Montana Molecular
Fluorescent Biosensors for Live Cell Discovery

Red Fluorescent Diacylglycerol (DAG) Assay

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Overview

The red fluorescent DAG sensors are used for measuring diacyl glycerol changes in live mammalian cells. This protocol is optimized for imaging live cells on a 96-well plate and has been validated in HEK293, CHO and NIH 3T3 cells. These sensors are suitable for live-cell imaging and for screening on automated fluorescence plate readers. This protocol can be adjusted and optimized for many different types of cells, including primary cultures, iPSC-derived cells, and pancreatic islet cells, and is easily scaled for 384 well format.

The vector carrying these sensors is a modified baculovirus. In mammalian cells, BacMam expresses only the fluorescent sensor and is a BSL-1 reagent. If you want to measure DAG in cells other than HEK 293, then the IU/mL needed to transduce a well of HEK 293 cells can be used as a starting point for assay optimization in other cell types. We recommend that you take the time to do a dilution series in your cells, to optimize in your particular cells and fluorescence detection system.

Relevant Products

Product	Description	Promoter	Recommended Use
D0300R	Red Downward DAG	CMV	Fluorescence imaging and plate reader assay (Z' > 0.7)
U0300R	Red Upward DAG	CMV	Fluorescence imaging and Plate reader assay (Z' > 0.5)

Materials included

- DAG sensor in BacMam under the control of a CMV promoter in TNM-FH Insect Culture Medium (Allele Biotech product #ABP-MED-10001).

  Red fluorescent sensor that changes in response to increases in DAG. Downward sensors decrease in fluorescence following activation of a Gq-coupled GPCR. Upward sensors increase following activation of a Gq-coupled GPCR. IU/mL are units that produce expression in mammalian cells, as distinct from plaque forming units (PFU), that for baculovirus, would be 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 our preferred plate for this assay.
- Dulbecco's Phosphate Buffered Saline [2].
- Complete culture media specific to your cells. (Please see Assay Performance Section)

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.

About these Assays

Many cell surface receptors couple to the heterotrimeric G protein Gq, which in turn activates phospholipase C (PLC). PLC produces two different second messengers, diacyl glycerol (DAG) and inositol triphosphate ($IP_{3)}$, causes an increase in Ca^{2+}. This coordinated increase of both DAG and Ca^{2+} triggers the activation of conventional protein kinase C (cPKCs) to phosphorylate many different protein targets. Live cell assays that measure increases in Ca^{2+} have been used for many years to detect this pathway, but a rise in Ca^{2+} is an ambiguous signal: there are other signaling pathways that cause increases in intracellular Ca^{2+}. This assay for DAG can be used to unambiguously resolve PLC pathway activation in living cells, and it can be combined with a green fluorescent sensor for Ca^{2+} or PIP_2 to better understand the kinetics of these coordinated, parallel signaling processes [Tewson P, et.al. 2012].

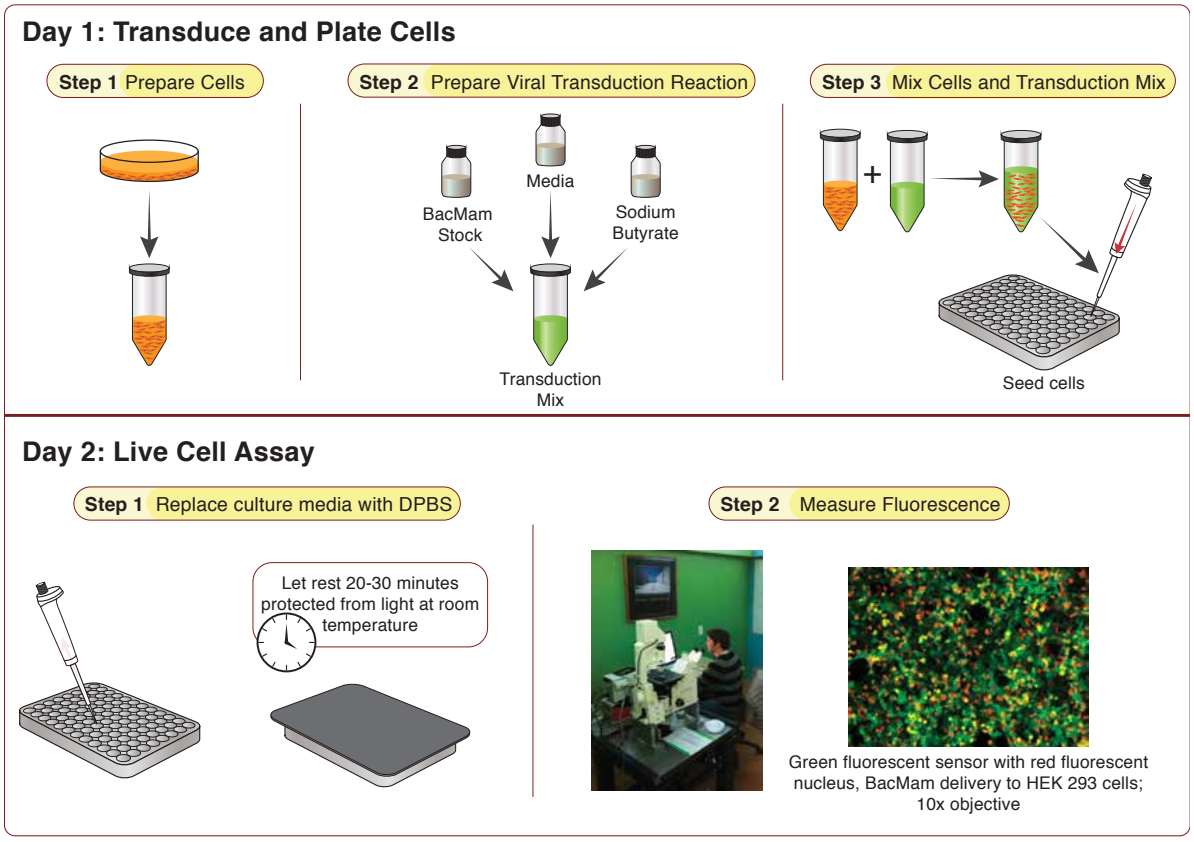
Depending on the kit, fluorescence either increases or decreases in response to activation. The Downward DAG sensor (#D0300R) decreases in fluorescence. One can imagine applications where this sensor might have advantages over a sensor that increases in fluorescence, for example, if background fluorescence is significant, the decreasing fluorescence from the sensor can be more easily separated from background fluorescence.

Related Products

Product	Description	Promoter	Recommended Use
D0400G	Green PIP2 sensor	CMV	Fluorescence imaging
U0200G	Green cADDis cAMP	CMV	Fluorescence imaging and Plate reader assay (Z' > 0.85)

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 uL 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.

* 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 reaction (i.e. one well in a 96-well plate), prepare the transduction solution by mixing 25 uL of the BacMam stock with 0.6 ul of the 500 mM stock solution of sodium butyrate* and 24.4 uL of the complete culture media for your cells, for a total volume of 50 uL. Mix gently.

* Concentration of sodium butyrate should be 6mM in this step. Following Step 3, final concentration of sodium butyrate will be 2mM.

Step 3. Mix Cells and Transduction Mix from above.

- Mix Tube A and Tube B (100 uL tube A + 50 uL tube B). Mix gently and then seed 150 uL 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 ≈ 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 FLUORESCENCE MEASUREMENT

- 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 RFP excitation and emission wavelengths.
- 50 uM carbachol (included in the kit) can be used to activate control wells transduced with M1 receptor control.

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.

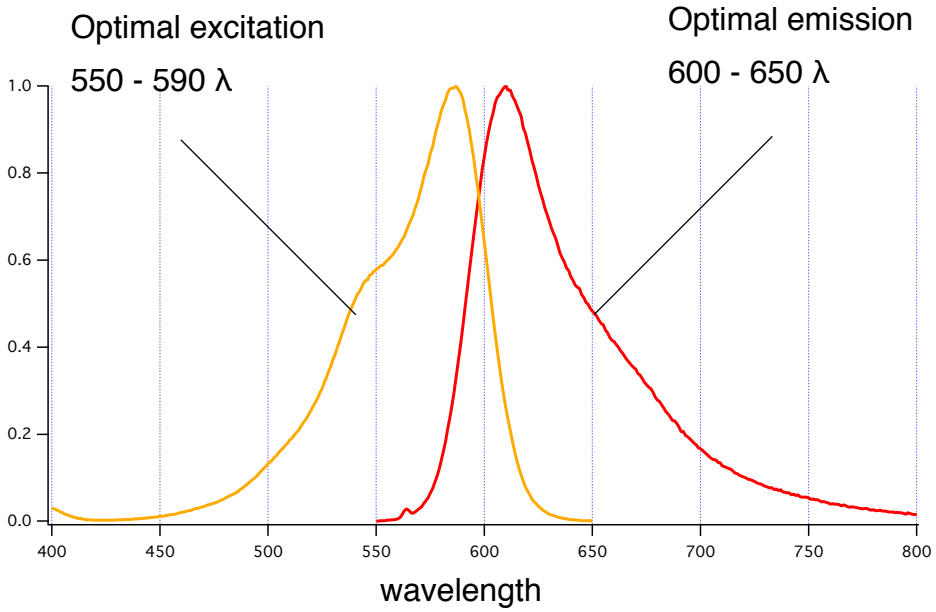
- Prepare a 500 mM stock solution of sodium butyrate in sterile water.
- For each transduction reaction (i.e. one well in a 96-well plate, containing 100uL culture media per well), prepare a transduction solution by mixing 25 uL of the BacMam stock with 24.4 uL of DPBS and 0.6 uL of the 500 mM stock solution of sodium butyrate for a total volume of 50 uL. 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. For example, a range of 10 uL to 80 uL , 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 uL complete growth medium with sodium butyrate at a concentration of 1-2 mM. Return cells to normal growth conditions for approximately 38-42 hrs before measuring fluorescence as described above. If cells will not tolerate a full media exchange, partial media exchanges can be done.

Detecting Fluorescence

Fluorescence Properties

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, and unlike many assays that measure accumulation of an analyte over time, this sensor detects, in real time, the DAG level in the living cell. Changes in DAG 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 so after the compound addition. Sampling the fluorescence at intervals of 2 to 5 seconds provides a good measurement of the response. In HEK293 cells, the

maximal response using the control receptor is reached at ~ 60 seconds after the addition of the agonist.

Validated detection systems.

This red 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. Before deploying in automated plate readers, we recommend that you optimize the assay on a microscope. The following sections provide more detail on automated measurement.

The simplest format: one drug, one sensor, one time point.

We have validated this assay on our BioTek Synergy MX fluorescence plate reader using the protocol described on the previous pages. Control receptor agonists are added by hand, and then the plate is inserted into the fluorescence plate reader to record the fluorescence from each well sequentially. This can all be done by hand because these sensors respond over several minutes to agonists in the well. While this protocol is simple, the drawback is that it does not capture the kinetics of the response, simply the sensor fluorescence before and after the addition of drug.

Two channel format: one drug, two sensors, one time point.

By combining green and red sensors, one can simultaneously record two limbs of second messenger signaling. In this case, standard fluorescence plate readers can be used, but they need to be fitted with the optics necessary to collect two different channels of fluorescence, which will involve specific filter sets and either two detectors or very fast filter switching. Most two channel plate readers can scan quickly, but the kinetics of the responses are lost. This can be quite limiting if the kinetics of the two second messenger systems are different. For example, the Gq stimulated release of Ca^{2+} stores can be quite rapid, while the DAG signaling occurs over a longer time frame. If the timing is off with a single point measurement, the DAG signaling might be detected, but without catching the Ca^{2+} transient.

Capturing the kinetics, one well at a time.

High content imaging systems offer an opportunity for capturing kinetic responses. In the models that have onboard liquid handling, fluorescence can be recorded before and after the addition of vehicle, and then continuously recorded after addition of the drug. This provides kinetic data, but each well in the plate is recorded over relatively long time frames. Our assays have been validated on the BioTek Cytation imaging system. However, the red fluorescent assays may not be suitable for use on some automated confocal-based systems because only the signal on the focal plane is collected.

Factors Influencing Assay Performance

Virus Titer

Typically, viruses are quantified in terms of plaque forming units (PFU). In the case of BacMam, PFU would be a measurement of the viruses that are capable of transducing an insect cell, the natural host. Since mammalian cell expression is the application of interest for these sensors, we quantify infectivity by measuring the units (IU/mL) that produce expression in mammalian cells. While this correlates with PFU, IU/mL is a direct measurement of transduction in mammalian cells. We make this measurement by

transducing HEK 293 cells with serial dilutions of the virus and then counting the fluorescent cells. Check the label on the BacMam stock to find IU/mL.

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. Too much virus, on the other hand, can reduce the magnitude of the sensor signal (i.e. the change in fluorescence that results from sensor activation).

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.

Culture Media Considerations

This assay has been validated with EMEM, DMEM, and F-12K complete growth media. Other types of media may affect results.

Trouble Shooting

If you encounter problems, there are several steps that you can consider when troubleshooting.

Are the 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. Do you know that the CMV promoter works in your cell line? Twenty-four to forty-eight hours after transduction, 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 expression of the sensors. While BacMam transduction alone will initially generate low levels of sensor expression, sodium butyrate or another HDAC inhibitor such as VPA or trichostatin A (TSA) will generate optimal levels of sensor expression and maintain this level of expression [Kost, T. et. al. 2007]. If cells look unhealthy, lower concentrations of HDAC inhibitor may be used. This may improve cell health, but it will also reduce sensor expression.

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.

Is the positive control working?

If the cells are expressing the sensor, and fluorescence is detectable on your instrument, then a good positive control is included in this kit. Adding 5 uL of the M1 muscarinic acetylcholine receptor virus to a set of positive control wells will ensure that a Gq-coupled receptor is present. You can double check to make sure the receptor is expressed by examining cells in a fluorescent microscope with filters for green fluorescence. You should see the red sensor fluorescence throughout the cell, and green nuclear fluorescence that marks the cells that also express the receptor control.

Addition of carbachol will then generate a change in fluorescence. If it does not, then it is important to use this positive control to optimize three aspects of your assay. First, a serial dilution series of the sensor with a constant amount of receptor virus can be used to optimize for your cells, receptor, and instrumentation. Second, find the amount of virus sufficient to transduce all of the cells in the well. Third, it is important to determine what the kinetics of the response is and whether your assay and instrument are capable of making the measurements in that time frame.

Contact

After following the suggested protocol and trouble shooting steps, if you still have questions or feedback please let us know. We strive to respond to emails sent to info@montanamolecular.com within 24 hours.

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

3. Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC: Green fluorescent protein as a marker for gene expression. Science 1994.

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.