Green Fluorescent PIP_2 Assay

May 10, 2022

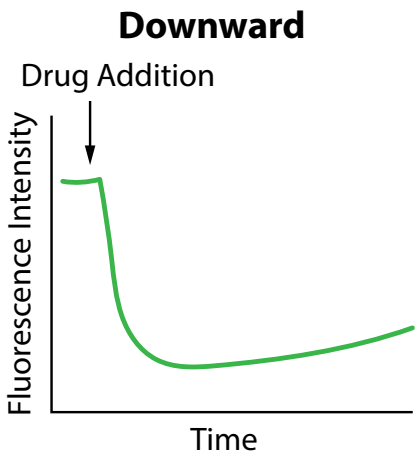
Product Info & Protocol

About these Assays

PIP_2, also known as Phosphatidylinositol 4,5-bisphosphate or PtdIns(4,5)P2, is a substrate for several signaling molecules. It is a precursor for the DAG/IP3 pathway, but also plays a key role in phospholipid signaling and is involved in the regulation of ion channels and transporters. The fluorescent sensors used in the assays described here can be combined with different colored sensors, such as the red GECO calcium sensor (#U0600R) or a red DAG sensor (#U0300R) to measure multiple signals simultaneously. The green PIP_2 sensor is based on a dimerization-dependent fluorescent protein. It will decrease in fluorescence in response to decreasing PIP_2 levels.

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 PIP_2 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
#D0400G	Green PIP_2 Sensor	CMV	Fluorescence imaging
#D0405G	Green PIP_2 Sensor	CAG	Fluorescence imaging

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
PIP_2 sensor BacMam ≅ 2×10^{10} VG/mL in ESF 921 Insect Culture Medium (Expression Systems, product #96-001-01)	Green fluorescent sensor that decreases in fluorescence intensity in response to decreases in PIP_2. 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
Rq **M1 muscarinic acetylcholine receptor BacMam** in ESF 921 Insect Culture Medium (Expression Systems, product #96-001-01)	A Gq-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.	4˚C
Aq **carbachol** (Sigma Aldrich product number C4382) 25 mM in H_20	Carbachol can be used to stimulate Gq signaling through the positive control, the M1 muscarinic acetylcholine 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. 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 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

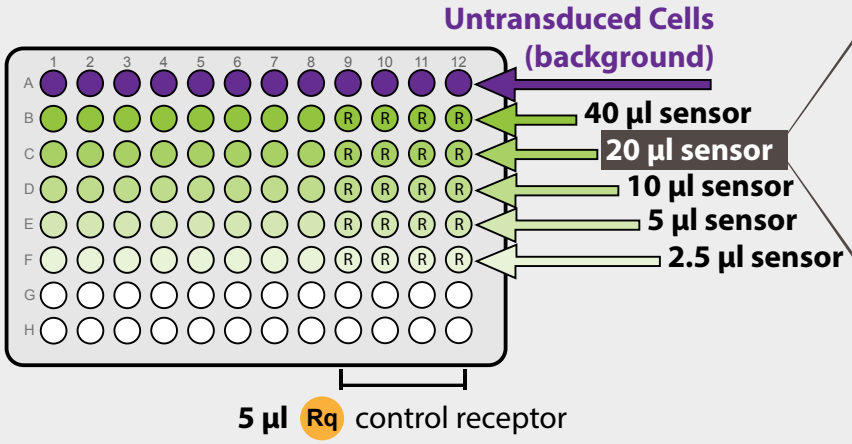
We recommend using a titration series to determine the best combination of signal above background, cell health, and response. Ideally, the signal in each well before drug addition should be at least 5 times above background.

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 **Rq** control receptor (included in your kit). These wells will be used for your control experiment on day 2.



5 µl Rq control receptor

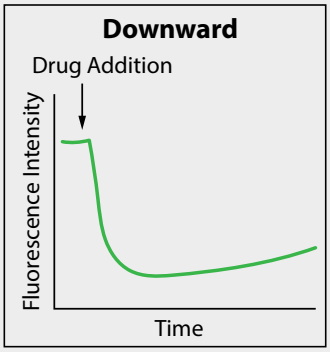
Transduction Reaction Per Well	
20 µL	PIP_2 sensor
5 µL	**Rq** (M1 muscarinic control receptor)
0.6 µL	500 mM **SB** (sodium butyrate)
24.4 µL	complete media
50 µL	**total volume per well**

Day 2

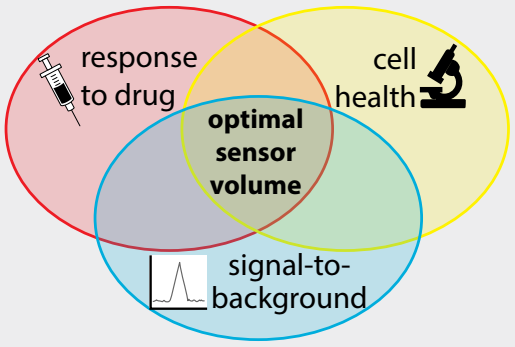
d. Measure fluorescence to evaluate sensor expression levels.

e. Add 10 µM **Aq** (carbachol, final concentration in well) to activate the set of control wells transduced with the M1 muscarinic receptor. Monitor the change in fluorescence.

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



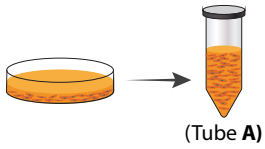
Check your cells for these 3 parameters to determine **optimal sensor volume.**



Day 1 – Detailed Procedure

Transduce and Plate Cells

Step 1)
Prepare Cells (Tube A)

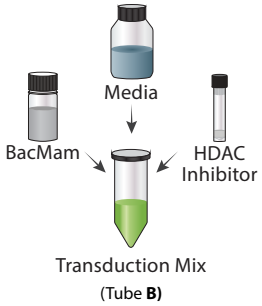


a. Detach cells using standard trypsinization protocol. Resuspend cells in complete culture media and determine cell count.

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.

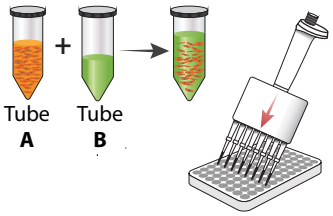
Step 2)
Prepare Viral Transduction Reaction (Tube B)

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 **Rq** 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.

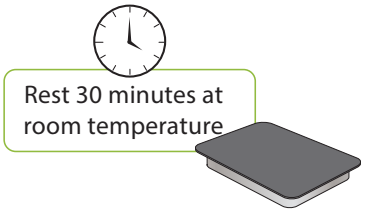


Step 3)
Mix Cells and Transduction Mix

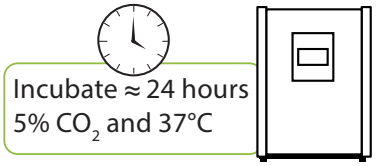
d. Mix Tube A and Tube B (100 µL tube A + 50 µL tube B). Mix gently and seed 150 µL of mix per well on the 96-well plate.



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	PIP_2 sensor
0.6 µL	500 mM **SB** sodium butyrate
5 µL	**Rq** 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	PIP_2 sensor
5 µL	**Rq** 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	PIP_2 sensor
5 µL	**Rq** 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 **Rq** 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)

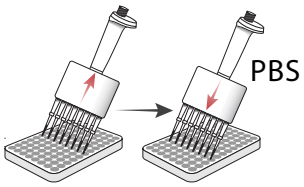
b. For each transduction reaction (i.e. one well in a 384-well plate), prepare the transduction solution by mixing 5 µL of the sensor BacMam stock with 0.3 µL of the 500 mM stock solution of **SB** sodium butyrate, 1 µL of the **Rq** control receptor (if needed), and 18.7 µL of the complete culture media 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 butyrate to 0.2 µL per well.*

Day 2 – Detailed Procedure



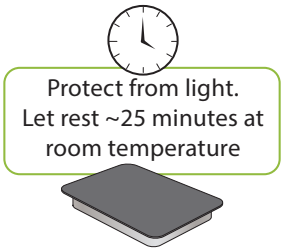
Measuring Fluorescence

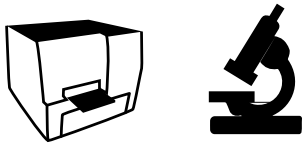
g. Prior to measuring fluorescence, replace culture media with PBS (1X, containing Ca^{2+} and Mg^{2+}). Wash gently so as not to dislodge cells. **Cover the cells and allow to rest at room temperature in PBS for ~25 minutes before measuring fluorescence so cells can adjust to the new environment.** 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 decrease levels of PIP_2. Capture the change in fluorescence intensity using standard GFP excitation and emission wavelengths. For wells transduced with the M1 muscarinic control receptor, add 10 µM **Aq** (carbachol, final concentration in well) to activate the receptor. The optimal dose of carbachol may need to be determined for a given cell line.





*** Important: Important:** Make sure that the final concentration of any drug solvents in the well (e.g. DMSO) does not exceed 1%. The ideal concentration for DMSO is 0.1%.

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

This sensor is constructed with a green fluorescent protein. The optimal excitation wavelength for imaging the green fluorescent PIP$_2$ sensor is 480 nm, but the absorption band of this protein is quite broad, so broad bandpass filters that pass 450 to 480 nm light can be used effectively. On the emission side, the green light spans 510 to 550 nm, so broad band pass emission filters can also collect much of the emission. These filter properties are similar to many of the FITC filter sets commonly available on most microscopes and plate readers. Preferred excitation and emission wavelengths are 488/525..

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.*

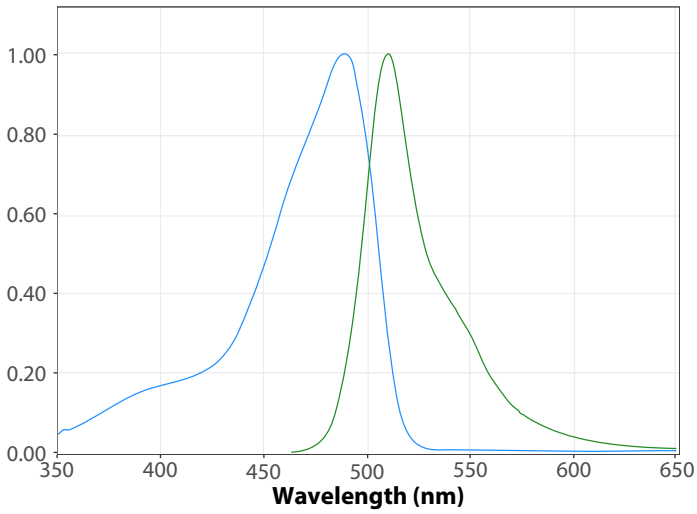


Figure 1. Absorption and emission properties of a typical green fluorescent protein plotted as a function of wavelength. Optimal excitation light ranges from 450 to 490 nm, while optimal emission filters should select the emitted light between 510 and 550 nm.

Timing

Unlike many assays that measure accumulation of PIP$_2$ in cell lysates, this sensor measures PIP$_2$ changes in living cells, in real time. For best results, be sure to capture changes in PIP$_2$ 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 within ~60 seconds after the addition of the agonist and in HEK 293T cells, begins to return to baseline after ~100 seconds.

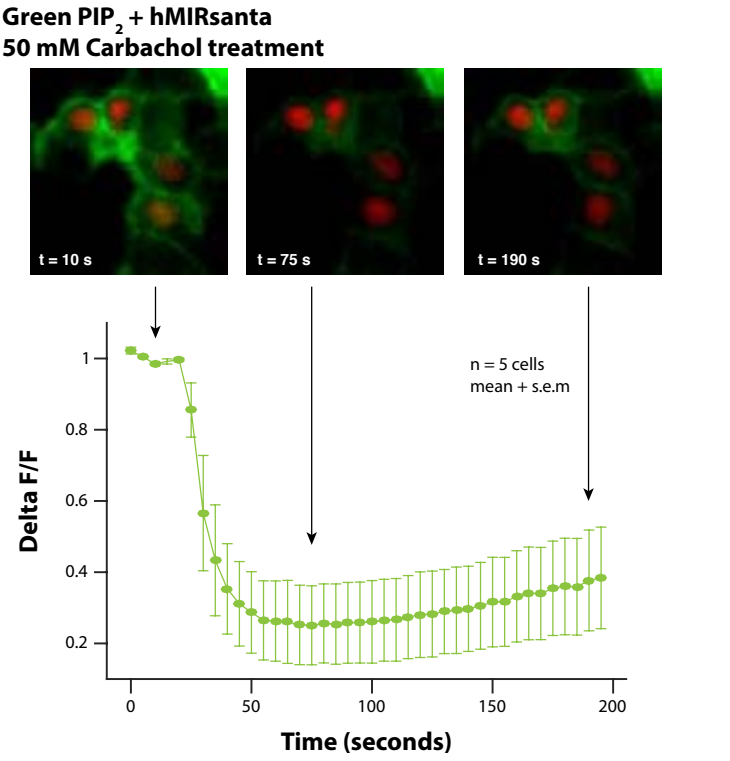


Figure 2. Green fluorescent PIP$_2$ sensor in HEK 293 cells, co-transduced with human muscarinic acetylcholine control receptor M1, and a red nuclear label. Fluorescence was captured from cells before the addition of 50 µM carbachol, and sampled at regular intervals. The maximal response is reached at ~60 seconds after the addition of the drug, and the response begins its return to baseline ~100 seconds after drug is added.

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 [3]. 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 **Rq** (M1 control receptor) to a set of control wells and activate with carbachol included in the kit **(see Figure 2).**

Optimizing Receptor Expression

If you have titrated the PIP_2 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 PIP_2 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**

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 - 2 mM valproic acid - 5 mM trichostatin A - 0.25 µM * 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 - 0.25 µM * 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.
	Cells are sensitive to acidity from the insect cell culture media present in BacMam virus. The insect cell culture media is more acidic than typical mammalian cell culture media	Wash transduction mix off of cells after 4-6 hours. Replace with fresh cell culture media, maintaining concentration of sodium butyrate or other HDAC inhibitor.

PIP2 Sensors - in the Literature

1. L. Liu, et al. **Gαq sensitizes TRPM8 to inhibition by PI(4,5)P2 depletion upon receptor activation.** J.Neurosci. May 2019.
2. Alexey N. Pronin, Qiang Wang and Vladlen Z. Slepak **Teaching an Old Drug New Tricks: Agonism, Antagonism, and Biased Signaling of Pilocarpine through M3 Muscarinic Acetylcholine Receptor.** Molecular Pharmacology Nov. 2018
3. Q. Wang, et al. **Regulator of G protein signaling Gβ5-R7 is a crucial activator of muscarinic M3 receptor-stimulated insulin secretion.** FASEB J. Jul. 7,2017.
4. Y. Ding, et al. **Ratiometric biosensors based on dimerization dependent fluorescent protein exchange.** Nature Methods, 2015.
5. P. Tewson, et al. **A multiplexed fluorescent assay for independent second-messenger systems: decoding GPCR activation in living cells.** J. Biomolecular Screening 18, 2013.

References

1. Graham FL, Smiley J, Russell WC, Nairn R: **Characteristics of a human cell line transformed by DNA from human adenovirus type 5**. J Gen Virol 1977, 36(1):59-74.
2. Dulbecco R and Vogt M: **Plaque formation and isolation of pure lines with poliomyelitis viruses**. The Journal of experimental medicine 1954.
3. Kost T, Condreay J, Ames R, Rees S, Romanos M: **Implementation of BacMam virus gene delivery technology in a drug discovery setting**. Drug Discovery Today 2007, 12(9-10):396-403
4. Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC: **Green fluorescent protein as a marker for gene expression.** Science 1994.
5. Tewson, et al. **Simultaneous Detection of Ca^{2+} and Diacylglycerol Signaling in Living Cells.** PLoS One, 2012.

Related Products			
Product	**Sensor Description**	**Promoter**	**Recommended Use**
#U0300R	Red Up DAG	CMV	Fluorescence imaging and plate reader assay (Z' > 0.5)
#D0300R	Red Down DAG	CMV	Fluorescence imaging and plate reader assay (Z' > 0.5)
#U0600R	Red GECO Calcium	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.

certified WBENC WOMEN'S BUSINESS ENTERPRISE

WOMEN OWNED™