Montana Molecular
Fluorescent Biosensors for Live Cell Discovery

cADDis™ cAMP Assay for Gi

February 2, 2022

Product Info & Protocol

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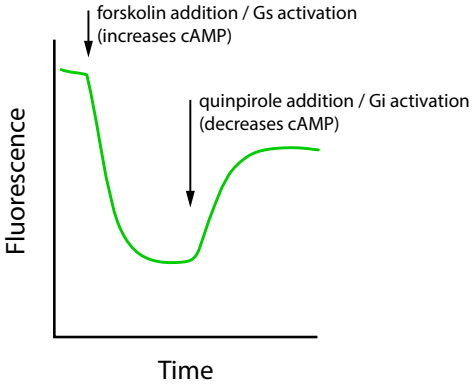
About these Assays

Cyclic AMP (cAMP) is an essential second messenger for many cellular processes. The messages carried by cAMP are tightly regulated within cells. The cADDIs assay for Gi detects changes in cAMP in living cells. This assay can be targeted to specific populations of cells in mixed cultures. The cADDis assay for Gi can be combined with different colored sensors to measure multiple signals simultaneously.

When cAMP is increasing in the cell, the cADDis assay for Gi decreases in fluorescence intensity. When a Gi-coupled receptor is activated, cAMP production is inhibited and fluorescence increases.



The cADDis assay for Gi **decreases** fluorescence intensity when cAMP is **increasing in the cell** and **increases in fluorescence in response to activation of Gi.** To detect the activation of Gi, cAMP levels in the cell should first be increased with the addition of forskolin or by activating a Gs-coupled receptor. Alternatively, a constitutively active Gs (product #X0250X) can be added during the transduction process to increase cAMP (see **alternative approach #3** on page 9).

The following protocol is optimized for measuring cAMP responses in rapidly dividing, immortalized cell lines on a 96-well plate, and has been optimized for HEK 293T cells [1]. This assay can be detected using live cell imaging or with automated fluorescence plate readers. For use in iPSC-derived or adherent cells, see **Suggestions for Assays in Adherent Cells** section. For use in CHO cells, see **Protocol Adjustments for CHO Cells** section.

This protocol applies to the products in Table 1:

Table 1. Relevant Products

Product	Description	Promoter	Recommended Use
#X0200G	Green Gi cADDis cAMP	CMV	Fluorescence imaging and plate reader assay (Z' > 0.7)
#X0205G	Green Gi cADDis cAMP	CAG	Fluorescence imaging and plate reader assay

Our goal is to make your workflow easy and reproducible.

We'd love to hear about your research.

Questions?
Call us, we can help!
+1 406-200-8321
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We also have a **Troubleshooting Guide** *at the end of this document*

Assay Kit Materials and Storage

BacMam stocks should be stored at 4°C protected from light in the original package. Store **HDAC inhibitor at 4°C. Store control agonist at -20°C. Avoid repeated freeze/thaw cycles.** We recommend re-testing BacMam stock after storing for more than 12 months. If your BacMam stock has been purified, use it within 30 days for best results.

Table 2 Materials in Kit	Details	Storage
Green Downward cADDis cAMP sensor BacMam ≅ 2 ×10^{10} VG/mL in ESF 921 Insect Culture Medium (Expression Systems, product #96-001-01)	Green fluorescent sensor that changes in fluorescence intensity in response to increases or 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.	4˚C
SB **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.	4˚C
Ri **hD2 receptor BacMam** in ESF 921 Insect Culture Medium (Expression Systems, product #96-001-01)	A Gi-coupled receptor provided as a positive control for the purpose of assay optimization.	4˚C
Ai **quinpirole hydrochloride** (Sigma Aldrich product #Q102) 2 mM in sterile water	Quinpirole hydrochloride can be used to stimulate Gi signaling through the positive control, the hD2 receptor.	-20˚C

Additional Materials Required (not included in kit)

1. Black, clear bottom microplate coated with a cell attachment factor. Greiner Cell Coat (#655946) is our preferred 96-well plate available from VWR.
2. Dulbecco's Phosphate Buffered Saline with Ca^{2+} and Mg^{2+} (PBS) available from VWR **[2]**.
3. Forskolin (Cayman Chemical, #11018).
4. Cells and cell media of your choice.
5. Optional: β2 adrenergic receptor in BacMam product #Z0500N is used for **Alternative Approach #2** (pg. 9).
6. Optional: constitutively active Gαs (product #X0250X) is used for **Alternative Approach #3** (pg. 9).

Biosafety Considerations

The BacMam vector carrying the fluorescent biosensor in this assay is a modified baculovirus, used for delivery to, and expression in, a wide variety of mammalian cell types including primary cultures.

BacMam is a modified baculovirus, *Autographa californica*, AcMNPV. The natural host of baculovirus is larvae of the order *Lepidoptera*. The BacMam vector in the kit is produced in the lab using Sf9 insect cells and 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.

Other types of viruses are quantified in terms of plaque forming units (PFU) in cells from the natural host. Since BacMam is modified to produce expression in mammalian cells, we quantify the virus by measuring viral genes (VG) per milliliter (mL). Viral samples are prepared to release viral genomic DNA, then multiple dilutions of the preparation are run in qPCR using primers that are specific to the VSVG gene in the BacMam genome. Results are compared 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 stock.

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

Terms of Sale

All materials in this kit are provided without warranty, express or implied. 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. Use of materials is restricted to the intended purpose described in this protocol. Reverse engineering or modification of materials is not permitted. User agrees to accept these Terms of Sale before using materials.

Protocol for Use

This protocol is optimized for use in HEK 293T cells, however, it can be adjusted for use with practically any cell type.

Take the time to optimize the assay for your cell type and your particular conditions.

See our suggestions for **Assays in Adherent Cell Types, Protocol Adjustments for CHO Cells,** and **Scaling for 384-well Plates**.

OPTIMIZE SENSOR EXPRESSION

First Experiment - Optimize by Titrating the Sensor

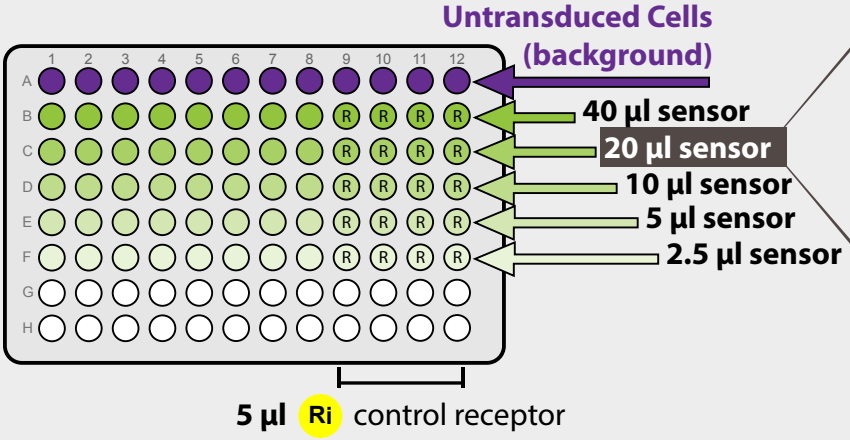
We recommend using a titration series to determine the best combination of signal above background, cell health, and response. Ideally, the signal in each well before drug addition should be at least 5 times above background.

Day 1

a. Set up your plate. Be sure to include control wells (untransduced cells) in order to calculate signal-to-background. **Refer to detailed protocol Day 1 procedure on page 5.**

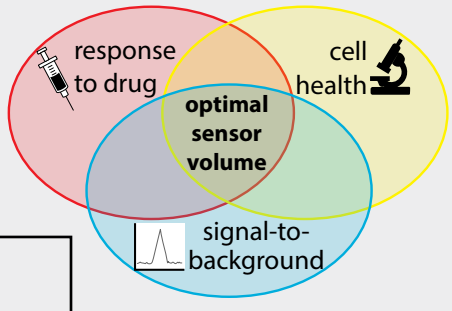
b. Perform titration to determine optimal sensor volume for your cells.

c. For each sensor volume, include a subset of wells that also has 5 µl of **Ri** control receptor (included in your kit). These wells will be used for your control experiment on day 2.



Transduction Reaction Per Well	
20 µL	cADDis cAMP for Gi sensor
5 µL	**Ri** (D2 control receptor)
0.6 µL	500 mM **SB** (sodium butyrate)
24.4 µL	complete media
50 µL	**total volume per well**

Check your cells for these 3 parameters to determine **optimal sensor volume.**

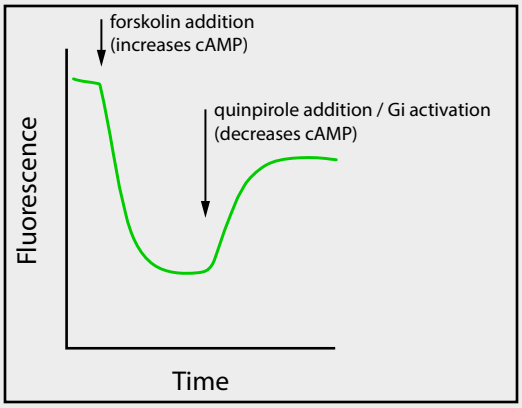


Day 2

d. Measure fluorescence to evaluate sensor expression levels.

e. After adding 50 µM forskolin and monitoring a decrease in fluorescence that should stabilize in 15-20 minutes, add 10 µM **Ai** (quinpirole, final concentration in well) to activate the set of control wells transduced with the D2 receptor.

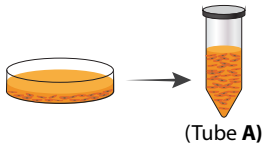
f. **Refer to detailed protocol Day 2 procedure on page 8.**



Day 1 – Detailed Procedure

Transduce and Plate Cells

Step 1)
Prepare Cells (Tube A)

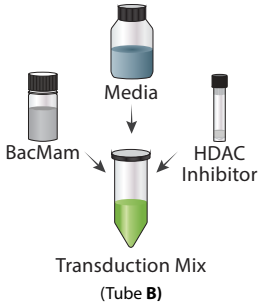


a. Detach cells using standard trypsinization protocol. Resuspend cells in complete culture media and determine cell count.

b. 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.

Step 2)
Prepare Viral Transduction Reaction (Tube B)

c. 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 **SB** (stock solution of sodium butyrate), 5 µL of **Ri** control receptor (if needed), and 24.4 µL of the complete culture media for your cells, for a total volume of 50 µL. Mix gently. Following Step 3, the final concentration of sodium butyrate will be 2 mM.

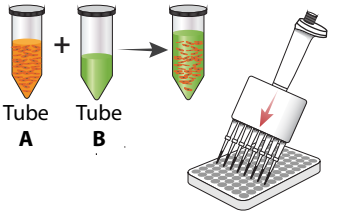


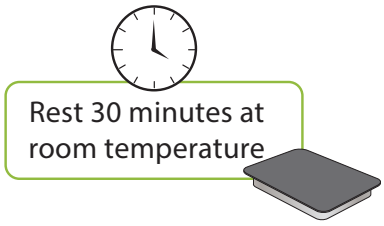
Step 3)
Mix Cells and Transduction Mix

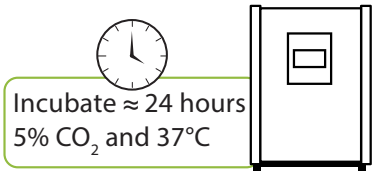
d. Mix Tube A and Tube B (100 µL tube A + 50 µL tube B). Mix gently and seed 150 µL of mix per well on the 96-well plate.

e. Cover plate to protect from light and let rest at room temperature for 30 minutes.

f. Incubate ≈ 20-24 hours under normal cell growth conditions (5% CO_2 and 37°C), protected from light.







Cells (Tube A)

Per Well (96-well plate)	
100 µL	cells

Tip: *500,000 cells/mL works well for HEK 293T cells. This will result in 50,000 cells/well in a 96 well plate. But remember, your optimal cell density is cell type dependent*

Transduction Reaction (Tube B)

Per Well (96-well plate)	
20 µL	cADDis cAMP for Gi sensor
0.6 µL	500 mM **SB** sodium butyrate
5 µL	**Ri** control receptor (if needed)
24.4 µL	complete media
50 µL	**total volume**

Tip: *When preparing a master mix, scale up by 10-15% of the number of wells needed to avoid coming up short.*

(Tube A) + (Tube B)

Cells + Transduction Reaction

Per Well (96-well plate)	
100 µL	cell suspension
50 µL	transduction reaction
150 µL	**total volume per well**

Protocol Adjustments for CHO Cells

For best results in CHO cells on a fluorescence plate reader, we recommend a modified transduction protocol. To boost expression, **replace sodium butyrate with valproic acid** in the transduction reaction and use the **following protocol modifications** to set up the assay in 96-well format:

Tip: *For best results when using valproic acid, prepare 50-100 µL aliquots and store at -20°C.*

a. Detach cells using standard trypsinization protocol. Resuspend cells in complete culture media and perform cell count.

b. Prepare a dilution of cells at your desired concentration (we recommend 22,500 cells/well in a 96-well plate, as a starting point). **50 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.

c. Prepare transduction reaction (detailed at right).

d. Mix the cells with the transduction reaction (**50 µL** cells + **50 µL** transduction mix). Mix gently, then seed **100 µL** of this mix per well on a 96-well plate.

e. Let cells sit at room temperature, protected from light, for 20 minutes.

f. **Spin the plate at 1,500 x g for 1.5-2 hours at room temperature.**

* We recommend sealing the plate with Breathe-Easy® (Cat. No. 70536-10) during this step to avoid contamination.

Transduction Reaction

Per Well (96-well plate)	
15 µL	cADDis cAMP for Gi sensor
5 µL	**Ri** control receptor (if needed)
1.7 µL	300 mM valproic acid (5.1 mM in well)
0.7 µL	1M HEPES
27.6 µL	cell culture media
50 µL	**total volume**

g. After spinning the plate, **remove the transduction mix** and replace with fresh media containing **5 mM valproic acid** (100 µL-150 µL per well).

h. Return plate to normal growth conditions and incubate for 20-24 hours.

If the above protocol does not result in acceptable expression levels, we recommend using **System Biosciences' Spinoculation protocol**, which adds SBI's TransDux Max and MAX enhancer reagents to the transduction reaction. Please make the following adjustments to your transduction reaction:

Transduction Reaction + Enhancer Reagents

Per Well (96-well plate)	
15 µL	cADDis cAMP for Gi sensor
5 µL	**Ri** control receptor (if needed)
0.34 µL	TransDux
17 µL	Max Enhancer
1.7 µL	300 mM valproic acid (5.1 mM in well)
0.7 µL	1M HEPES
10.26 µL	cell culture media
50 µL	**total volume**

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.

Transduce Adherent Cells (Day 1)

Step 1)
Prepare Cells

a. For each transduction reaction (i.e. one well in a 96-well plate, containing 100 µL culture media per well), prepare a transduction solution by mixing 20 µL of the sensor BacMam stock with 5 µL of **Ri** control receptor (if needed), 24.4 µL of culture media, and 0.6 µL of the 500 mM stock solution of **SB** 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 PBS in the step above. See the **Assay Optimization section** for more information.

b. 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 20-24 hours.

c. Optional step (cell type dependent): After 4-8 hour incubation with sensor BacMam (6 hours is optimal), aspirate transduction solution and add 100 µL complete growth medium with sodium butyrate at a concentration of 2 mM. Return cells to normal growth conditions for approximately 16-20 hours before measuring fluorescence as described above. If cells will not tolerate a full media exchange, partial media exchanges can be done.

Step 2)
Measure Fluorescence

d. Reference **Day 2 Detailed Procedure** steps on page 8

Scaling for 384 Wells (1 plate)

To set up the assay in 384-well plates, follow all of the protocol steps outlined above, adjusting reagent volumes as follows:

Step 1) Prepare cells (Tube A)

a. 50 µL of the cell resuspension will be required for a single well in a 384-well plate. A plating density of 12,500 cells per well is a good starting point, so prepare the cell suspension at 250,000 cells/mL. Depending on the cell type and plate type, 10,000-15,000 cells per well may be optimal.

Step 2) Prepare Viral Transduction Reaction (Tube B)

b. For each transduction reaction (i.e. one well in a 384-well plate), prepare the transduction solution by mixing 5 µL of the sensor BacMam stock with 0.3 µL of the 500 mM stock solution of **SB** sodium butyrate, 1 µL of the **Ri** control receptor (if needed), and 18.7 µL of the complete culture media for your cells, for a total volume of 25 µL. Mix gently.

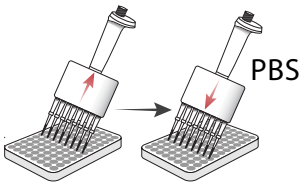
Step 3) Mix Cells and Transduction Mix

c. Mix Tube A and Tube B (50 µL tube A + 25 µL tube B). Mix gently and seed 75 µL of the mix per well on the 384-well plate.

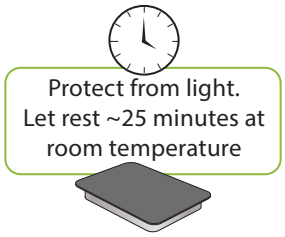
Tip: *In step 1, the volume of the cell suspension per well can be reduced from 50 µL to 25 µL if desired, for a final plating volume of 50 µL per well after the completion of step 3. Make sure that the well is still receiving 12,500 cells and that you reduce the volume of sodium butryate to 0.2 µL per well.*

Day 2 – Detailed Procedure

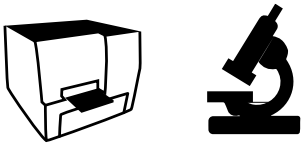
Measuring Fluorescence



g. Prior to measuring fluorescence, replace culture media with PBS (1X, containing Ca^{2+} and Mg^{2+}). Wash gently so as not to dislodge cells. **Cover the cells and allow to rest at room temperature in PBS for ~25 minutes before measuring fluorescence so cells can adjust to the new environment.** Experiments are performed at room temperature.



h. Visually inspect cells on a microscope to confirm cell health, sensor expression, and transduction efficiency.



i. Measure signal-to-background. Transduced cells should be at least 5× brighter compared to untransduced cells.

j. Drug addition: After acquiring 5-10 baseline measurements, with 30 second-to-1 minute intervals, add 10 µM forskolin (final concentration in well) to increase cAMP levels **(Figure 1)**. Measure the decrease in fluorescence intensity over time, capturing the change in fluorescence intensity using standard GFP excitation and emission wavelengths. After approximately 15-20 minutes, when the signal has reached a stable plateau, add compounds that activate your Gi receptor and decrease levels of cAMP. This will produce an increase in fluorescence intensity. Continue to measure fluorescence for an additional 15-20 minutes after addition of Gi receptor agonists. For wells transduced with **Ri** (D2 control receptor), add 10 µM **Ai** (quinpirole, final concentration in well) to activate the receptor. The optimal dose of quinpirole may need to be determined for a given cell line.

*** Note:** 10 µM forskolin is a good staring point, but the optimal concentration may be cell-type dependent and will likely fall in the range of 3-30 µM. If 10 µM does not produce a robust signal, it is important to take the time to perform a concentration-response experiment in your cells. Forskolin should be used at a concentration that is just below saturating (i.e. EC90-EC95).

*** Important:** Make sure that the final concentration of any drug solvents in the well (e.g. DMSO) does not exceed 0.3%. The ideal concentration is 0.1% for DMSO.

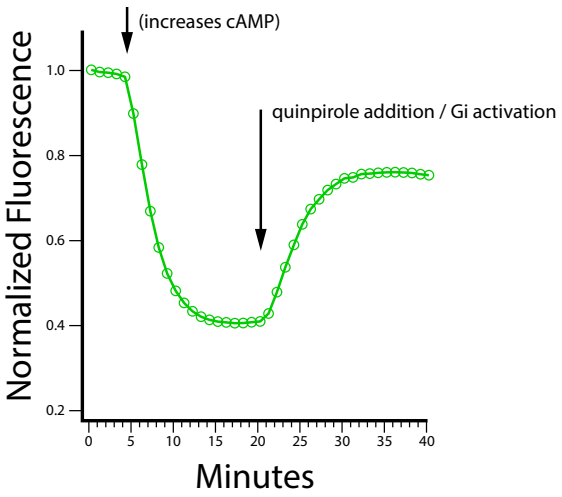


Figure 1. HEK 293T cells transduced with green downward cADDis sensor and D2R BacMam, responding to addition of forskolin and the resulting increase in cAMP. Quinpirole is then added to activate D2R.

Tip: *Add agonists at a concentration of 3-4× (in a volume of 50 µL PBS) to wells containing 100-150 µL PBS. This will result in a 1:3 or 1:4 dilution, and allow for adequate diffusion.*

Tip: *Always test the addition of the vehicle alone (i.e. PBS without drug).*

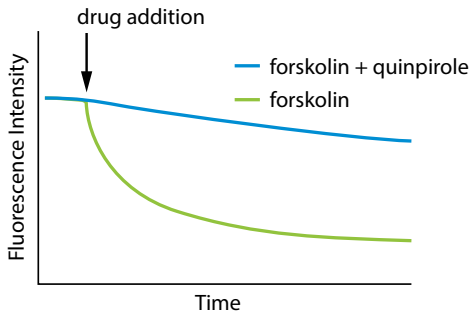
Tip: *The timing of the responses may differ depending on your cell type. For example, it may take longer than 15-20 minutes for the signal to plateau after the addition of forskolin.*

Tip: *Once you have established the kinetics of the forskolin response, you do not need to measure fluorescence for this portion of the experiment each time. You may add forskolin in the hood and allow the plate to incubate for the appropriate amount of time. The experiment can then begin with the acquisition of 5-10 baseline reads, followed by addition of the Gi receptor agonists.*

Alternative Approach 1:

Forskolin + Gi agonist simultaneous addition.
Suppression of forskolin-induced cAMP accumulation.

If you do not wish to use the assay format above, which utilizes sequential addition of forskolin and the Gi receptor agonists, you may add a mix of the forskolin and Gi agonist (i.e. simultaneous addition). A lack of cAMP accumulation can then be measured, as compared to the condition that receives forskolin only.



Alternative Approach 2:

Alternative to forskolin: use isoproterenol activation of β2 adrenergic receptor to elevate cAMP.

This method may be used in place of forskolin as a means of elevating cAMP levels. (If needed, order the β2 adrenergic receptor in BacMam product #Z0500N). Follow the **detailed protocol for Day 1**, making adjustments to the transduction reaction, as shown in table, at right:

Adjusted Transduction Reaction

Per Well (96-well plate)	
20 µL	cADDis cAMP for Gi sensor
0.6 µL	500 mM **SB** sodium butyrate
5 µL	**Ri** control receptor (D2 receptor)
5 µL	**Rs** control receptor (β2 adrenergic receptor)
19.4 µL	complete media
50 µL	**total volume**

On **Day 2 of the experiment** (detailed on page 8), add 10 µM isoproterenol (final concentration in well) instead of forskolin, to elevate cAMP levels **(Figure 2).** As with forskolin, wait until the fluorescence signal has decreased and reached a stable plateau, then add 10 µM **Ai** (quinpirole, final concentration in well) to activate the D2 receptor in wells that were transduced with **Ri** (D2 control receptor). The optimal dose of quinpirole may need to be determined for a given cell line.

* **Note:** You may still test forskolin, alongside isoproterenol in these conditions

* **Note:** Other Gs-coupled GPCRs and agonists may be used to elevate cAMP.

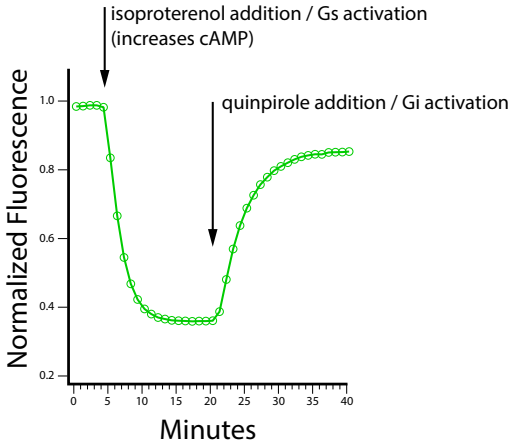


Figure 2. HEK 293T cells transduced with green downward cADDis, β2AR, and D2R BacMam, responding to addition of isoproterenol and the resulting increase in cAMP. Quinpirole is then added to activate D2R.

Alternative Approach 3:

No forskolin addition or Gs activation.

Montana Molecular offers a constitutively active Gαs (product #X0250X), which can be used to increase the steady state levels of cAMP. Since cAMP levels will already be elevated, adding forskolin or a Gs agonist isn't necessary. The constitutively active Gαs is simply co-transduced with the cADDis sensor at the time of transduction, on Day 1 of the protocol. Please **contact us** for more details on this method if you are interested.

Fluorescence Detection

Our assays are compatible with automated fluorescent plate readers and imaging systems.

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, mNeonGreen fluorescent protein [6]. While the peak excitation and emission wavelengths are 506 nm and 517 nm, respectively, a range of 485-505 nm (excitation) and 515-535 nm (emission) may be used if your instrument does not allow measurement at the peak ex/em. For example, on the BioTek Synergy MX™, the preferred ex/em is 488/525. If using filters, we recommend Chroma's Catalog set #49003 for optimal results.

Tip: *We offer* **mNeon BacMam Kits** *(products* **#F0500G** *and* **#F0505G***). This test kit is a good way to determine BacMam transduction efficiency, evaluate promoter systems, and optimize expression in your cells of choice.*

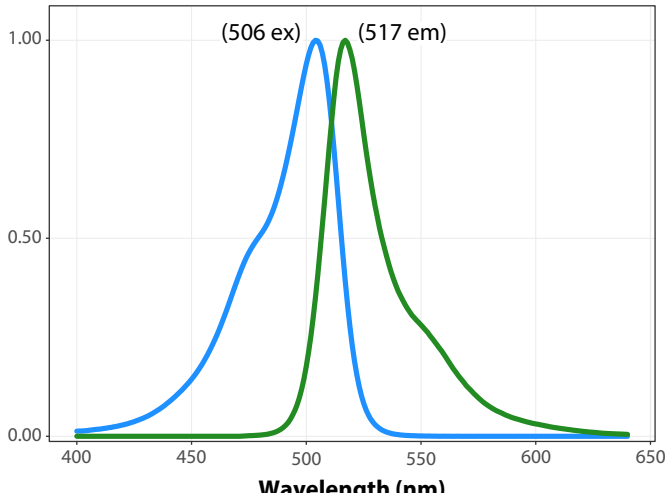


Figure 3. Absorption and emission properties of the mNeonGreen fluorescent protein plotted as a function of wavelength.

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 4, fluorescence was captured from cells before the addition of forskolin or isoproterenol, and then sampled at regular intervals after drug addition. The maximal response is reached at 10-15 minutes after the addition of the drugs, at which time the Gi agonist quinpirole can be added to decrease cAMP.

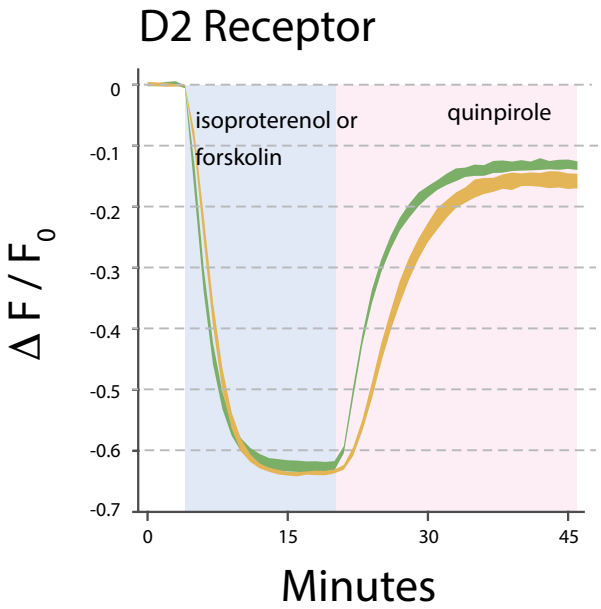


Figure 4. HEK 293T cells co-transduced with green downward cADDis sensor, the D2 receptor, and β2-adrenergic receptor, activated with forskolin or isoproterenol followed by quinpirole addition to activate D2. This graph shows the expected timing and duration of cAMP signals when using the positive control provided in your kit.

Assay Optimization

Optimizing Fluorescence

Twenty-four hours after transduction, check your cells for fluorescence. Wells that were transduced with the sensor should be 5-10 times brighter than control wells that were not treated with the sensor.

HDAC inhibitors may be used to maintain expression of the sensors. While BacMam transduction alone will result in sensor expression, sodium butyrate or another HDAC inhibitor, such as valproic acid (VPA) or trichostatin A (TSA), will generate higher levels of expression and will maintain this level of expression [4]. If cells look unhealthy, use lower concentrations or no HDAC inhibitor.

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, McCoy's 5A, and F12K culture media.

Optimizing Expression for Your Cell Type

To determine optimal conditions for your cell type, prepare a dilution series of transduction reactions by varying the amount of BacMam. For example, a range of 2.5 µL to 40 µL is a good starting range in a 96-well plate.

Varying the cell density, concentration of sodium butyrate, or trying a new HDAC inhibitor (VPA or TSA) may boost expression as well.

Please **contact us** if you would like to use the sensor under the control of a specific promoter system. Sensors under weak promoters may be limited to detection on imaging systems. To maintain strong expression in specific cell types, we recommend ordering a Cre-inducible, floxed sensor.

Purified viral preparations, which can increase expression in particularly sensitive or difficult to transduce cell types, are available upon request.

Use the Positive Control

If the cells are expressing the sensor, and fluorescence is detectable on your instrument, then check the sensor using the positive control receptor included in your kit. Add 5 µL of the **Ri** (D2 control receptor) to a set of control wells and activate with quinpirole included in the kit (**see Figure 1**).

Optimizing Receptor Expression

If you have titrated the cADDis sensor and determined the optimal volume, but fail to see a receptor-mediated signal, the receptor expression level may be the issue. Try titrating the receptor with a fixed amount of the cADDis sensor.

If you need further help, see the **Troubleshooting Guide** or let us know, we're happy to help!

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Troubleshooting Guide

Problem	Possible Cause	Solution
1. Low sensor expression and/or poor transduction efficiency	Suboptimal sensor BacMam volume is being used.	**Perform titration of the sensor BacMam stock**, testing a large range (i.e. 2.5-40 µL in 96-well plate format) to identify optimal volume. Too little can result in low expression, too much can cause cells to become sick.
	Transducing adherent cells.	Transduce cells while in suspension. If this isn't possible, try doing a media exchange on adherent cells after 4-6 hours, in addition to leaving the virus on overnight. See **Suggestions for Assays in Adherent Cells.**
	Suboptimal cell density; too few or too many cells added.	Transduce cells so that the cells will be around 75-80% confluent at the time of transduction. Also, when transducing cells in suspension, make sure that cells in the source flask are < 100% confluent (approximately 80% confluent is ideal).
	HDAC inhibitor was not added to the transduction mix, or the concentration was wrong.	Add HDAC inhibitor at the proper concentration: sodium butyrate - 2mM valproic acid - 5mM trichostatin A - 0.25µM * Perform a titration to determine optimal concentration for the cell type being used.
	HDAC inhibitor being used is not optimal for cell type.	Test other HDAC inhibitors (e.g. sodium butyrate, valproic acid, trichostatin A.)
	Cell type being used transduces poorly.	• After adding transduction mix to cells, let cells sit at room temperature for 30-40 min. before placing back in incubator (longer incubation times at room temperature may further improve transduction). • Perform media exchange after various incubation times with the transduction mix, in addition to leaving the virus on overnight. • Try high-titer, purified BacMam stock. • Validate assay in a different cell type (e.g. HEK 293T cells) • Transduce cells multiple times (e.g. on Day 1, and again on Day 2). • Incubate cells for 48 hours post transduction, before performing assay. • Consider using a different viral vector, such as lentivirus or AAV.
	Cell culture media is inhibiting transduction.	Remove media during transduction, preparing the transduction mix in PBS and adding to cells. Replace transduction mix with media after 2-4 hours.

Problem	Possible Cause	Solution
(continued) **Low sensor expression and/or poor transduction efficiency**	BacMam stock was not stored properly (i.e. not stored at 4°C, exposed to light for long periods, subjected to multiple freeze-thaw cycles), or the shelf life has been exceeded.	Follow **guidelines for product storage**. BacMam stocks are stable for at least 12 months when stored properly. After this time period, the stock should be re-evaluated and compared to previous experiments. Purified BacMam stocks should be used within 30 days for best results.
	BacMam stock was not mixed adequately before transducing cells.	Mix BacMam stock thoroughly before transduction, especially after being stored for long periods.
	Promoter is not optimal for cell type being used.	Identify promoters that work best in the cell type being used. If promoter is not on product list, **consult Montana Molecular** for custom production services.
	Cells are contaminated	Monitor cells for bacteria, fungi, etc.
2. Low fluorescence signal on microscope/plate reader.	Low sensor expression, low transduction efficiency.	See solutions for **Problem 1**.
	Excitation/emission settings are not optimal for the sensor being used.	Refer to protocol for the **fluorescence spectra** of the sensor. Make sure that filter sets or monochromators are aligned with the peak excitation and emission wavelengths of the sensor.
	Cells are in cell culture media, and the media is producing a large fluorescent signal (autofluorescence).	Exchange media so that cells are in PBS at the time of experiment. If cell culture media must be used, try using FluoroBrite media.
	Wrong microplate type is being used.	Use black, clear-bottom microplates with low autofluorescence.
	Exposure time or gain setting on instrument is suboptimal.	Test different exposure and gain settings, monitoring how the signal-to-background and noise in the measurement changes. Too high can result in saturation and/or photobleaching; too low can result in noisy data.
	Cells were dislodged during media exchange/plate washing.	Make sure that media exchange or plate washing is done gently and does not dislodge cells. **Confirm with visual inspection on a microscope**.
3. Signal-to-background is low (i.e. cells/wells with sensor are not much brighter than control cells/wells without sensor).	Low sensor expression, low transduction efficiency.	See solutions for **Problem 1**.
	Excitation/emission settings are not optimal for the sensor being used.	Refer to protocol for the **fluorescence spectra** of the sensor. Make sure that filter sets or monochromators are aligned with the peak excitation and emission wavelengths of the sensor.
	Exposure time or gain setting on instrument is suboptimal.	Test different exposure and gain settings, monitoring how the signal-to-background and noise in the measurement changes. Too high can result in saturation and/or photobleaching; too low can result in noisy data.
	Media exchange was not performed before running the assay; cells are in media rather than PBS. Cell culture media being used has high autofluorescence.	Perform media exchange so that cells are in PBS at the time of experiment. If cell culture media must be used, try using FluoroBrite media.
	Cells were dislodged during media exchange/plate washing.	Make sure that media exchange or plate washing is done gently and does not dislodge cells. **Confirm with visual inspection on a microscope**.

	Problem	Possible Cause	Solution
4.	**Signal is noisy.**	Low sensor expression, low transduction efficiency.	See solutions for **Problem 1.**
		Gain setting or exposure time on instrument is too low.	Increase gain setting or exposure time.
		Media exchange was not performed, or plate washing was inadequate causing high well-to-well variability. Cells are not in PBS at the time of experiment.	Exchange media so that cells are in PBS at the time of experiment. If cell culture media must be used, try using FluoroBrite media. Make sure that plate washing is highly consistent from well to well.
		Cells were dislodged during media exchange/plate washing.	Make sure that media exchange or plate washing is done gently and does not dislodge cells. **Confirm with visual inspection on a microscope**.
5.	**Good fluorescence signal, but sensor is not responding to drug as expected. No change in fluorescence observed, or signal is in the wrong direction.**	Photobleaching	Reduce exposure time, sampling rate, and/or light intensity.
		Drug is at the wrong concentration	Confirm drug concentration and solubility.
		Drug was not stored properly.	Confirm drug **storage** conditions.
		Drug was added to the cells in a volume that was too low relative to the volume of PBS/media in the well, resulting in improper mixing.	Add drug in a volume that will allow for sufficient diffusion (i.e. 1:3 or 1:4 drug to total volume)
		Drug was not added in the same solution as the solution in the well/culture dish.	Make sure that the drug preparation and cells are in the same solution.
		Drug addition is producing an artifact.	Make sure to add a vehicle-only control. Make sure drug is added in a solution that is the same as the solution in the well. Do not exceed 1% DMSO final in the well (0.5% or less is ideal).
		Compounds being tested are fluorescent.	Scan compounds for fluorescence to confirm. If possible, dilute compounds in order to reduce the fluorescence artifact of the compound.
		Drug addition was too forceful and dislodged cells.	Add drugs manually or with an on-board dispense function, but do so gently, so as not to dislodge cells.
		Baseline reads were not acquired before adding drug.	Acquire 5-10 baseline fluorescence reads before adding drug. Monitor for a change in fluorescence intensity upon addition of drug.
		Gain setting on instrument is too high, and signal is saturating. Gain setting is too low, and signal cannot be detected.	Adjust gain setting.
		Too much sensor has been added to cells and the signal is saturated (i.e. not enough analyte for the amount of sensor in the cell).	Titrate the amount of sensor to determine maximum signal for your cell type. See protocol recommendations for **HEK 293T** and **CHO cells**.
		Target receptor was not added, or expression levels are suboptimal (too little or too much, or receptor has high level of constitutive activity).	Titrate the amount of receptor to optimize the signal for your cell type and receptor.
		Sampling rate is not consistent with sensor kinetics.	Acquire 5-10 baseline measurements before adding drug. Resume measurement quickly after adding drug (within 5-10 seconds for DAG/PIP_2, 60 seconds for cADDis and cGMP, and 1-2 seconds for GECO Ca^{2+}). Measure long enough to capture max response of sensor.

Problem	Possible Cause	Solution
6. Poor cell health, cells detaching from plate.	Too much BacMam stock was added to cells (e.g. sensor, receptor, Gs mutant).	**Titrate lower amounts of BacMam stock** to identify the optimal volume for your cells.
	Concentration of HDAC inhibitor is too high, or cells are sensitive to the HDAC inhibitor being used.	Confirm concentration of HDAC inhibitor being used. Make new stock solution. Try a different HDAC inhibitor. Confirm that they are being used at the proper concentration: sodium butyrate - 2 mM valproic acid - 5 mM trichostatin A - 0.2 5µM * Perform a titration to determine optimal concentration for the cell type being used.
	Plate surface is not coated with a cell attachment factor.	Coat plates with a cell attachment factor (e.g. PDL, laminin, collagen, fibronectin etc.) to enhance attachment.
	Edge wells are being used, and cells in the edge wells may be subject to conditions that are not conducive to growth.	Do not use edge wells.
	Cells were dislodged during media exchange/plate washing.	Make sure that media exchange or plate washing is done gently and does not dislodge cells. **Confirm with visual inspection on a microscope**.
	PBS being used does not contain Ca^{2+} and Mg^{2+}.	Use PBS containing Ca^{2+} and Mg^{2+}
	Cells are contaminated.	Monitor cells for bacteria, fungi, mycoplasma.
	Cells were not grown under proper growth conditions (i.e. 5% CO_2, 37°C).	Incubate transduced cells at 37°C, in 5% CO_2.

cADDis in the Literature

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PCT/US2014/063916 Patent Pending

Related Products			
Product	**Description**	**Promoter**	**Recommended Use**
#D0300R	Red Down DAG	CMV	Fluorescence imaging and plate reader assay (Z' > 0.7)
#U0300R	Red Up DAG	CMV	Fluorescence imaging and plate reader assay (Z' > 0.5)
#U0600R	Red GECO $Ca2^{+}$	CMV	Fluorescence imaging and plate reader assay (Z' > 0.5)

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** or call us at **+1 406-200-8321** and we'll respond as quickly as we can.

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European Patent Number: EP3065754B1