



Green cADDIs™ cAMP Assays (PCT/US2014/063916 Patent Pending)

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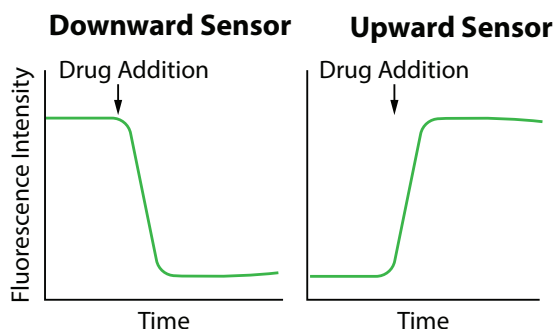


About these Assays

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

Depending upon the sensor in your kit, fluorescence either increases or decreases. For example, #D0200G Green Down cADDIs **decreases in fluorescence** when cAMP levels **increase** in the cell. Both Upward and Downward cADDIs are bright, robust, and easy to detect on fluorescence plate readers (**Figure 3- page 10**). For sensitive and/or difficult to transduce cell types, such as primary neurons, we offer purified, high-titer BacMam stock. Please **contact us** to discuss whether purified BacMam is the right product for you.

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 293T 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. 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
#D0200G	Green Down cADDIs cAMP	CMV	Fluorescence imaging and plate reader assay ($Z' > 0.9$)
#D0205G	Green Down cADDIs cAMP	CAG	Fluorescence imaging and plate reader assay
#U0200G	Green Up cADDIs cAMP	CMV	Fluorescence imaging and plate reader assay ($Z' > 0.85$)
#U0205G	Green Up 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

info@montanamolecular.com



*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
cADDIS cAMP sensor BacMam $\approx 2 \times 10^{10}$ 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 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 ₂ O	Sodium butyrate is added to the culture to maintain BacMam expression. Other HDAC inhibitors may work as well.	4°C
Rs	β2 adrenergic receptor BacMam in ESF 921 Insect Culture Medium (Expression Systems, product #96-001-01)	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. (The control receptor included in CAG-promoter kits does not have a separate fluorescent protein.)	4°C
As	isoproterenol 10 mM (Sigma Aldrich Product Number I2760) in 10 mM HCl	Isoproterenol can be used to stimulate Gs signaling through the positive control, the β2 adrenergic 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 Ca²⁺ and Mg²⁺ (PBS) available from VWR [\[2\]](#).
3. Cells and cell media of your choice.

Biosafety Considerations

The **BacMam vector carrying the fluorescent biosensor** in these assays 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 pseudo-typed 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**

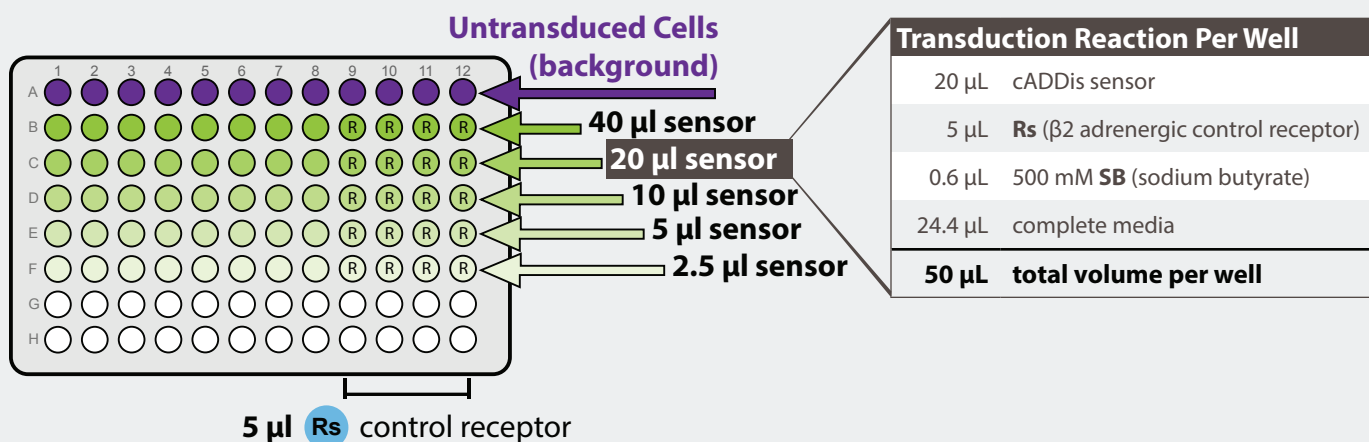
First Experiment - Optimize by Titrating the Sensor

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

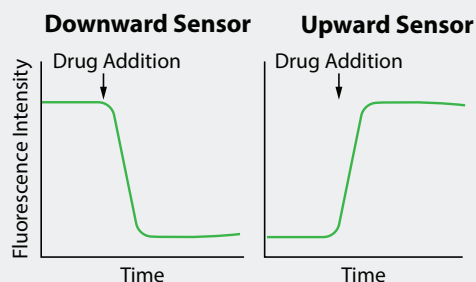
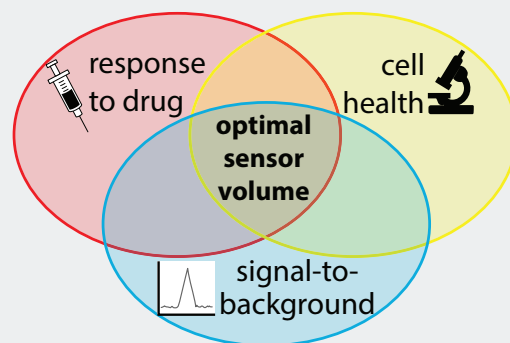
- Set up your plate. Be sure to include control wells (untransduced cells) in order to calculate signal-to-background. **Refer to detailed protocol Day 1 procedure on page 5.**
- Perform titration to determine optimal sensor volume for your cells.
- For each sensor volume, include a subset of wells that also has 5 μ L of **Rs** control receptor (included in your kit). These wells will be used for your control experiment on day 2.

Day 2



- Measure fluorescence to evaluate sensor expression levels.
- Add 10 μ M **As** (isoproterenol, final concentration in well) to activate the set of control wells transduced with the β 2 adrenergic receptor. Monitor the change in fluorescence.
- Refer to detailed protocol Day 2 procedure on page 8.**

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





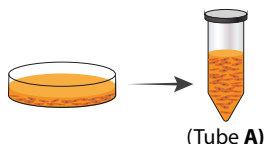
Day 1 – Detailed Procedure

Transduce and Plate Cells

Step 1)

Prepare Cells (Tube A)

- Detach cells 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.



Cells (Tube A)

Per Well (96-well plate)

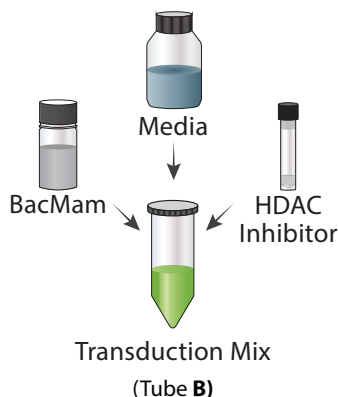
100 μL cells

Tip: 500,000 cells/mL works well for HEK293T cells. This will result in 50,000 cells/well in a 96 well plate. But remember, your optimal cell density is cell type dependent

Step 2)

Prepare Viral Transduction Reaction (Tube B)

- 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 **Rs** 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.



Transduction Reaction (Tube B)

Per Well (96-well plate)

20 μL cADDIS cAMP sensor
0.6 μL 500 mM **SB** sodium butyrate
5 μL **Rs** control receptor (if needed)
24.4 μL complete media

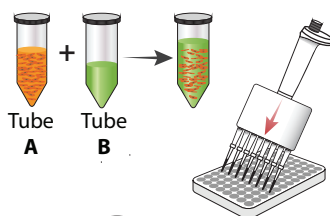
50 μL total volume

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

Step 3)

Mix Cells and Transduction Mix

- 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 let rest at room temperature for 30 minutes.
- Incubate \approx 20-24 hours under normal cell growth conditions (5% CO_2 and 37°C), protected from light.



Rest 30 minutes at room temperature

Incubate \approx 24 hours
5% CO_2 and 37°C

(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 .

- Detach cells using standard trypsinization protocol. Resuspend cells in complete culture media and perform cell count.
- 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.
- Prepare transduction reaction (detailed at right).
- 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.
- Let cells sit at room temperature, protected from light, for 20 minutes.
- 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.
- After spinning the plate, **remove the transduction mix** and replace with fresh media containing **5 mM valproic acid** (100 μL -150 μL per well).
- Return plate to normal growth conditions and incubate for 20-24 hours.

Transduction Reaction

Per Well (96-well plate)	
15 μL	cADDIs cAMP sensor
5 μL	Rs 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

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 sensor
5 μL	Rs 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

- 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 **Rs** 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** (see page 4). Cell culture media may be used in place of PBS in the step above. See the **Assay Optimization section** (page 9) for more information.
- 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.
- 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

- 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)

- 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)

- 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 **Rs** 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

- 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.

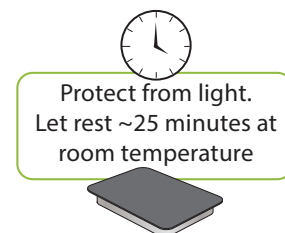
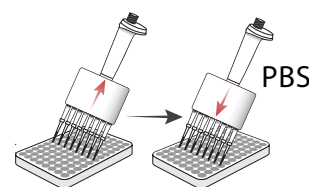
Tip: In step 1, the volume of the cell suspension per well can be reduced from 50 μL to 25 μL if desired, for a final plating volume of 50 μL per well after the completion of step 3. Make sure that the well is still receiving 12,500 cells and that you reduce the volume of sodium butyrate to 0.2 μL per 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 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. Add compounds that activate your receptor and increase levels of cAMP. Capture the change in fluorescence intensity using standard GFP excitation and emission wavelengths. For wells transduced with the β_2 adrenergic control receptor, add 10 μM **As** (isoproterenol, final concentration in well) to activate the receptor. The optimal dose of isoproterenol may need to be determined for a given cell line.
- * **Important:** Make sure that the final concentration of any drug solvents in the well (e.g. DMSO or Ethanol) does not exceed 1.0%.



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).

Fluorescence Detection

Our assays are compatible with automated fluorescence 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

Green 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). These test kits are a good way to determine BacMam transduction efficiency, evaluate promoter systems, and optimize expression in your cells of choice.

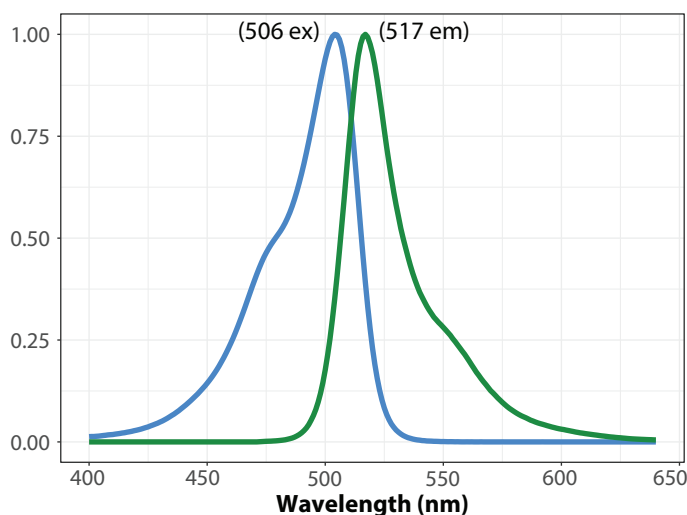


Figure 1. Absorption and emission properties of the mNeon-Green fluorescent protein plotted as a function of wavelength.

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 5–10 minutes after the addition of the drug.

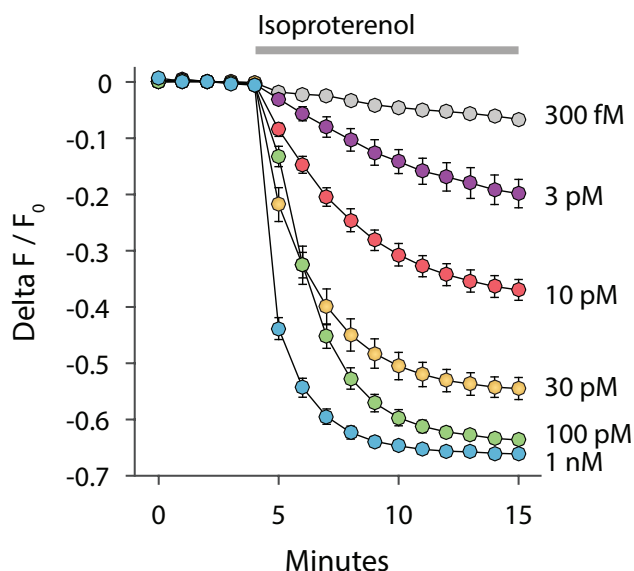


Figure 2. HEK 293T cells transduced with 20 µL of BacMam carrying Green Down cADDIS cAMP biosensor, activated with isoproterenol. The graph shows the expected timing and duration of cAMP when using the positive controls provided in your kit.

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 (see **Figure 3**).

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 **Rs** (β_2 adrenergic control receptor) to a set of control wells and activate with isoproterenol included in the kit (see **Figure 2, page 9**).

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!

+1 406-200-8321, info@montanamolecular.com

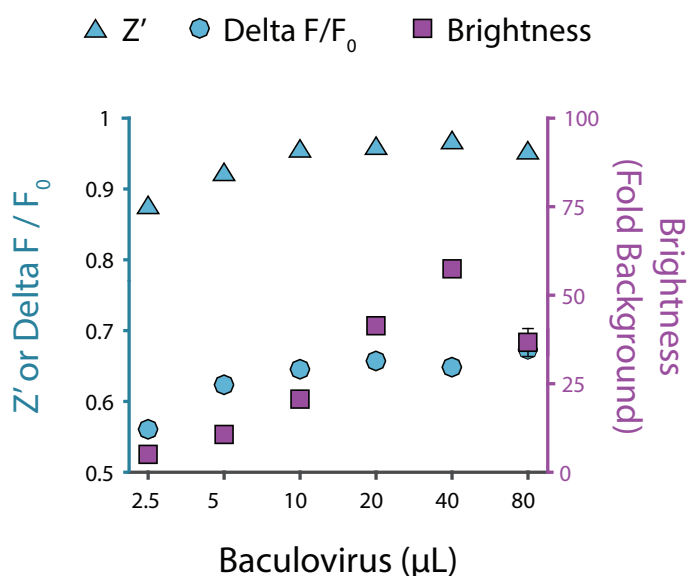


Figure 3. As the amount of cADDIs added to the wells increases, so does the baseline fluorescence, plotted in purple. The change in fluorescence when cAMP changes also increases with more virus, but reaches the maximum possible change and remains constant over a broad range of virus concentration (circles).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



Troubleshooting Guide

1.

Low sensor expression and/or poor transduction efficiency

Problem	Possible Cause	Solution
	Suboptimal sensor BacMam volume is being used.	Perform titration of the sensor BacMam stock , testing a large range (i.e. 2.5-40 μ L 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 - 0.25 μ M * 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.	<ul style="list-style-type: none"> • 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 HEK293T 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 - 2mM valproic acid - 5mM trichostatin A - 0.25µM * 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 .



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PCT/US2014/063916 Patent Pending



Related Products

Product	Description	Promoter	Recommended Use
#U0200R	Red Up cADDIs cAMP	CMV	Fluorescence imaging and plate reader assay ($Z' < 0.5$)
#X0200G	Gi cADDIs cAMP	CMV	Fluorescence imaging and plate reader assay ($Z' > 0.7$)
#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 Ca ²⁺	CMV	Fluorescence imaging and plate reader assay ($Z' > 0.5$)

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