Montana Molecular
Fluorescent Biosensors for Live Cell Discovery

cADDis cAMP Assay for Gi

Overview	2
Relevant Products	2
Materials in the Kit	2
Storage	3
Additional materials not supplied.	3
BioSafety Considerations	3
Warranty	3
About these Assays	3
Sample Reaction Setup for Optimizing Gi-assay with Gαs	4
Protocol for Use	4
Suggestions for Assays in Adherent Cells	6
Fluorescence Detection	7
Assay Performance Considerations	8
Trouble Shooting	10
Contact Us	10
Related Products	11
References	11

Overview

The following protocol is optimized for measuring cAMP inhibition, via a Gi-coupled receptor, in rapidly dividing, immortalized cell lines on a 96-well plate. The protocol has been validated in live CHO, NIH 3T3 and HEK 293 cells [1]. This assay is very robust and can be used for live-cell imaging or for screening on automated fluorescence plate readers. For use in iPSC-derived or adherent cells, see Suggestions for Assays in Adherent Cells section.

The BacMam vector carrying these sensors is a modified baculovirus, which can be used for delivery to, and expression in, a wide variety of mammalian cell types including primary cultures.

Relevant Products

Product	Description	Promoter	Recommended Use
X0200G	Green cADDis Gi Assay	CMV	Fluorescence imaging and Plate reader assay (Z' > 0.7)

Materials in the Kit

- cADDis cAMP sensor BacMam ≅ 2 x10^{10} VG/mL in TNM-FH Insect Culture Medium (Allele Biotech product #ABP-MED-10001).

  Green fluorescent sensor that increases in fluorescence intensity in response to decreases in cAMP. VG/mL is the number of viral genes per milliliter, as distinct from plaque forming units (PFU), that for baculovirus, would be measured in insect cells.

- Sodium Butyrate (Sigma Aldrich product # B5887) 500 mM in H_2O.

  Sodium Butyrate is added to the culture to maintain BacMam expression. Other HDAC inhibitors may work as well.

- hD2 Receptor BacMam in TNM-FH Insect Culture Medium (Allele Biotech product #ABP-MED-10001).

  The Gi-coupled hD2 Receptor provided as a positive control for the purpose of assay optimization. Your own Gi-coupled receptor of interest may either be present in your cell line, or delivered via transduction/viral vector, or via plasmid/transfection.

- Quinpirole hydrochloride, 2 mM in Sterile Water

  Activates Gi signaling through the hD2 positive control receptor.

- $G_{\alpha s}$

  Constituitively active $G_{\alpha s}$, increases steady-state levels of cAMP and eliminates the need for forskolin.

Storage

BacMam stocks should be stored at 4°C and protected from light. Avoid repeated freeze/thaw cycles.

Additional materials not supplied.

- Greiner CellCoat (#655946) is our preferred 96-well plate available from VWR.
- Dulbecco's Phosphate Buffered Saline (DPBS) available from VWR [2].

BioSafety Considerations

BacMam is a modified baculovirus, Autographa californica, AcMNPV. The virus in this kit is pseudotyped to infect mammalian cells. In mammalian cells, the baculovirus genome is silent, and it cannot replicate to produce new virus in mammalian cells. While it should be handled carefully, in a sterile environment, it is classified as a Biosafety Level 1 (BSL-1) reagent.

This product is for research use only and is not recommended for use or sale in human or animal diagnostic or therapeutic products.

Warranty

Materials are provided without warranty, express or implied. End user is responsible for making sure product use complies with applicable regulations. No right to resell products or any components of these products is conveyed.

About these Assays

Cyclic AMP is an essential second messenger used in many important cellular processes. The messages carried by cAMP are tightly regulated in space and time. The cADDis assay contains a genetically-encoded biosensor with a cAMP binding domain fused to a fluorescent protein such that cAMP activity is converted into robust changes in fluorescence. cAMP changes are measured in the living cell, in contrast to accumulation assays measured in cell lysates. cADDis can be combined with different colored sensors to measure multiple signals simultaneously. For example, green cADDis can be combined with the red GECO Ca^{2+} assay (#U0600R)

The cADDis assay decreases fluorescence intensity when cAMP is increasing in the cell and increases in fluorescence in response to activation of $G_{\alpha i}$. To detect the activation of $G_{\alpha i}$, the steady state levels of cAMP should first be increased with the constitutively active $G_{\alpha s}$ provided in your kit.

The optimal ratio of cADDis BacMam to the constitutively active $G_{\alpha s}$ BacMam must be determined for a given cell line. In HEK 293 cells, much less $G_{\alpha s}$ is needed, relative to cADDis. Too little or too much of the $G_{\alpha s}$ mutant will limit the ability to detect the decrease in cAMP. To optimize the ratio of cADDis to the $G_{\alpha s}$ mutant for your particular cell line and/or receptor type, a good starting point is to keep the amount of sensor constant, while gradually increasing the amount of $G_{\alpha s}$ mutant.

To use the provided positive control, begin by keeping the volume of the receptor control constant. A typical preliminary experiment is to select two amounts of cADDis, each combined with the same amount of hD2 receptor control, and vary the amounts of Gs mutant (see Sample Reaction Set Up on the next page).

Sample Reaction Setup for Optimizing Gi-assay with $G_{\alpha s}$

For a single 50 µl rxn (i.e. 1 well in a 96-well plate):

20 µl sensor
5 µl hD2 receptor control
2 µl, 4 µl, or 8 µl $G_{\alpha s}$ mutant
0.6 µl 500 mM Sodium Butyrate (2 mM final concentration in well)
X µl surrounding solution to bring final volume to 50 µl (DPBS, EMEM, or Media of choice)

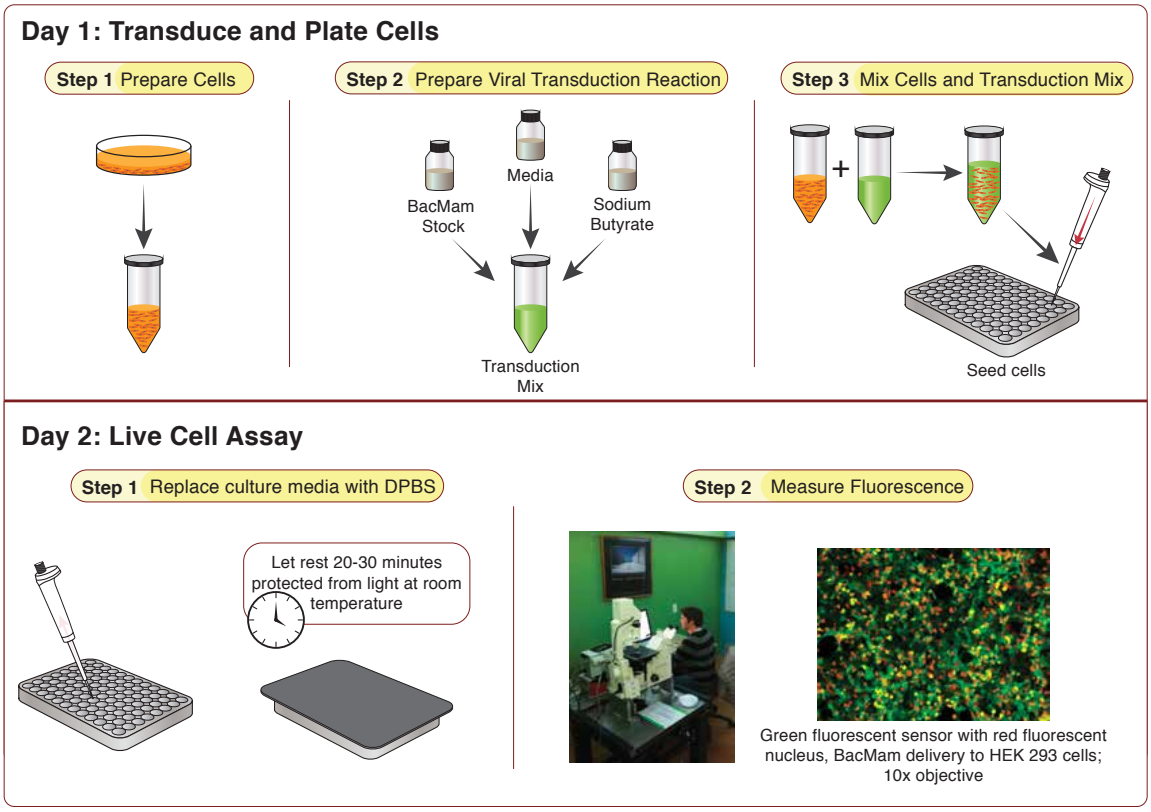
25 µl sensor
5 µl hD2 receptor control
2 µl, 4 µl, or 8 µl $G_{\alpha s}$ mutant
0.6 µl 500 mM Sodium Butyrate (2 mM final concentration)
X µl surrounding solution to bring final volume to 50 µl (DPBS, EMEM, or Media of choice)

In our experience, when using the assay in HEK93T cells, the optimal amounts of the sensor, $G_{\alpha s}$ mutant, and hD2 receptor are 25µl, 4µl, and 5µl, respectively.

An alternative to using the constitutively active $G_{\alpha s}$ provided in your kit, is to first activate a Gs-coupled receptor to increase basal cAMP, and then activate the $G_{\alpha i}$-coupled receptor and measure the increase in fluorescence.

Using forskolin to stimulate adenyl cyclase is an option, however it is not recommended, as forskolin binds adenyl cyclase and can disrupt the Gi pathway.

Protocol for Use



DAY 1 TRANSDUCE AND PLATE CELLS

Step 1) Prepare cells (Tube A)

- Detach cells from flask using normal trypsinization protocol. Resuspend cells in culture media (EMEM) and determine cell count. Prepare a dilution of cells at 500,000 cells/ml. 100 µl of this cell resuspension will be required for a single well in a 96-well plate, so prepare enough of the dilution to seed the desired number of wells in the plate. Let cells sit in hood and move on to preparation of the viral transduction reaction.

Step 2) Prepare Viral Transduction Reaction (Tube B)

- Prepare a 500 mM stock solution of sodium butyrate in sterile water (provided in kit).
- For each transduction reaction (i.e. one well in a 96-well plate), prepare the transduction solution by mixing 25 µl of the BacMam sensor stock with 5 µl of the hD2 Receptor Control, optimized amount of $G_{\alpha s}$ mutant, (in HEK 293 cells, with 5 µl hD2 receptor control, 4 µl $G_{\alpha s}$ mutant works well) 0.6 µl of the 500 mM stock solution of sodium butyrate, and enough Complete Media (EMEM, 10% FBS) for a total volume of 50 µl. Mix gently.

Step 3) Mix Cells and Transduction Mix from above.

- Mix Tube A and Tube B (100 µl tube A + 50 µl tube B). Mix gently and then seed 150 µl of the mix per well on the 96-well plate.
- Place plate in dark (e.g. cover with aluminum foil) and incubate at room temperature for 30 minutes. After this incubation period, place plate back in cell incubator. Incubate the cells under normal growth conditions, protected from light, for ≈ 24 hrs (5% CO^2 and 37°C).

Step 4 (Optional)*

After 4-8 hrs incubation time with virus (6 hrs is optimal), remove transduction solution and add 100 µl complete growth medium containing 2mM sodium butyrate**.

Return cells to normal growth conditions for approximately 18 hrs.

* This protocol does not *require* a media exchange step. Media exchange can be done to reduce well-well variability, which may be necessary for some automated plate readers.

** 1 mM sodium butyrate may improve cell health, but results in dimmer fluorescence.

DAY 2 FLUORESCENCE MEASUREMENT

- Cells are now ready for assay. Prior to imaging, replace culture media with DPBS. Wash gently so as not to dislodge cells. Cover the cells and allow them to rest at room temperature in DPBS for 30-35 minutes before measuring fluorescence. Experiments are performed at 25°C using standard GFP excitation and emission wavelengths.
- Add 1 µM quinpirole (final concentration in well) to activate D2 receptor control wells and measure an increase in fluorescence intensity when cAMP levels decrease. The optimal dose of quinpirole may need to be determined for a given cell line. Add quinpirole in a volume of 50 µl of DPBS, to wells containing 100-150 µl DPBS.

Suggestions for Assays in Adherent Cells

The protocol above is optimized for rapidly dividing immortalized cells. However, these assays are compatible with screening primary cultures and iPSC-derived lines, where the cells are plated before transduction. Specific details of the protocol will vary by cell type, so it is important to take the time to titrate BacMam for optimal results. For expression in rare cell types, or specific cells in mixed cultures, Cre-dependent and specific promoter systems are available for many of our sensors.

- Prepare a 500 mM stock solution of sodium butyrate in sterile water.
- For each transduction reaction (i.e. one well in a 96-well plate, containing 100µL culture media per well), prepare a transduction solution by mixing 25 µL of the Sensor BacMam stock with 5 µL of Receptor control, 4 µL of $G_{\alpha s}$ mutant, 0.6 µL of the 500 mM stock solution of sodium butyrate, and 15.4 µL of DPBS, for a total volume of 50 µL. Mix the solution gently.
- Sensor expression and cell health can be controlled by titrating the virus, so it is worth taking the time to optimize the assay for your particular cell type. Cell Culture media may be used in place of DPBS.
- Prepare a dilution series of transduction reactions by varying the amount of BacMam. For example, a range of 10 µL to 80 µL , adjusting the amount of DPBS accordingly.
- Add the transduction reaction directly to the plated cells (no aspiration of cell medium necessary). Gently rock the plate 4-5 times in each direction to mix throughout the well. Incubate the cells under normal growth conditions (5% CO_2 and 37°C), protected from light, for 4-8 hours (6 hrs is optimal).
- Aspirate transduction solution and add 100 µL complete growth medium with sodium butyrate at a concentration of 1-2 mM. Return cells to normal growth conditions for approximately 18-22 hrs before measuring fluorescence as described above. If cells will not tolerate a full media exchange, partial media exchanges can be done

Fluorescence Detection

Our assays are compatible with automated fluorescent plate readers. Our customers have reported good results on:

- Hamamatsu FDSS
- Molecular Devices FLIPR
- Molecular Devices Flexstation
- Perkin Elmer Enspire

We have validated on:

- Biotek Synergy MX
- Biotek Cytation
- BMG CLARIOstar
- Epifluorescence microscopes

Fluorescence Properties

cADDis is constructed with the very bright, mNeon green fluorescent protein [6]. We recommend Chroma's Catalog set #49003 for optimal results. The fluorescence properties are compatible with many of the FITC filter sets commonly available on most microscopes and plate readers.

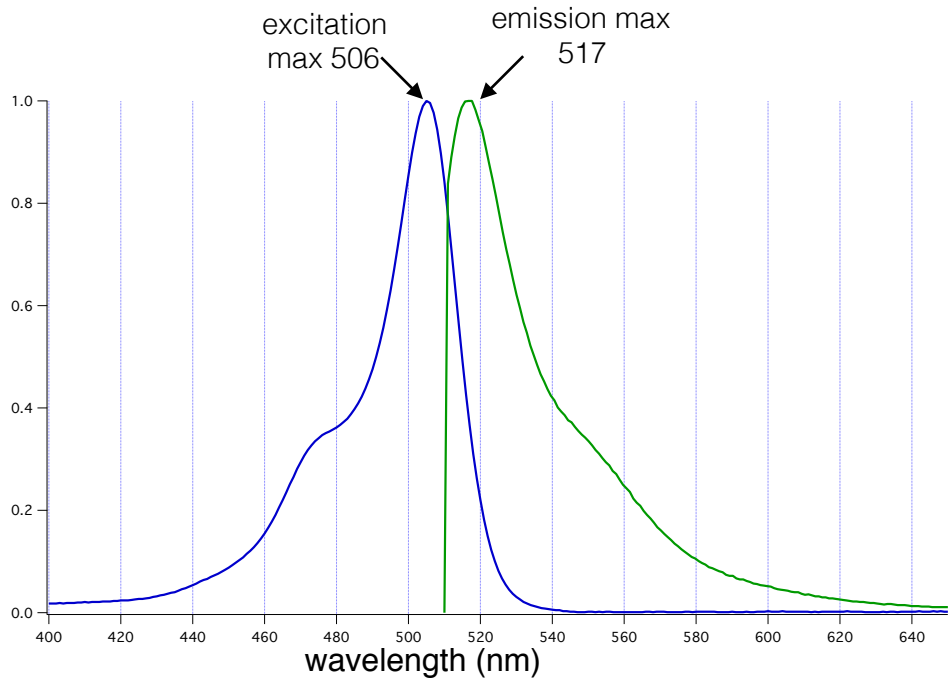


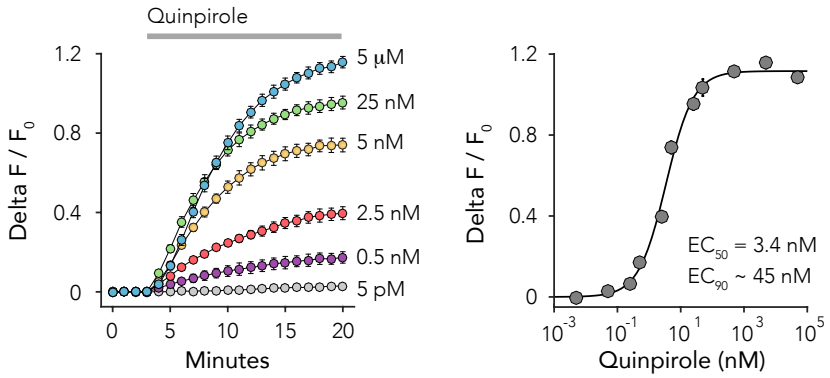
Figure 1. Absorption and emission properties of the mNeon green fluorescent protein plotted as a function of wavelength.

Assay Performance Considerations

Timing

Unlike many assays that measure accumulation of cAMP in cell lysates, the cADDis assay measures cAMP in living cells, in real time. For best results, be sure to capture changes in cAMP during the peak response. In Figure 2, fluorescence was captured from cells before the addition of the drug and then sampled at regular intervals. The maximal response is reached at ~ 10 minutes after the addition of the drug.

Figure 2. Downward cADDis can be co-expressed with a constitutively active Gs Alpha subunit to detect Gi-coupled receptor activation. The fluorescence of the sensor increases in response to decreasing cAMP levels. The D2 receptor was activated with quinpirole.



How we measure the infectivity of the viral stock.

Typically, viruses are quantified in terms of plaque forming units (PFU). In the case of baculoviruses, this would be a measurement of the viruses that are capable of transducing an insect cell, the natural host. Since mammalian cell expression is the goal for this assay, we quantify infectivity by measuring viral genes (VG) per milliliter (mL) of the BacMam stock. We use primers specific to the VSVG gene present in the BacMam genome. Viral samples are prepared to release viral genomic DNA, then multiple dilutions of the preparation are run in qPCR against a standard curve to generate an average titer for each viral stock. Check the label on the tube to find VG/mL for your cAMP sensor stock.

Expression levels of the sensor.

To optimize the assay in your particular cell type, it is important to optimize the amount of virus used in the transduction. Too little virus will produce variable results, particularly if the sensor expression levels are low and difficult to detect on your instrument. In the case of HEK 293 cells, the baseline fluorescence goes up as you add more virus, and when a particular threshold is reached, the absolute change in sensor fluorescence, as well as the Z' for the assay, becomes constant. Figure 3 illustrates this relationship, where the change in fluorescence is in response to activation of Gs. [Tewson PH et.al, 2015].

Receptor expression

The magnitude of the sensor response can be affected by the level of GPCR expression in your cells. We have found that low levels of receptor expression produce the largest signals, while high levels of receptor expression often produce smaller responses. This is consistent with the observation that over expression of some GPCRs can change the levels of second messengers due to low levels of spontaneous activity.

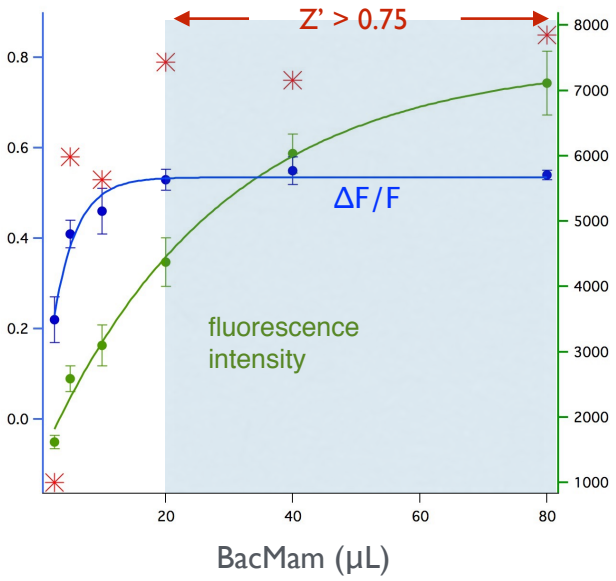


Figure 3. As the amount of cADDis sensor BacMam added to the wells increases, so does the baseline fluorescence, plotted in green. The change in fluorescence in response to Gs signaling also increases with more virus, but reaches the maximum possible change and remains constant over a broad range of virus concentration (plotted in blue). Once the maximum change in sensor response is obtained, Z' is 0.75 to 0.8 over a broad range of virus concentration (red stars).

Our green fluorescent sensors under CMV promoter control have been validated on many automated fluorescence plate readers. In this section, we summarize some of the considerations in the use of various types of fluorescence detection instruments and the trade offs between these systems.

The simplest format: one drug, one sensor, one time point.

We have validated our sensors on a BioTek Synergy MX fluorescence plate reader using the sample protocol described on the previous pages. Baseline fluorescence is measured first, control receptor agonists are added by hand, and then the plate is inserted back into the fluorescence plate reader to record the fluorescence from each well sequentially. This can all be done by hand because these sensors respond over several minutes to agonists in the well (see the plotted response in Figure 2). While this protocol is simple, the drawback is that it does not capture the kinetics of the response, simply the sensor fluorescence before and after the addition of drug.

Two channel format: one drug, two sensors, one time point.

By combining green and red sensors, one can simultaneously record two limbs of second messenger signaling. In this case, standard fluorescence plate readers can be used, but they need to be fitted with the optics necessary to collect two different channels of fluorescence, which will involve specific filter sets and either two detectors or very fast filter switching. Most two channel plate readers can scan quickly, but the kinetics of the responses are lost. This can be quite limiting if the kinetics of the two second messenger systems are different. For example, the Gq stimulated release of Ca^{2+} stores can be quite rapid, while the DAG signaling occurs over a longer time frame. If the timing is off with a single point measurement, the DAG signaling might be detected, but without catching the Ca^{2+} transient.

Capturing the kinetics, one well at a time.

High content imaging systems offer excellent opportunities for capturing kinetic responses. In the models that have onboard liquid handling, fluorescence can be recorded before and after the addition of vehicle, and then continuously recorded after addition of the drug. This provides kinetic data, but each well in the plate is recorded over relatively long time frames. Confocal systems are not recommended, as the signal outside of the focal plane is eliminated in these systems..

Capturing the kinetics in parallel, multi-well recording.

There are instruments that can collect fluorescence data from all of the wells of a plate simultaneously and have automated liquid handling. These are perfectly suited for recording the kinetics of the response from every well. Because the recordings are all done in parallel, the speed of the assay is considerably faster than a single channel instrument. An example of such an instrument is the Molecular Devices “Flex Station” and “FLIPR” series of plate readers, and our assays have been validated on these instruments.

Trouble Shooting

Here are a few simple steps that may help you trouble shoot if needed.

Are Your cells fluorescent?

Different types of promoters drive expression in mammalian cells. The CMV promoter in our BacMam vectors is an effective promoter in many cell lines, but not all. Twenty four hours after transduction, you should see bright green fluorescent cells in a typical epifluorescence microscope, or the transduced wells in a 96 well plate should be significantly more fluorescent than untransduced cells in wells on the same plate.

HDAC inhibitors are important to expression of the sensors. While BacMam transduction alone will initially generate low levels of sensor expression, sodium butyrate or another HDAC inhibitor such as VPA or trichostatin A (TSA) will generate optimal levels of sensor expression and maintain this level of expression [Kost, T. et. al. 2007]. If cells look unhealthy, lower concentrations of HDAC inhibitor may be used. This may improve cell health, but it will also reduce sensor expression.

Finally, the type of cell culture media used in your experiment can affect the transduction efficiency of BacMam. Our assays have been validated in EMEM, DMEM, and F12K culture media.

Is the positive control working?

If the cells are expressing the sensor, and fluorescence is detectable on your instrument, then the next step is to test the assay in your cells. A positive control receptor is included in this kit. Add 5 µl of the hD2 receptor control to a set of positive control wells as described in the protocols.

Addition of quinpirole will cause a change in fluorescence, as shown in Figure 2. If it does not, then it is important to use this positive control to optimize three aspects of your assay. First, a serial dilution series of the sensor with a constant amount of receptor virus can be used to find the optimal sensor expression for your instrument (See Figure 3). Second, it is important that you find the amount of virus sufficient to transduce all of the cells in the well. Third, it is important to determine what the kinetics of the response is and whether your instrument can measure in the appropriate time frame.

Contact Us

If you have any questions about the protocols described here, or if you have ideas about how we can improve these tools, then we want to hear from you. Your feedback is extremely valuable. Please send an email to info@montanamolecular.com, and we’ll respond as quickly as we can.

Related Products

Product	Sensor Description	Promoter	Recommended Use
U0300R	Red Upward DAG	CMV	Fluorescence imaging and Plate reader assay (Z' > 0.5)
D0300R	Red Downward DAG	CMV	Fluorescence imaging and Plate reader assay (Z' > 0.7)
U0600R	Red GECO Ca^{2+}	CMV	Fluorescence imaging and Plate reader assay (Z' > 0.5)

References

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4. Kost T, Condreay J, Ames R, Rees S, Romanos M: Implementation of BacMam virus gene delivery technology in a drug discovery setting. Drug Discovery Today 2007, 12(9-10): 396-403.

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PCT/US2014/063916 Patent Pending