Montana Molecular
Fluorescent Biosensors for Live Cell Discovery

Cilia-targeted cADDis Sensors

About these Assays

Cyclic AMP is an essential second messenger for many cellular processes. The messages carried by cAMP are tightly regulated within cells. To measure cAMP within the ciliary space, we fused cADDis, a genetically encoded sensor for cAMP, to the 5HT6 receptor. The cilia-targeted cADDis sensor decreases in fluorescence intensity when localized cAMP is increasing in the cilia. The following protocol assumes cilia formation in your cells (Delling 2013).

Overview

The green cADDis sensor (Tewson, 2015) is a fluorescent protein-based sensor for live cell measurement of cAMP in the primary cilium of mammalian cells. 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
#D0201G	Green cilia-targeted cADDis cAMP sensor	CMV	Fluorescence imaging
#D0211G	Ratiometric cilia-targeted green cADDis cAMP sensor with red fluorescent tag	CMV	Fluorescence imaging

Materials in the Kit

- Green Cilia-Targeted cADDis cAMP sensor BacMam in ESF 921 Insect Culture Medium (Expression Systems, product # 96-001-01).

  Green fluorescent sensor that decreases in fluorescence intensity in response to increases in cAMP. The ratiometric cilia-targeted sensor also expresses a red fluorescent protein for ratiometric measurements.

- 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.

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].
- 2-methyl-5hydroxytryptamine (2-M-5HT) a 5HT6 receptor agonist
- L-858051 a forskolin derivative

BioSafety Considerations

The BacMam vector carrying the fluorescent biosensors used in these assays is a modified baculovirus, which can be 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.

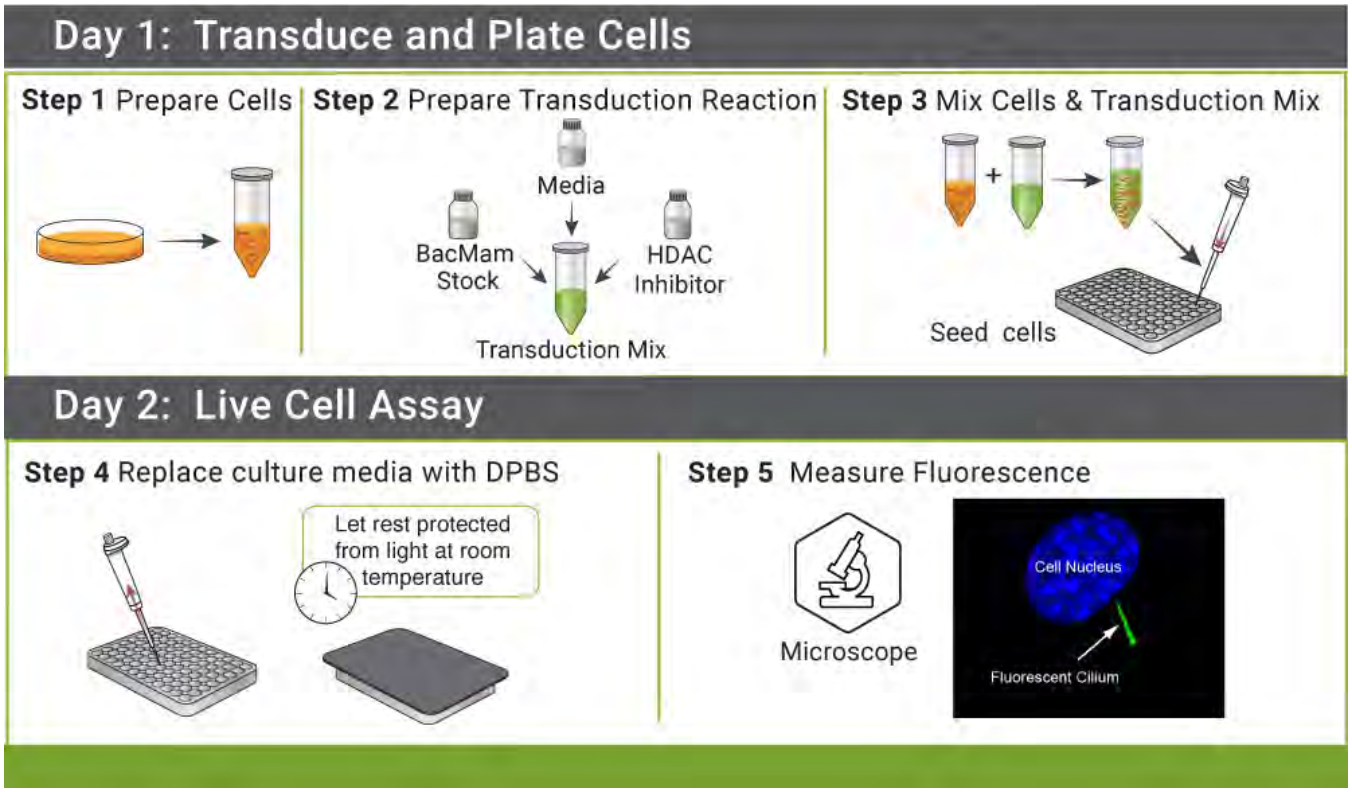
This product is for research use only and is not recommended for use or sale in human or animal diagnostic or therapeutic products.

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.

Protocol for Use

This protocol is optimized for rapidly dividing, immortalized cell lines in 96 well plates. We recommend that you take the time to optimize the assay for your particular cell type. For transducing non-dividing adherent cells such as neurons, islets, cardiomyocytes, and iPSC-derived lines, see our Suggestions for Other Cell Types and Plate Formats following this protocol.



DAY 1 TRANSDUCE AND PLATE CELLS

Step 1) Prepare cells (Tube A)

- Detach cells from flask using standard trypsinization protocol. Re-suspend 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.

Example:

For **96** wells (1 plate)

100 µL cell suspension (500,000 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 BacMam sensor stock with 0.6 µl of the 500 mM stock solution of sodium butyrate* and 29.4 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
0.6 µL 500 mM Sodium Butyrate	x **110** = 66 µL
29.4µL Complete Media	x **110** = 3234 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 then 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 ≈ 20-24 hrs under normal cell growth conditions (5% CO_2 and 37°C), 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 FLUORESCENCE MEASUREMENT

- Prior to measuring fluorescence, replace culture media with DPBS (containing calcium and magnesium). 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. Forskolin will cause an increase in cAMP throughout the cell. A Gs-coupled receptor agonist can also be used. 2-M-5HT, a 5HT6 receptor agonist, is one example.
- 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 cilia.

Fluorescence Detection

Fluorescence Properties

cADDis is constructed with the very bright, mNeon green fluorescent protein [6], and the constitutively expressed red fluorescent protein is SantakaRFP. Preferred excitation and emission wavelengths for mNeon Green are 485/528 and preferred excitation and emission wavelengths for SantakaRFP are 558/603.

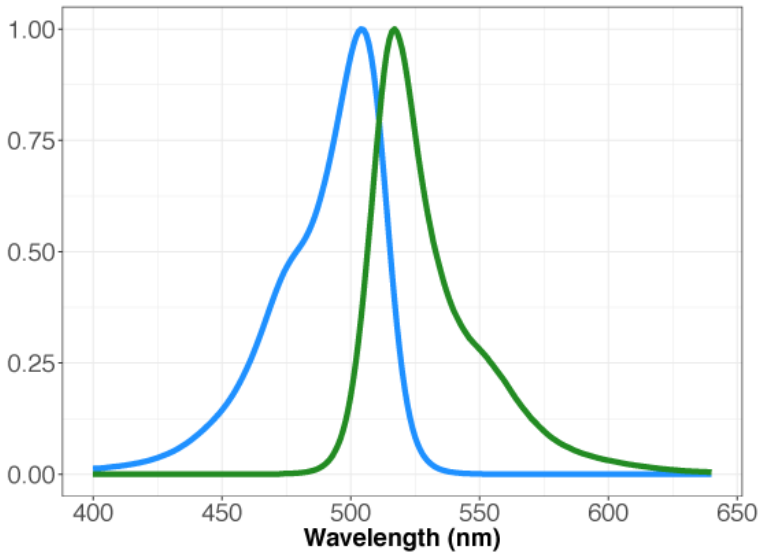


Figure 1A. Absorption and emission properties of the mNeon green fluorescent protein plotted as a function of wavelength.

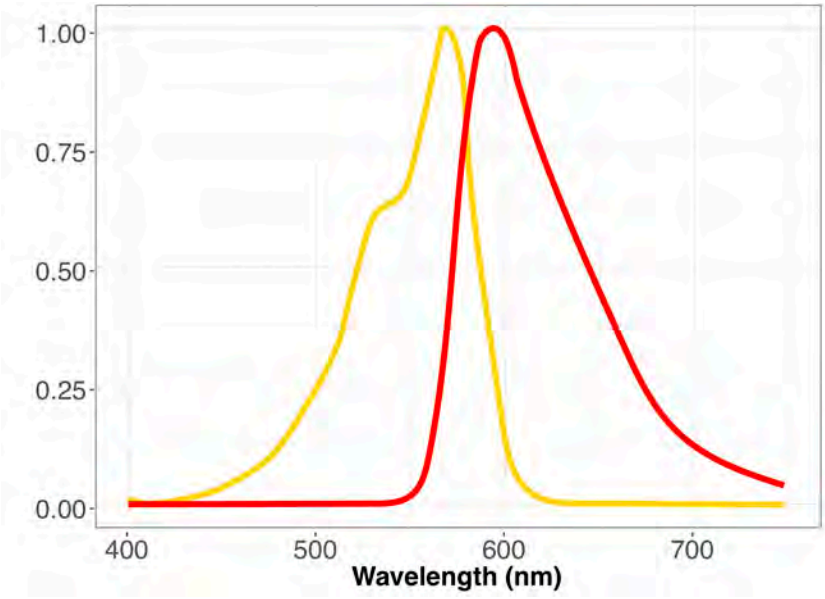


Figure 1B. Absorption and emission properties of a red fluorescent protein plotted as a function of wavelength.

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.

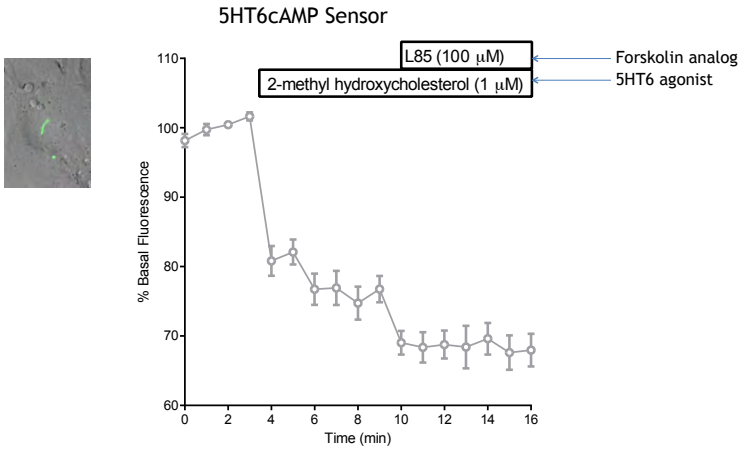


Figure 2. An example of the sensor response in gray in IMCD3 cells, imaged on the left (Moore 2016).

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 (provided in your kit).
- 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 in the step above. See the assay optimization section 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 hrs.
- Optional step (cell type dependent): After 4-8 hr incubation with Sensor BacMam (6 hrs 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 hrs before measuring fluorescence as described above. If cells will not tolerate a full media exchange, partial media exchanges can be done.

Assay Optimization

Are your cells fluorescent?

Twenty four hours after transduction, you should see green fluorescence localized to the cilia. HDAC inhibitors may be important 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 [Kost, T. et. al. 2007]. If cells look unhealthy, use lower concentrations or no HDAC inhibitor.

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.

Optimizing expression of the fluorescent sensor

It is important to optimize expression for your particular cell type. Too little virus will produce variable results, particularly if the sensor expression levels are low and difficult to detect on your instrument. Baseline fluorescence goes up as you add more virus, and when a particular threshold is reached the absolute change in sensor fluorescence become constant.

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 5 µL to 50 µL is a good starting range.

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.

We also provide a more highly refined and concentrated viral preparation upon request, which can increase expression in particularly sensitive or difficult to transduce cell types.

Optimizing 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.

Trouble Shooting

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. 5-50µL 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 4-6hrs, in addition to leaving the virus on overnight.
	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 *A titration can be performed 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 RT 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. HEK293 cells) - Transduce cells multiple times (e.g. on Day 1, and again on Day 2). - Incubate cells for 48 hrs post transduction, before performing assay. - Consider using a different viral vector, such as lentivirus or AAV.

Problem	Possible Cause	Solution
(continued) 1) Low sensor expression and/or poor transduction efficiency.	Cell culture media is inhibiting transduction.	Remove media during transduction, preparing the transduction mix in DPBS and adding to cells. Replace transduction mix with media after 2-4 hours.
	BacMam stock was not stored properly (i.e. not stored at 4°C, exposed to light for long periods, or 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 reevaluated and compared to previous experiments. Purified BacMam stocks have a shelf life of 3 months.
	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 above.
	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 monochromotors 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 DPBS 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.

Problem	Possible Cause	Solution
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 above.
	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 DPBS. Cell culture media being used has high autofluorescence.	Perform media exchange so that cells are in DPBS 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.
4) Signal is noisy.	Low sensor expression, low transduction efficiency.	See solutions for problem 1 above.
	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 DPBS at the time of experiment.	Exchange media so that cells are in DPBS 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.

Problem	Possible Cause	Solution
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 DPBS/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.
	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 for recommendations for HEK293 and CHO cells.

Problem	Possible Cause	Solution
(continued) 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.	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 R-GECO). Measure long enough to capture max response of sensor.
	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.
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 *A titration can be performed 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 harmed or 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
(continued) 6) Poor cell health, cells detaching from plate.	DPBS being used does not contain calcium and magnesium.	Use DPBS containing calcium and magnesium.
	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.

Contact Us

If you have ideas about how we can improve our products or protocols, then we would like to hear from you. Your feedback is extremely valuable. Please send an email to info@montanamolecular.com. We'll respond as quickly as we can.

References

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PCT/US14/63916 Patent Pending