Montana Molecular
Fluorescent Biosensors for Live Cell Discovery

R-GECO Red Fluorescent Ca^{2+} Assay

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Overview

The red fluorescent Ca^{2+} sensor, R-GECO, increases in fluorescence intensity in response to increases in intracellular calcium. R-GECO was developed in Robert Cambell's lab at the University of Alberta [1]. Changes in fluorescence in living cells can be detected with epifluorescence microscopy or on automated fluorescence plate readers. There is no need to fix or lyse the cells before measuring fluorescence.

The vector carrying these sensors is a modified BSL-1 baculovirus (BacMam) [2]. In mammalian cells, only the fluorescent sensor is expressed. BacMam has high transduction efficiency in most primary cultures and iPSC-derived cells, and expression levels in the cell can be adjusted by titrating the virus.

The following protocol is optimized for screening in HEK 293 cells [3] in a 96-well plate. If you measure Ca^{2+} in cells other than HEK 293, or on a different type of plate or dish, then the following protocol can be used as a starting point to optimize for your cells or experimental set up. For cells other than HEK 293 cells, we recommend that you take the time to do a dilution series, to optimize expression in your particular cells. Publications using BacMam expression in many different cell types is available here.

Relevant Products

Number: #U0600R

Materials Included

- R-GECO sensor in BacMam under the control of a CMV promoter.

  Red fluorescent Ca^{2+} sensor. $3.1x10^{10}$ VG/mL in TNM-FH Insect Culture Medium (Allele Biotech product #ABP-MED-10001) VG/mL (viral genes per mL) is measured in mammalian cells, as distinct from plaque forming units (PFU), measured in insect cells.

- Sodium Butyrate (Sigma Aldrich product number B5887) 500 mM.

  Sodium Butyrate is added to the culture to maintain BacMam expression. Other HDAC inhibitors may work as well.

- hM1 muscarinic acetylcholine receptor BacMam in TNM-FH Insect Culture Medium (Allele Biotech product #ABP-MED-10001).

  A Gq-coupled GPCR in a BacMam vector, provided as a positive control for assay optimization. Separately expresses a green fluorescent protein that is targeted to the nucleus.

- Carbachol 25 mM in H_2O

  Carbachol is used to stimulate Gq signaling through the positive control receptor.

Storage

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

Additional Materials Not Supplied

- Greiner CellCoat (#655946) is a good 96 well plate for this assay.
- Dulbecco's Phosphate Buffered Saline [4].

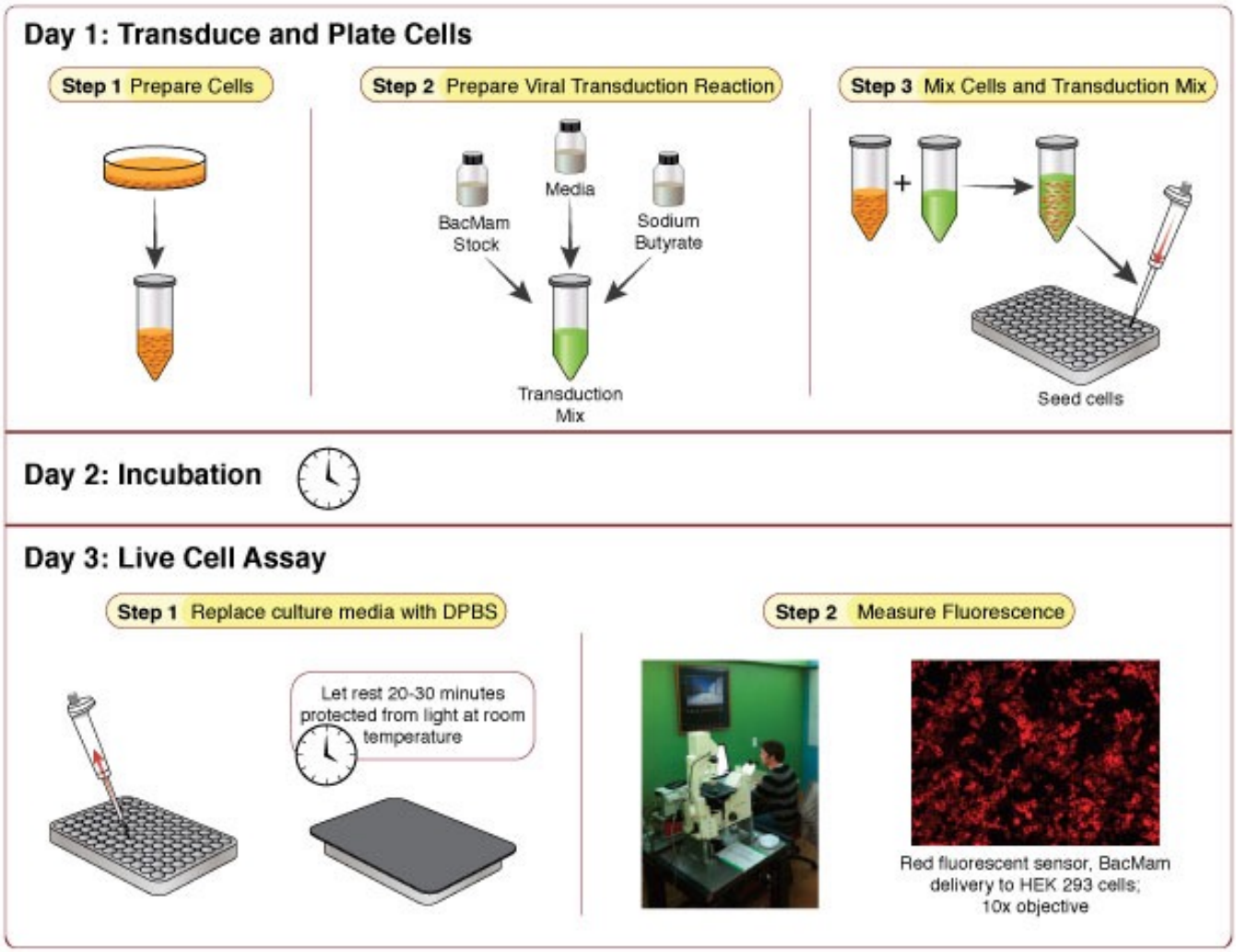
BioSafety Considerations

BacMam is the modified baculovirus, Autographa californica, AcMNPV. The baculovirus 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.

Protocol for Use



The following protocol is optimized for rapidly dividing immortalized cell lines. However, this assay also works well with non-dividing cells, including neurons, pancreatic islets, cardiomyocytes and iPSC-derived cells. We recommend that you take the time to optimize the assay for your particular cell type. See our Suggestions for Adherent Cells following this protocol.

DAY 1 TRANSDUCE AND PLATE CELLS

Step 1) Prepare cells (Tube A)

- Detach cells from flask using normal 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 while preparing the viral transduction solution.

* 450,000 cells/mL works well for HEK293 cells.

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 (i.e. one well in a 96-well plate), prepare the transduction solution by mixing 15-25 µL of BacMam stock with 0.6 µl of the 500 mM stock solution of sodium butyrate*. Add 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.

** Control wells with a mixture of the sensor BacMam and the receptor control BacMam is recommended.

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 ≈ 36-48 hrs under normal cell growth conditions, protected from light. 24 hr incubation periods may be used, but sensor fluorescence will be much lower.

** 1 mM sodium butyrate may improve cell health but also reduce fluorescence.

DAY 2 INCUBATION

Red fluorescent sensors take longer than green fluorescent sensors to mature.

DAY 3 FLUORESCENCE MEASUREMENT

- 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 RFP excitation and emission wavelengths.
- 25 mM carbachol is included in the kit for positive control. Activative control wells expressing the M1 receptor control with 30 uM carbachol.

Suggestions for Assays in Adherent Cells

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 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.
- Sensor expression and cell health can be controlled by titrating the virus, so it is worth taking the time to set up wells with titrated amounts of the sensor to optimize for your particular cell type.
- Prepare a dilution series of transduction solutions by varying the amount of BacMam. For example, amounts ranging from 5 µL to 60 µL , adjusting the amount of DPBS accordingly.
- For each transduction (i.e. one well in a 96-well plate, containing 100µL culture media per well), prepare a transduction solution by mixing BacMam stock with 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.
- 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 36-48 hrs before measuring fluorescence as described above. If cells will not tolerate a full media exchange, partial media exchanges can be done.

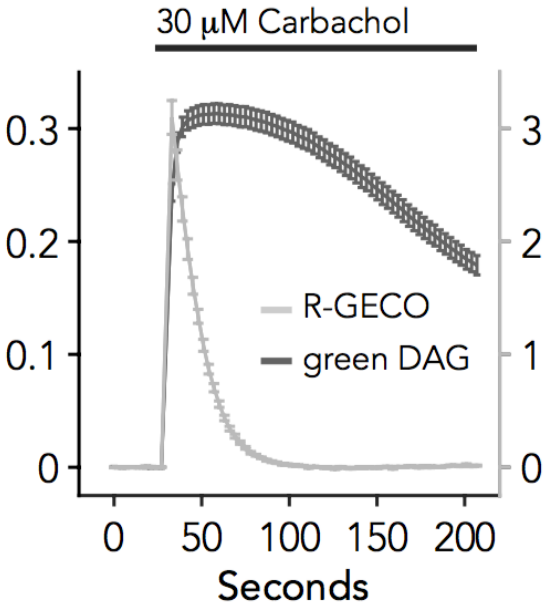
Detecting Fluorescence

Our assays are compatible with automated fluorescent plate readers. Our customers have reported good results on:

- Hamamatsu FDSS
- Molecular Devices FLIPR
- Molecular Devices Flexstation
- Perkin Elmer Enspire

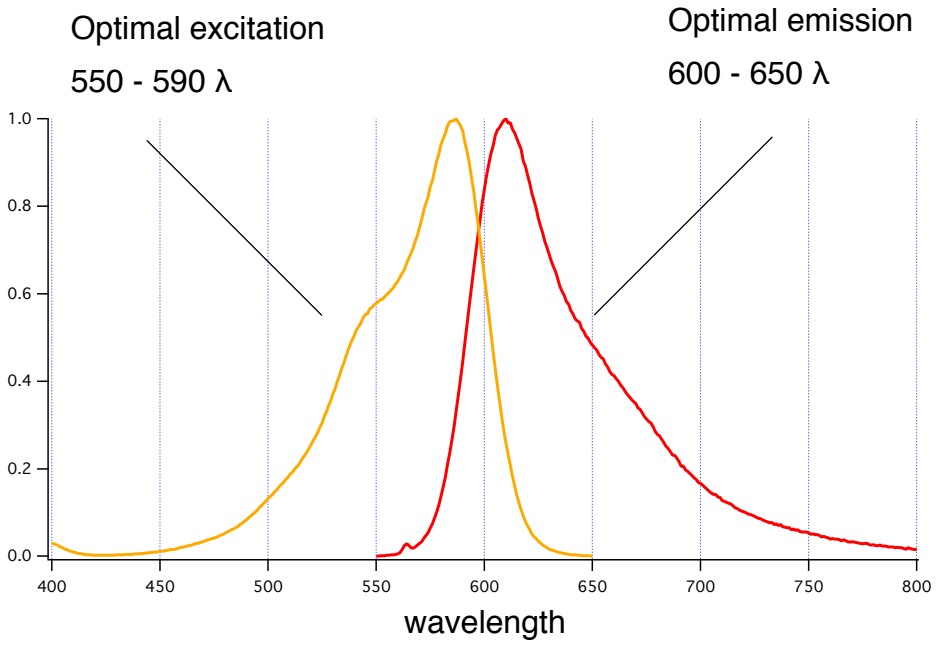
We have validated on:

- Biotek Synergy MX
- Biotek Cytation
- BMG CLARIOstar
- Epifluorescence microscopes



R-GECO fluorescent sensor has been validated on a variety of epifluorescence microscopes with lenses ranging from 20 X, 0.9 N.A. to 63X, 1.4 N.A. The data to the left was acquired on a BMG CLARIOStar fluorescence plate reader. Green Upward DAG sensor was co-transduced with R-GECO and measured in the same cells. Change in green fluorescence is indicated on these left axis, Change in red fluorescence on the right axis.

This sensor is constructed using a red fluorescent protein. The optimal excitation wavelength is 590 nm, but the absorption band of this protein is quite broad, so broad bandpass filters that pass 550 to 590 nm light can be used quite effectively. On the emission side, the red light spans 600 to 700 nm, so broad band pass emission filters can also help to collect much of the emission.



Timing

This is a live cell assay that detects in real time, the Ca^{2+} level in the living cell. Changes in Ca^{2+} can occur quite rapidly, so the application of drug and resulting changes should be captured as quickly as possible. The best possible experimental setup involves capturing the fluorescence from a well of cells first, before the addition of any compound, and then again a second or two after the compound addition. The maximal response is reached ~ 3 seconds after the addition of the agonist and in HEK293 cells, returns to baseline after 10-12 seconds [5].

Factors Influencing Assay Performance

Viral Titer

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 cAMP sensor stock.

Expression levels of the sensor.

To optimize the assay for your particular cell type, it is important to titrate the amount of 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.

Level of 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 often 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

Are the cells fluorescent?

This virus drives expression in mammalian cells because a CMV promoter has been positioned in front of the sensor coding region. CMV is an effective promoter in many cell lines, but not all. When you add the virus, are you seeing bright fluorescent cells in a microscope? Thirty hours after transduction with the R-GECO virus, you should see red fluorescent cells in a typical fluorescent microscope.

HDAC inhibitors are important to expression of the sensors. While BacMam alone will initially generate low levels of sensor expression, it is important to include sodium butyrate (provided in the kit) to generate optimal levels of sensor expression and maintain this level of expression [5]. Other HDAC inhibitors may be used too.

Contact us

If you have questions or feedback, please let us know. We strive to respond to emails sent to info@montanamolecular.com within 24 hours and usually respond much sooner.

References

1. Zhao Y, Araki S, Wu J, et al. An Expanded Palette of Genetically Encoded Ca2+ Indicators. Science 2011; 333(6051):1888-1891. doi:10.1126

2. Boyce, FM; Bucher, NL (1996). "Baculovirus-mediated gene transfer into mammalian cells". Proceedings of the National Academy of Sciences of the United States of America 93 (6): 2348–52. doi:10.1073/pnas.93.6.2348. PMC 39799. PMID 8637876.

3. 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.

4. Dulbecco R and Vogt M: Plaque formation and isolation of pure lines with poliomyelitis viruses. The Journal of experimental medicine 1954.

5. Tewson, Paul et al. “Simultaneous Detection of Ca2+ and Diacylglycerol Signaling in Living Cells.” *PLoS ONE* 7.8 (2012): e42791.

6. 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.