Montana Molecular
Fluorescent Biosensors for Live Cell Discovery

Red Fluorescent PIP_2 Assay

Overview

The red PIP_2 sensor is based on a dimerization-dependent fluorescent protein. This protocol is optimized for imaging rapidly dividing, immortalized cell lines on a 96-well plate and has been validated in live HEK293, CHO and NIH 3T3 cells. For imaging live iPSC-derived or primary cultured cells, see Suggestions for 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
D0400R	Red PIP_2	CMV	Fluorescence imaging

Materials in the Kit

- PIP_2 sensor BacMam ~ 1 x 10^{10} VG/mL in TNM-FH Insect Culture Medium (Allele Biotech product #ABP-MED-10001).

  Red fluorescent sensors that decrease in fluorescence intensity when PIP_2 levels decrease. VG/mL is the titer determined by qPCR, and is the average number of viral genes per mL of the BacMam stock.

- Sodium Butyrate (Sigma Aldrich product # B5887) 500 mM in H_2O.

  Sodium Butyrate is added to the culture to maintain BacMam expression. Other histone deacetylase (HDAC) inhibitors, such as Trichostatin A (TSA) may work too.

- HM1 Muscarinic Receptor BacMam with nuclear green label in TNM-FH Insect Culture Medium (Allele Biotech product #ABP-MED-10001).

  A Gq-coupled receptor in BacMam provided as a positive control for the purpose of assay optimization. Contains a separate green fluorescent protein that is targeted to the nucleus.

- Carbachol 25 mM in H_2O

  Carbachol can be used to stimulate Gq signaling through the positive control, the HM1 Muscarinic acetylcholine Receptor.

Storage

BacMam stocks should be stored at 4°C and protected from light. Avoid freeze/thaw cycles.

Additional Materials Not Supplied

- Greiner CellCoat (#655946) is our preferred 96-well plate available from VWR.
- Dulbecco's Phosphate Buffered Saline (DPBS) available from VWR [Dulbecco, R. and Vogt, M. 1957].

BioSafety Considerations

BacMam is the modified baculovirus, Autographa californica, AcMNPV. The baculovirus contained in this kit is pseudotyped to infect mammalian cells. In mammalian cells, the baculovirus genome is silent and it cannot replicate to produce new virus in mammalian cells. While it should be handled carefully, in a sterile environment, it is classified as a BSL-1 reagent.

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

Warranty

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

About these Assays

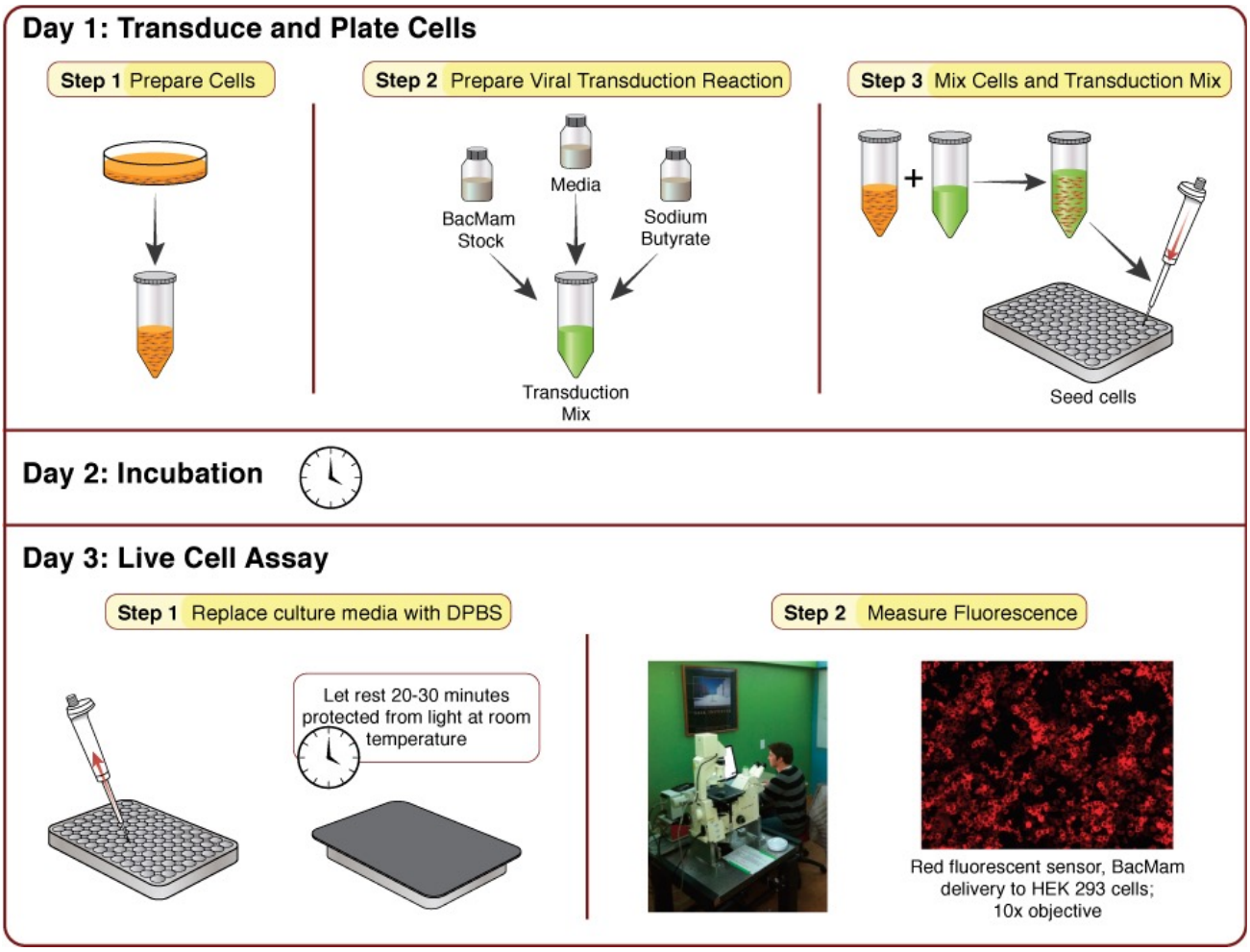
PIP_2, also known as Phosphatidylinositol 4,5-bisphosphate or PtdIns(4,5)P2, is a substrate for several signaling molecules. It is a precursor for the DAG/IP3 pathway, but also plays a key role in phospholipid signaling and is involved in the regulation of ion channels and transporters. The fluorescent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

Related Products

Product	Sensor Description	Promoter	Recommended Use
U0300G	Green Upward DAG	CMV	Fluorescence imaging and Plate reader assay (Z' > 0.8)
D0300G	Green Downward DAG	CMV	Fluorescence imaging and Plate reader assay (Z' > 0.8)

Protocol for Use

This protocol is optimized for rapidly dividing, immortalized cell lines. However, the protocol can be adjusted for transducing non-dividing adherent cells such as neurons, islets, cardiomyocytes, and iPSC-derived lines. We recommend that you take the time to optimize the assay for your particular cell type. See our Suggestions for Adherent Cells section 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.

* 420,000-440,000 cells/mL works well for HEK293 cells.

Example:

96 wells (1 plate)

100 µL cell suspension (420,000 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 20-25 µL of the BacMam stock with 0.6 µL of the 500 mM stock solution of sodium butyrate**, 5 µL of receptor control, and enough complete culture media 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. 1 mM sodium butyrate may improve cell health, but also reduce fluorescence.

Example:

96 wells needed (1 plate). The number of wells desired, in bold, corresponds to the number in Step 1 above.

Single Well	*Master Mix*
20 µL Sensor	x **110** = 2200 µL
5 µl HM1 Receptor Control	x **110** = 550 µL
0.6 µL 500 mM Sodium Butyrate	x **110** = 66 µL
24.4µL Complete Media	x **110** = 2684 µL
50 µL Total Volume	x 110 = **5500 µL** transduction mix (96 wells)

Step 3) Mix Cells and Transduction Mix from above.

- Mix Tube A and Tube B (100 µL tube A + 50 µL tube B). Mix gently and 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 for ≈ 44-48 hrs under normal cell growth conditions, protected from light.

Example:

96 wells needed (1 plate)

Single Well	*Master Mix*
100 µL cell suspension	x 110 = 11000 µL
50 µL transduction reaction	x 110 = 5500 µL
150 µL total volume per well	x110 = 16,500 µL total reaction volume

DAY 2 INCUBATION

- (Optional) A partial media exchange during this incubation period may improve cell health. With approximately 150 µL total volume in the well, remove 75 µL and add 75 µL of fresh culture media, making sure to maintain the concentration of sodium butyrate.

DAY 3 FLUORESCENCE IMAGING

- Cells are now ready for assay. Prior to imaging, replace culture media with DPBS. Wash gently so as not to dislodge cells. Cover the cells and allow them to rest at room temperature in DPBS for 20-30 minutes before measuring fluorescence. Experiments are performed at 25°C using standard RFP excitation and emission wavelengths.
- Add 50µL of 200µM carbachol (50 µM final concentration per well) to activate the DAG/IP_3 pathway in a set of control wells. A decrease in the fluorescence intensity will be observed after addition of the carbachol when PIP_2 is hydrolyzed to produce IP_3 within the cell.

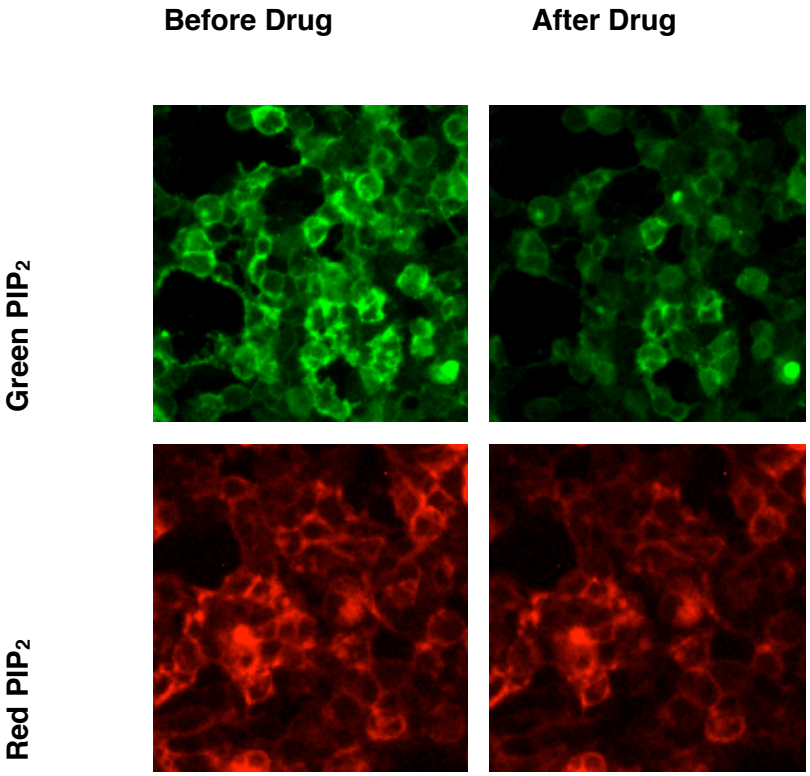
Suggestions for Adherent Cells

The protocol above is optimized for rapidly dividing immortalized cells, however, this product is compatible with screening primary cultures and iPSC-derived lines, where the cells are plated before transduction. Specific protocols will vary by cell type, so it is important to take the time to titrate the BacMam stock for best results. For expression in rare cell types, or specific cells in mixed cultures, Cre-dependent and specific promoter systems are available for many of our sensors.

- Prepare a 500 mM stock solution of sodium butyrate in sterile water.
- For each transduction reaction (e.g. one well in a 96-well plate, 100 µL per well), prepare the transduction solution by mixing 20-25 µL of the BacMam stock with 5 µL of receptor control, 0.6 µL of the 500 mM stock solution of sodium butyrate, and enough DPBS 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.
  - Prepare a dilution series of transduction reactions by varying the amount of BacMam ranging from 10 µL to 80 µL and adjusting the amount of DPBS accordingly.
- Add the transduction reaction directly to the plated cells (no aspiration of cell medium necessary). Gently rock the plate 4-5 times in each direction to mix throughout the well. Incubate the cells under normal growth conditions (5% CO_2 and 37°C), protected from light, for 4-8 hours (6 hours is optimal).
- Aspirate transduction solution and add 100 µL complete growth medium with sodium butyrate at a concentration of 1-2 mM. Return cells to normal growth conditions for approximately 44-48 hrs.

Fluorescence Detection

This sensor is constructed using a red fluorescent protein. Use 572/20 nm excitation and 630/30 nm emission filters to collect the red fluorescence from the red PIP2 sensor. These filter properties are similar to many of the FITC filter sets commonly available on most microscopes.



Timing

The PIP_2 assay measures PIP_2 in living cells, in real time. Be sure to capture fluorescence during the peak response as shown in Figure 2, which provides an example experiment using the Green PIP_2 sensor.

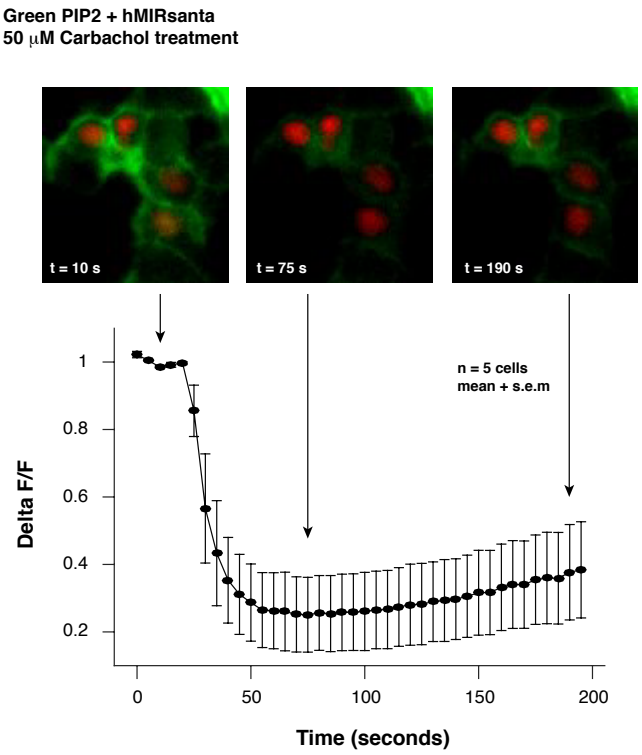


Figure 2. Green fluorescent PIP_2 sensor in HEK 293 cells, co-transduced with human muscarinic acetylcholine control receptor M1, and a red nuclear label. Fluorescence was captured from cells before the addition of 50 μM carbachol, and sampled at regular intervals. The maximal response is reached at ~60 seconds after the addition of the drug, and the response begins its return to baseline ~100 seconds after drug is added.

Assay Performance Considerations

How we measure the infectivity of the viral stock.

Typically, viruses are quantified in terms of plaque forming units (PFU). In the case of BacMam, this would be a measurement of the viruses that are capable of transducing an insect cell, the natural host. Since mammalian cell expression is the goal for this assay, we quantify infectivity by measuring (VG) per milliliter (mL) of the BacMam stock. We use primers specific to the VSVG gene present in the BacMam genome. Viral samples are prepared to release viral genomic DNA, then multiple dilutions of the preparation are run in qPCR against a standard curve to generate an average titer for each viral stock. Check the label on the tube to find VG/mL for your PIP_2 sensor stock.

Expression levels of the sensor.

To optimize the assay in your particular cell type, it is important to optimize the amount of BacMam virus used in the transduction. Too little virus will produce variable results, particularly if the sensor expression levels are low and difficult to detect on your instrument.

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 receptors can artificially change the resting levels of second messengers.

Trouble Shooting

Here are a few simple steps that may help you trouble shoot if needed.

Are your cells fluorescent?

Different types of promoters drive expression in mammalian cells. The CMV promoter in our BacMam vectors is an effective promoter in many cell lines, but not all. If your cells are not

fluorescent, check that the CMV promoter works in your cell line. 40-48 hours after transduction with the downward PIP_2 sensor, you should see red fluorescent cells in a typical epifluorescence microscope, or the transduced wells in a 96 well plate should be significantly more fluorescent than untransduced cells in wells on the same plate.

HDAC inhibitors are important to maintain expression of the sensors. While BacMam transduction without the HDAC inhibitor will initially generate low levels of sensor expression, it is important to follow protocols and include sodium butyrate or another HDAC inhibitor such as VPA, or trichostatin A (TSA) to generate optimal levels of sensor expression.

The type of cell culture media used in your experiment can affect the transduction efficiency of BacMam. Our assays have been validated in EMEM, DMEM, and F12K culture media.

Is the positive control working?

If the cells are expressing the sensor, and fluorescence is detectable on your instrument, then check the sensor using the positive control receptor included in this kit. Adding 5 µL of the hM1 receptor stock to set of control wells will ensure that a Gq coupled receptor is present in all of the cells. You can double check to make sure the M1 receptor is expressed by examining the cells in a fluorescent microscope with filters for green fluorescence. You should see green nuclear fluorescence that marks the cells that express the exogenous M1 receptor as shown in Figure 2.

Addition of carbachol will cause a change in fluorescence, when the receptor control is present in the cells, as shown in Figure 2. This positive control can be used to optimize three aspects of your assay. First, a serial dilution series of the sensor with a constant amount of PIP_2 sensor can be used to determine the optimal sensor expression for your instrument and cell type. Second, it is important to titrate the amount of BacMam sufficient to transduce all of the cells in the well. Third, it is important to determine whether your instrument can measure the peak response in the appropriate time frame.

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 hope to hear from you. Your feedback is extremely valuable. Please send an email to info@montanamolecular.com, and we'll respond as quickly as we can.

References

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.

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4. 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.

5. Tewson P, Quinn AM, Hughes TE:. A Multiplexed Fluorescent Assay for Independent Second-Messenger Systems: Decoding GPCR Activation in Living Cells. Journal of Biomolecular Screening 2013 vol. 18 no. 7 797-806.