 20**) for expression of the cAMP biosensor in mammalian cells, described below.

3.4 $^{T}Epac^{VV}$ Biosensor Protein Expression

1. Seed mammalian cells (expressing the desired recombinant GPCR) on petri dishes 2–3 days before transduction at about

$(0.8–1.5) \times 10^6$ cells/dish in complete cell culture medium without the selection antibiotics (*see* **Note 21**).

2. On the day of transduction, aspirate the medium from cells and add 4 mL of serum-free RPMI medium (*see* **Note 22**) and 250–500 μL of the ten times concentrated virus stocks of BacMam $^{T}Epac^{VV}$ baculovirus at approximately MOI 100–400 (*see* **Note 23**).
3. After 2–3 h of incubation in serum-free RPMI, remove the virus solution and add 8–10 mL of routine culture medium, supplemented with sodium butyrate at 5–10 mM final concentration (*see* **Note 24**). Before the assay the cells are further incubated for 20–40 h to allow the expression of $^{T}Epac^{VV}$ protein (*see* **Notes 25–27**).

3.5 cAMP Assay

1. On the day of assay, remove cells from the dish by using trypsin (or use rubber policeman to detach the cells).
2. Seed the cells on black clear-bottom 96-well cell culture plates (*see* **Note 28**) in 80 or 90 μL/well of DPBS supplemented with Ca^{2+} (1.2 mM) and Mg^{2+} (0.5 mM) at a cell density ranging from 30,000 to 60,000 cells/well (typically cells from one petri are seeded on one or two 96-well plates). Allow cells to adhere for approximately 1–2 h and use them in the assay. All reactions are carried out in a final volume of 100 μL (*see* **Note 29**). Measure the fluorescence using a fluorescence plate reader at 37 °C.
3. First detect the background fluorescence intensities for the non-stimulated cells by exciting at 427(20) nm (mTurquoise excitation) and measuring simultaneous dual emission of the excited fluorophores at 480(20) nm (mTurquoise emission) and 530(20) nm (Venus emission).
4. For the receptor agonist dose-response assays, add the ligand solutions (at 10× concentrations) to wells (a′ 10 μL/well) achieving the final volume of 100 μL.
5. Measure the responses over 30 min, measurements every 1–5 min.
6. If you want to measure the effect of antagonists to block agonist response, preincubate the cells with antagonist (a′ 10 μL/well) for 10 min before the addition of agonist (a′ 10 μL/well) in a final volume of 100 μL. Measure the responses over 30 min, measurements every 1–5 min.

3.6 Data Analysis

1. Calculate the change in FRET (ΔFRET) using equation

$$\Delta\frac{530\,\text{nm}}{480\,\text{nm}} = \frac{\frac{530\,\text{nm}_0}{480\,\text{nm}_0} - \frac{530\,\text{nm}}{480\,\text{nm}}}{\frac{530\,\text{nm}_0}{530\,\text{nm}_0}}$$

as described by Mazina et al. [4].

2. Analyze pharmacological data by means of nonlinear least squares regression analysis using the commercial program GraphPad Prism™ 5.00 (GraphPad Software Inc., CA). Plot the data and calculate the pEC_{50} values that are calculated from a sigmoidal dose-response curve (Fig. 1).

3.7 Time Frame

The entire protocol takes approximately 3–4 weeks to complete.

1. Cloning the cAMP biosensor gene into pFastBac1 vector and purification of plasmid DNA of pFastBacMam
(restriction + agarose-gel electrophoresis to isolate and purify donor and recipient DNA (*1 day*))
(ligation + transformation of the recombinant pFastBacMam plasmid (*1 day*) + purification of DNA (*1 h*)).
2. Transforming plasmid DNA of pFastBacMam into DH10BAC cells and purification of bacmid DNA
(transformation (*4 h*) + color-selection of colonies (*2 days*) + purification of bacmid (*2 h*)).
3. Transfection of Sf9 cells with bacmid DNA
(transfection (*30 min*) + incubation (*3–5 days*)).
4. Harvesting of P1 BacMam virus, determination on virus titer
(harvesting by centrifugation (*10 min*) + preparing 24-well plates with Sf9 for titration experiment (*30 min*) + incubation with virus (*24 h*) + titration experiment (*30 min*)).
5. Amplification of BacMam virus, harvesting of P2 virus, determination on virus titer, concentration of the P2 virus
(transduction with P1 virus (*5 min*) + incubation with virus (*3–5 days*) + harvesting by centrifugation (*10 min*) + titration experiment (*24 h*) + concentration by centrifugation (*1 h*/by TFF *2 h*)) (Larger volumes are easier to concentrate using TFF, but the preparation for TFF requires a little bit more time compared to concentration by centrifugation).
6. Transduction of mammalian cells with P2 BacMam virus
(transduction (*5 min*) + incubation with virus (*2–3 h*) + exchange of media (*5 min*) + incubation with complete medium and 5–10 mM sodium butyrate (*20–40 h*)).

7. Performing of cAMP assay
   (seeding the cells on the assay plate (*15 min*) + attaching of the cells (*1–2 h*) + the assay (*10 min to 2 h*)).
8. Data analysis
   (quick analysis (*30 min*), deeper analysis depends on the assay design and questions asked).

4 Notes

1. Sf9 insect cells are cultured in suspension with EX-CELL 420 growth medium in a 27 °C incubator in a nonhumidified environment.
2. Mammalian cells used for GPCR studies are grown as an adherent monolayer on polylysine-coated plastic dishes and maintained at 37 °C and 5 % CO_2 in a humidified incubator in complete growth medium.
3. For the work with cells and baculoviruses, use aseptic technique in laminar flow cabinets (sterile conditions).
4. Baculoviruses and Sf9 cell culture require biosafety level 1 (BSL 1); some common mammalian cell lines (e.g., HEK293 cells) may require BSL 2 laboratory.
5. If you use another construct or another expression vector, you will need to search for suitable restriction enzymes.
6. The polyhedrin promoter is removed from the pFastBac1 vector during cloning to ensure low promoter interference during virus amplification.
7. DH10BAC cells contain lacZ gene cassette that is disrupted during in vivo insertion of the recombinant DNA between the transposon sequences. If lacZ gene is not intact, the enzyme β-galactosidase is not expressed in the cells, and its added substrate X-gal cannot be cleaved. The colony selection is based on the fact that the characteristic blue dye product is not present in the colonies containing the inserted recombinant gene of interest.
8. You can also use other types of transfection reagent or procedures for the initial P1 virus production, e.g., Lipofectamine and Cellfectin, calcium phosphate precipitation, DNA microinjection, electroporation, etc.
9. Often baculovirus stocks are filtered through a 0.20 μm syringe filter to ensure sterile stock solution. Be aware that this results in significant loss of virus, since already the capsid of the budded baculovirus is 200–400 nm in length and 40–50 nm in diameter.

10. You can add a small percentage of FBS or DMSO to stored viral stocks to freeze them for a longer time at –90 °C. Depending on the virus, this addition may even not be necessary (we have seen that baculoviruses in EX-CELL 420 medium stay active for years when stored at –90 °C); just determine the titer of your stock after thawing once and use this titer for MOI calculation in future experiments.
11. Another amplification may be required for obtaining high-titer baculovirus stock of 10^8 till 10^9 pfu/mL.
12. Cells will stop dividing a couple of days after infection with MOI 0.01–1, and the viability usually drops down on days 3–5.
13. The number of infectious baculovirus particles drops down when conglomeration is initiated. The viral titer tends to be stable for up to 2 months and then rapidly drops down due to aggregation of virus.
14. We found that when a cassette with smaller nominal weight cutoff is used (100 kDa), the obtained baculovirus concentrate is less stable, probably due to concentrating also some enzyme proteases and increasing so the protease enzymatic activity.
15. When performing TFF in a cold room, viscosity of liquids is enhanced; hence lower pumping speeds should be used. If your virus seems to be unstable after concentration, consider adding protease inhibitors to the storage solution and in to the assay buffer.
16. The desired concentration factor depends on the virus application (for the assay, for storage, etc.) and on the holdup volume of the TFF system (i.e., what is the lowest volume of retentate that you can recover from your system). With higher starting volume of the viral suspension, you can perform TFF more efficiently.
17. The waste vessel should be graduated to better control the volume of collected permeate solution.
18. This step can be skipped if you don't want to increase the overall volume of baculovirus preparation.
19. You can use any other equipment that allows measuring of cell diameter.
20. The coefficients in correlation Eq. 2 should be adjusted for your laboratory after performing the comparative titer determinations with both cell size-based assay and plaque assay.
21. You can also perform the transduction directly on the assay plate, but exchanging the media is tedious (you should use 8–12 channel pipettes), and we have thus opted for transduction on petri dishes.

22. The volume at the time of transduction should be as low as possible because apart from MOI, also the higher concentration of the required infectious viral particles improves the probability of virus-cell interaction. The culture medium is suggested to be serum-free, because FBS increases the viscosity of the solution and reduces the mobility of virus particles, hence lowering the probability of virus-cell interaction. However if your cells tolerate the conditions with serum present better, you can just increase MOI and get sufficient expression nonetheless.
23. Here you can adjust the expression level of cAMP biosensor protein in a virus dose-dependent manner. Just begin with our suggested MOI, and if your cells are too bright or too dim, use lower or higher MOI, respectively.
24. 10 mM sodium butyrate (histone deacetylase inhibitor) is generally well tolerated by most cell lines, but you can also titer down and find an optimal concentration to enhance protein expression and avoid any cytotoxic effects. You can also use valproate instead of butyrate for the same effect.
25. If the majority of cells are detached from the dish after 2–3 h incubation with BacMam virus, removing of the virus is optional. The complete medium can simply be added to the cells. The 3- to 4-fold increase in volume dilutes the virus concentration, and the FBS increases the viscosity of the solution; hence the subsequent transduction is extensively reduced. Also, baculoviruses are insect viruses and cause very low cytopathic effect in mammalian cells compared to adenovirus or lentivirus, which in their wild-type form infect mammalian cells. Thus it is not problematic if the cells are incubated in the presence of baculovirus for 24 h.
26. In some mammalian cell lines, protein expression is more efficient at 30 °C. Consider testing the optimal cAMP sensor expression temperature for your cells.
27. The assay can be performed 24–48 h after transduction.
28. You can reuse the assay plates. Clean them with 10 % NaOH and thoroughly rinse with deionized water.
29. Buffer composition may influence binding to your GPCR of interest, e.g., potencies of melanocortin receptor ligands have been shown to depend on bivalent cation concentration [4, 10].

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