Montana Molecular
Fluorescent Biosensors for Live Cell Discovery

Red Fluorescent cADDis cAMP Assay

About these Assays

Cyclic AMP is an essential second messenger for many cellular processes. The messages carried by cAMP are tightly regulated within cells. The red cADDis assay offers an alternative to measurements of cAMP accumulation in cell lysates, because it provides a kinetic measurement of cAMP and can be targeted to specific populations of cells in mixed cultures. The sensor uses a single red fluorescent protein, so it can be combined with green sensors to measure multiple signals simultaneously.

Overview

The red fluorescent cADDis is used for measuring cAMP changes in live mammalian cells. The following protocol has been validated in HEK293, CHO and NIH 3T3 cells and is appropriate for live-cell imaging and for screening on automated fluorescence plate readers.

Relevant Product

Product	Description	Promoter	Recommended Use
U0200R	Red Upward cADDis cAMP	CMV	Fluorescence imaging and plate reader assay (Z' < 0.5)

Materials Included

- Red fluorescent cAMP sensor in BacMam under the control of a CMV promoter.

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

- Sodium Butyrate (Sigma Aldrich product number B5887) 500 mM.

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

- β2 Adrenergic Receptor BacMam in TNM-FH Insect Culture Medium (Allele Biotech product #ABP-MED-10001).

  A Gs-coupled receptor provided as a positive control for the purpose of assay optimization.

- Isoproterenol 10 mM

  Isoproterenol can be used to stimulate Gs signaling through the positive control, the β2 adrenergic receptor.

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 plate for this assay.
- Dulbecco's Phosphate Buffered Saline (Dulbecco, R; Vogt, M 1954).

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

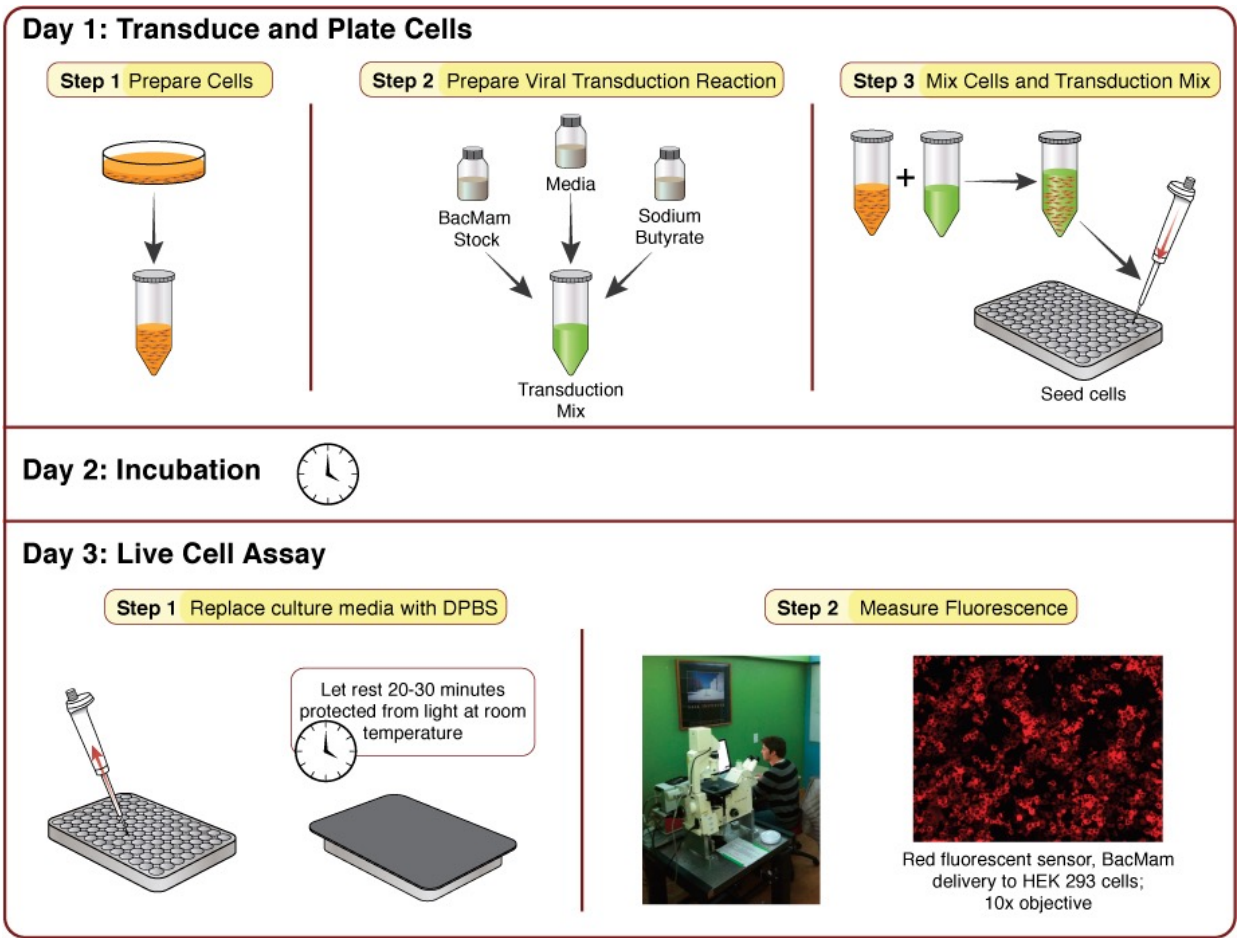
Materials are provided without warranty, express or implied. End user is responsible for making sure product use complies with applicable regulations. No right to resell or reverse-engineer products or any components of these products is conveyed.

Related Products

Product	Description	Promoter	Recommended Use
D0200G	Green Downward cADDis cAMP	CMV	Fluorescence imaging and Plate reader assay (Z' > 0.9)
U0200G	Green Upward cADDis cAMP	CMV	Fluorescence imaging and Plate reader assay (Z' > 0.85)
D0700G	Green ArcLight Voltage	CMV	Fluorescence imaging
U0300G	Green Upward DAG	CMV	Fluorescence imaging and Plate reader assay (Z' > 0.8)

Protocol for Use

This protocol is optimized for rapidly dividing, immortalized cell lines. However, the protocol can be adjusted for transducing non-dividing adherent cells such as neurons, islets, cardiomyocytes, and iPSC-derived lines. We recommend that you take the time to optimize the assay for your particular cell type. See our Suggestions for Adherent Cells following this protocol.



DAY 1 TRANSDUCE AND PLATE CELLS

Step 1) Prepare cells (Tube A)

- Detach cells from flask using standard trypsinization protocol. Resuspend cells in complete culture media and determine cell count.
- Prepare a dilution of cells at your desired concentration*. 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.

  * 420,000-440,000 cells/mL works well for HEK293 cells.

Example:

For **96** wells (1 plate)

100 µL cell suspension (420,000 cells/mL) per well.

100 µL cells x **110 (96** wells + 10% scale) = **11000 µL** cell suspension.

- When preparing the master mix, scale up by 10-15% to avoid coming up short. To seed a 96-well plate, multiply amounts in Step 1 and Step 2 by 110-120.

Step 2) Prepare Viral Transduction Reaction (Tube B)

- Prepare a 500 mM stock solution of sodium butyrate in sterile water (in your kit).
- For each transduction reaction (i.e. one well in a 96-well plate), prepare the transduction solution by mixing 25 µL of the Sensor BacMam stock with 0.6 µL of the 500 mM stock solution of sodium butyrate*, 5 µL of Receptor control, and 19.4 µL of the complete culture media for your cells, for a total volume of 50 µL. Mix gently.

* Concentration of sodium butyrate should be 6mM in this step. Following Step 3, final concentration of sodium butyrate will be 2mM.

Example:

96 wells needed (1 plate). The number of wells desired, in bold, must correspond to the number in Step 1 above.

Single Well	*Master Mix*
25 µL Sensor	x **110** = 2750 µL
5 µL Receptor Control	x **110** = 550 µL
0.6 µL 500 mM Sodium Butyrate	x **110** = 66 µL
19.4µL Complete Media	x **110** = 2134 µL
50 µL total volume	x 110 = **5500 µL** transduction mix (96 wells)

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 seed 150 µL of mix per well on the 96-well plate.
- Cover plate to protect from light and incubate at room temperature for 30 minutes.
- Incubate ≈ 48 hrs under normal cell growth conditions, protected from light. 24 hr incubation periods may be used, but sensor fluorescence will be much lower.

Example:

96 wells needed (1 plate)

Single Well	*Master Mix*
100 µL cell suspension	x 110 = 11000 µL
50 µL transduction reaction	x 110 = 5500 µL
150 µL Total Volume per well	x110 = 16,500 µL total reaction volume

DAY 2 INCUBATION

DAY 3 MEASURING FLUORESCENCE

- Cells are now ready for assay. Prior to measuring fluorescence, 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 20-30 minutes before measuring fluorescence. Experiments are performed at 25°C . The optimal excitation wavelength is 590 nm. A broad band pass emission filter spanning 600-700 nm is ideal.
- Add 30µM isoproterenol to activate a set of wells transduced with Receptor Control.
- When monitoring the red fluorescence emitted by the sensor, an increase in fluorescence intensity will be observed after addition of compounds that increase levels of cAMP in the cell.

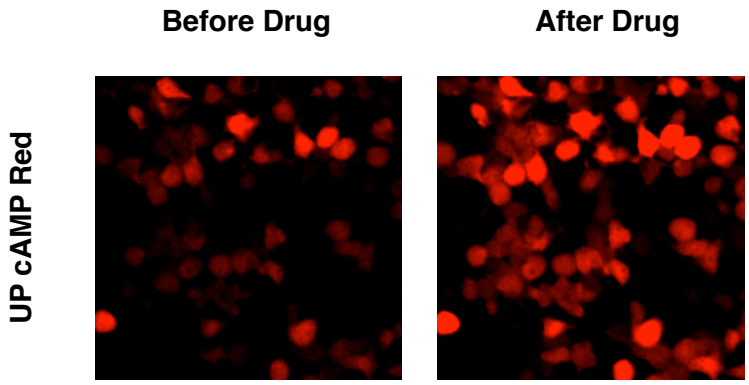
Suggestions for Assays in Adherent Cells

The protocol above is optimized for rapidly dividing immortalized cells. However, these assays are compatible with screening primary cultures and iPSC-derived lines, where cells are plated before transduction. Details will vary by cell type, so it is important to take the time to titrate BacMam for optimal results.

- 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, 19.4 µL of DPBS, and 0.6 µL of the 500 mM stock solution of sodium butyrate 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.
- 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 38-42 hrs before measuring fluorescence as described above. If cells will not tolerate a full media exchange, partial media exchanges can be done.

Detection

This sensor is constructed using a red fluorescent protein. Before attempting to deploy this sensor in automated readers, we recommend that you optimize the assay on a microscope. Red cADDis performs quite well in a variety of epifluorescence microscopes with lenses ranging from 20 X, 0.9 N.A. to 63X, 1.4 N.A.



Fluorescence Properties

The optimal excitation wavelength is 590 nm, but the absorption band of this protein is quite broad, so broad bandpass filters that pass 550 to 590 nm light can be used quite effectively. On the emission side, the red light spans 600 to 700 nm, so broad band pass emission filters can also help to collect much of the emission.

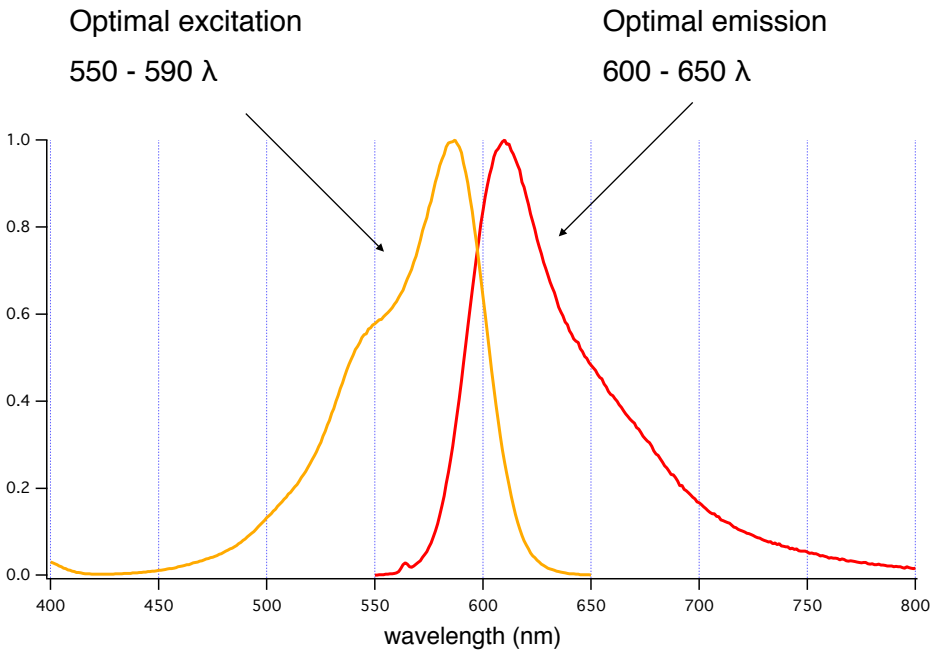


Figure 1. Red fluorescent protein spectra

Timing

This is a live cell assay, and unlike many assays that measure accumulation of cAMP over time, this sensor detects, in real time, the cAMP level in the living cell. Changes in cAMP can occur quite rapidly, so the application of drug and resulting changes should be captured as quickly as possible. The best possible experimental setup involves capturing the fluorescence signal from a well of cells first, before the addition of any compound, and then again a second or so after the compound addition. Sampling the fluorescence at intervals of 3 to 5 seconds provides a good measurement of the response.

Assay Performance Considerations

Virus Titer

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

Level of sensor expression

To optimize the assay for your particular cell type, it is important to titrate 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.

Level of receptor expression

The magnitude of the sensor response can be affected by the level of receptor expression. 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 receptors can change the level of second messengers.

Ratiometric Measurement

The red fluorescence emitted by the red cADDis sensor is not as bright as the green cADDis sensors. Because the red fluorescent sensor is not as bright, it may not be suitable for use alone on some imaging systems. However, red cADDis can be combined with green cADDis for ratiometric imaging applications as shown in Figure 2.

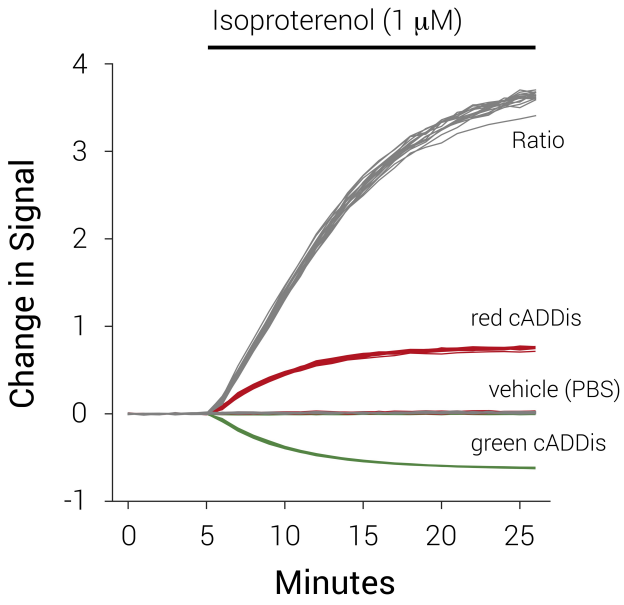
Troubleshooting

Are the cells fluorescent?

Different types of promoters drive expression in mammalian cells The CMV promoter in many of our BacMam vectors is an effective promoter in many cell lines, but not all. 24 to 48 hours after transduction with cADDis, you should see red 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 maintain expression of the sensors. While BacMam transduction alone will initially generate low levels of sensor expression, sodium butyrate or another HDAC inhibitor such as valproic acid (VPA) or trichostatin A (TSA) will optimize and maintain expression [Kost, T. et. al. 2007]. If cells look unhealthy, lower concentrations of HDAC inhibitor may be used.

Figure 2. HEK 293 cells expressing both red and green sensors measured in 16 wells of a 96 well plate on the BMG CLARIOStar plate reader. Change in signal is averaged over each well.



This may improve cell health, but it will also reduce sensor expression.

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.

Finally, if there is a specific promoter that you know drives expression in your cell type, please let us know. We are continually adding new promoter systems and may be able to help.

Is the sensor responding?

If fluorescence is detectable, then check cADDis using the positive controls included in this kit. Adding 5 ul of the β2 adrenergic receptor virus to a set of control wells will ensure that a Gs-coupled receptor is present in all of the cells. Addition of isoproterenol should produce a change in fluorescence.

Contact Us

We appreciate hearing from scientists who use these tools. Please let us know any feedback about the protocol or the assay, as we work to make them easier to understand and use. Send us email at info@montanamolecular.com and let us know what you need. We strive to respond within 24 hours and usually respond much sooner. Let there be light!

References

1. Dulbecco R and Vogt M: Plaque formation and isolation of pure lines with poliomyelitis viruses. The Journal of experimental medicine 1954.

2. Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC: Green fluorescent protein as a marker for gene expression. Science 1994.

3. 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.

4. Tewson PH, Martinka S, Shaner N, Hughes TE, Quinn AM: New DAG and cAMP sensors optimized for live cell assays in automated laboratories. Journal of Biomolecular Screening 2015.

5. PCT/US2014/063916 Patent Pending: Genetically-encoded Fluorescent Sensors for Detecting Ligand Bias and Intracellular Signaling throught cAMP pathways. Tewson et.al.