January 17, 2017

Montana Molecular
Fluorescent Biosensors for Live Cell Discovery

mNeon Green 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. These assays offer an alternative to measurements of cAMP accumulation in cell lysates, because they detect cell signaling in living cells and can be targeted to specific populations of cells in mixed cultures. The fluorescent sensors used in the assays described here, use one fluorescent protein, so they can be combined with different colored sensors to measure multiple signals simultaneously.

Depending upon the sensor in your kit, the fluorescence can either increase or decrease in response to receptor activation. For example, D0200G cADDis decreases in fluorescence when cAMP levels increase in the cell. One can imagine applications where a downward sensor might have advantages over an upward sensor, for example, if background fluorescence is high. Both Upward and Downward cADDis are bright, robust, and easy to detect on fluorescence plate readers (Figure 3). No need to fix or lyse your cells. Life is interesting!

Overview

The following protocol is optimized for measuring cAMP responses in rapidly dividing, immortalized cell lines on a 96-well plate, and has been validated in live CHO, NIH 3T3, and HEK 293 cells [Graham FL, 1977]. 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
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)
U0202G	Green Upward cADDis cAMP	synapsin	Targeted expression in neurons for fluorescence imaging
D0202G	Green Downward cADDis cAMP	synapsin	Targeted sensor expression in neurons for fluorescence imaging

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 sensors that change in fluorescence intensity in response to increases 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.

- 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. Contains a separate red fluorescent protein that is targeted to the nucleus.

- Isoproterenol 10 mM, in 10mM HCl

  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 96-well plate available from VWR.
- Dulbecco's Phosphate Buffered Saline (DPBS) available from VWR [Dulbecco, R. and Vogt, M.1957].

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

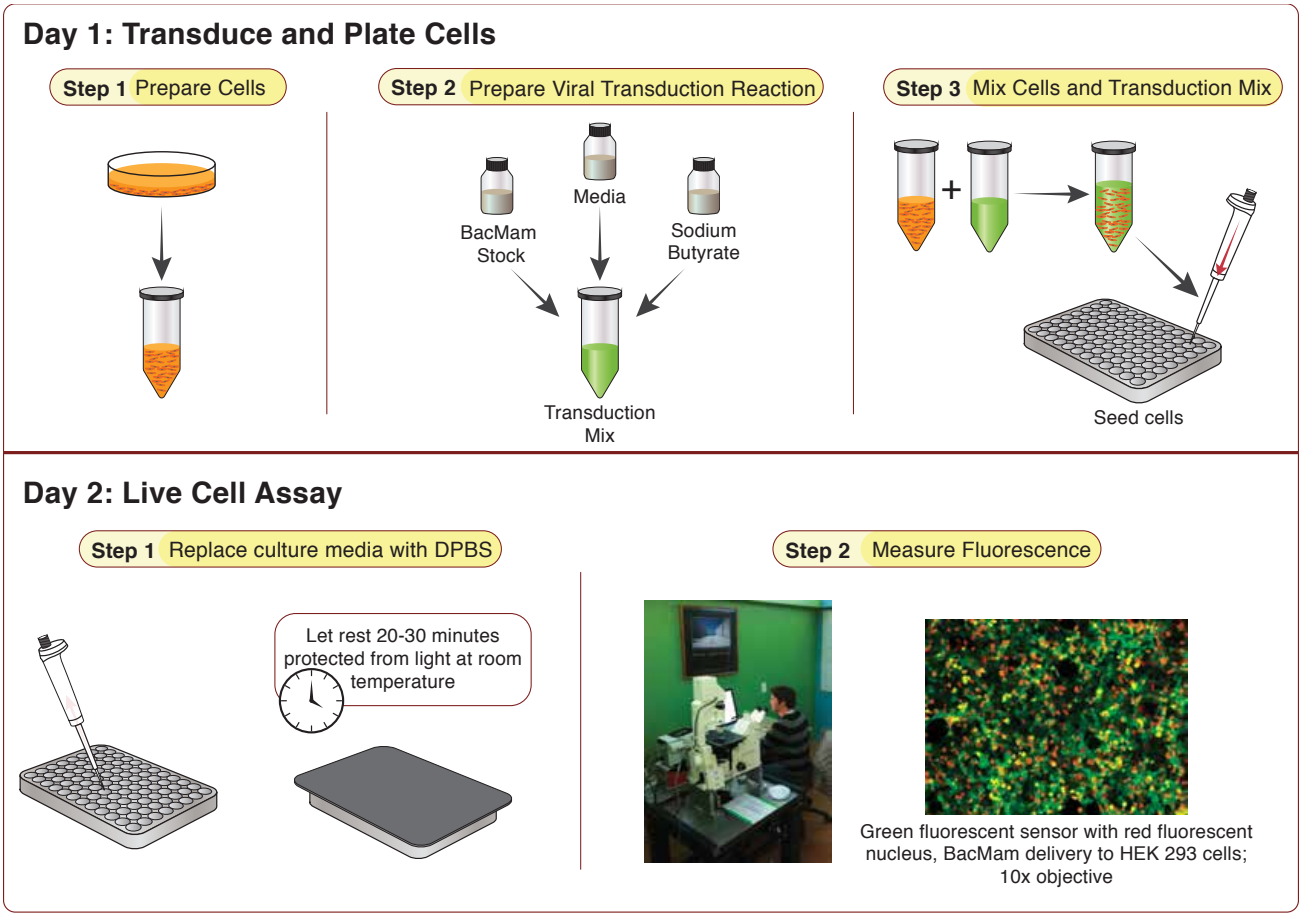
Materials are provided without warranty, express or implied. End user is responsible for making sure product use complies with applicable regulations. No right to resell products or any components of these products is conveyed.

Related Products

Product	Sensor Description	Promoter	Recommended Use
U0200R	Red Upward cADDis cAMP	CMV	Fluorescence imaging and Plate reader assay (Z' > 0.8)
U0300R	Red Upward DAG	CMV	Fluorescence imaging and Plate reader assay (Z' > 0.65)
D0300R	Red Downward DAG	CMV	Fluorescence imaging and Plate reader assay (Z' > 0.7)
D0700G	ArcLight Voltage	CMV	Fluorescence imaging only
U0600R	Red GECO Ca^{2+}	CMV	Fluorescence imaging and Plate reader assay (Z' > 0.5)

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.

* 480,000 cells/mL works well for HEK293 cells.

Example:

For **96** wells (1 plate)

100 µL cell suspension (480,000 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 20 µL of the Sensor BacMam stock with 0.6 µL of the 500 mM stock solution of sodium butyrate*, 5 µL of the Receptor control, and 24.4 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*
20 µL Sensor	x **110** = 2200 µL
5 µL Receptor Control	x **110** = 550 µL
0.6 µL 500 mM Sodium Butyrate	x **110** = 66 µL
24.4µL Complete Media	x **110** = 2684 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 with aluminum foil to protect from light and incubate at room temperature for 30 minutes.
- Incubate ≈ 24 hrs under normal cell growth conditions, protected from light.

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 MEASURING FLUORESCENCE

- 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 25-40 minutes before measuring fluorescence so they can adjust to their new environment. Experiments are performed at 25°C using standard GFP excitation and emission wavelengths.
- Use positive controls. Add 1-5µM isoproterenol (30% of total volume in well) to activate a set of wells transduced with the β2 Adrenergic receptor included in the kit.
- When monitoring the green fluorescence emitted by the sensor, a change 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 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 20 µL of the Sensor BacMam stock with 5 µL of Receptor control, 24.4 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. 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.

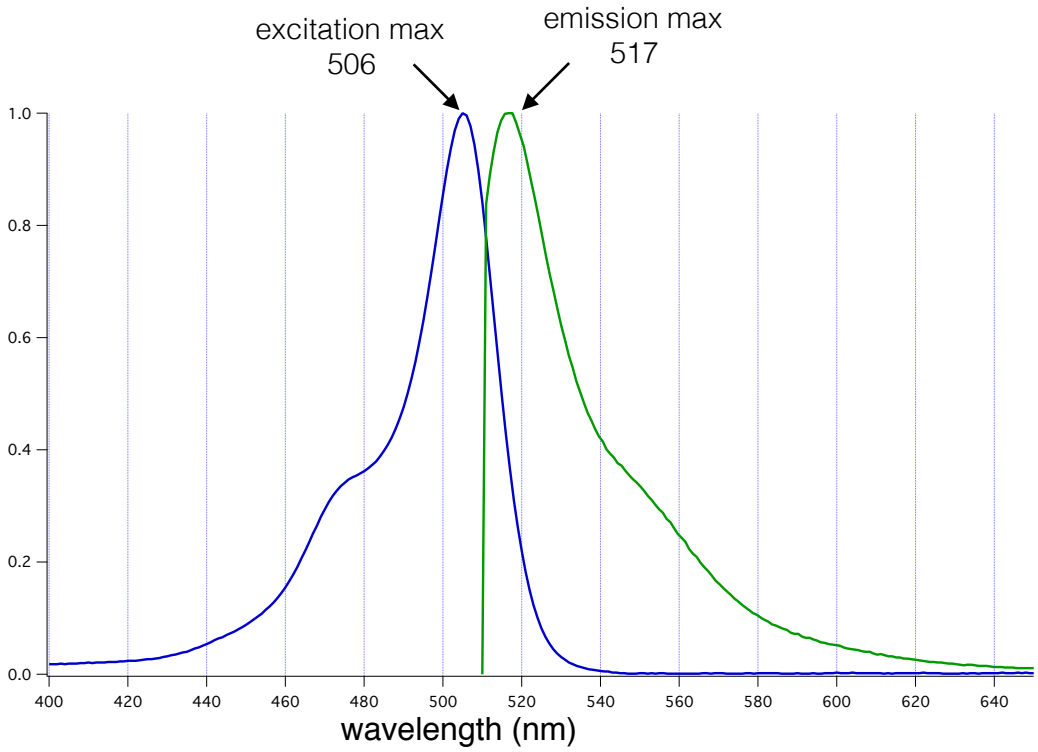


Figure 1. Absorption and emission properties of the mNeon green fluorescent protein plotted as a function of wavelength.

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 ~360 seconds after the addition of the drug.

Assay Performance Considerations

How we measure the infectivity of the viral stock.

Typically, viruses are quantified in terms of plaque forming units (PFU). In the case of BacMam, 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.

Level of sensor expression

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. As shown in the figure below and recently published [Tewson PH et.al, 2015].

Level of 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.

Our green fluorescent sensors under CMV promoter control have been validated on several automated fluorescence plate readers. However, sensors under synapsin or weaker promoters, may be limited to detection to imaging systems. 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.

By following the sample protocol described on the previous pages, you can expect to record a robust response on standard fluorescence plate readers. Fluorescence may be recorded sequentially from each well before adding drug, and again after adding the drug. The response to the receptor and agonist controls provided in your kit occurs over a few minutes. While this protocol is simple, the drawback is that it does not capture the kinetics of the response, only the 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 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.

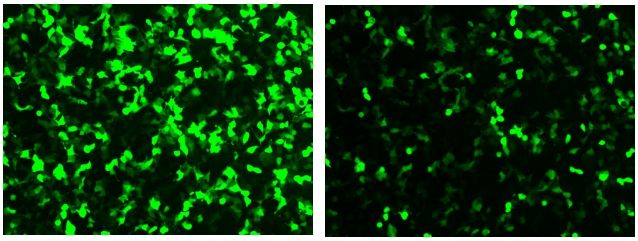
Capturing the kinetics, one well at a time.

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 recoding 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.

Before Agonist **After Agonist**



Trouble Shooting

The cADDis assays are robust and easy to use. There 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 check the sensor using the positive control included in this kit. Adding 5 µL 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. You can double check to make sure the receptor is expressed by examining the cells in a fluorescent microscope with filters for red fluorescence. You should see the green sensor fluorescence throughout the cell, and red nuclear fluorescence that marks the cells that also express the β2 adrenergic receptor.

Addition of isoproterenol 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 (see Figure 3). 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 and cell line. 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.

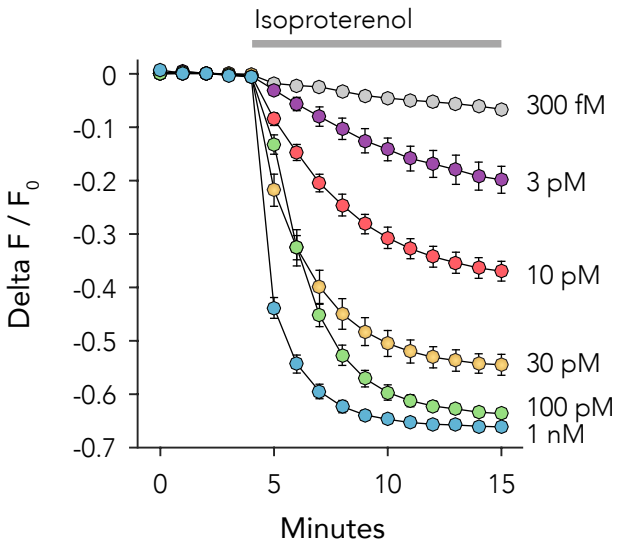


Figure 2. HEK 293 cells transduced with 20 µL of BacMam carrying green downward cAMP biosensor, activated with isoproterenol. The graph shows the expected timing and duration of cAMP when using the positive controls provided in your kit.

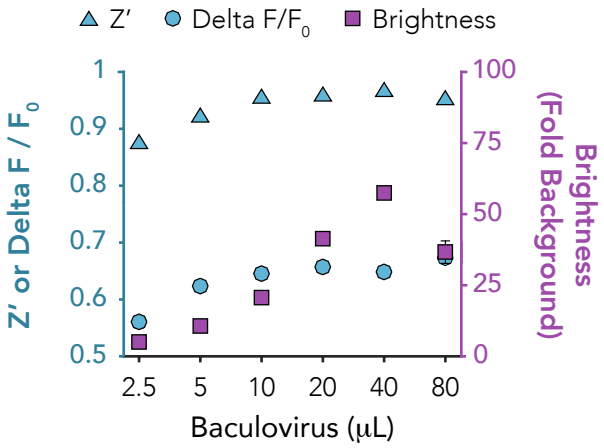


Figure 3. As the amount of cADDis added to the wells increases, so does the baseline fluorescence, plotted in purple. The change in fluorescence when cAMP changes also increases with more virus, but reaches the maximum possible change and remains constant over a broad range of virus concentration (circles). Z' is high over a broad range of virus concentration (triangles).

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.

References

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PCT/US2014/063916 Patent Pending