Montana Molecular
Fluorescent Biosensors for Live Cell Discovery

Green Cell Stress Assays

About these Assays

The cell stress sensor is a genetically-encoded fluorescent biosensor that produces very bright fluorescence when the cell endures endoplasmic reticulum (ER) stress or undergoes the unfolded protein response (UPR). A broad host of both chemical compounds and genetic mutations can induce ER stress. Furthermore, the UPR is one of the major stress pathways within the cell, which allows the cell stress sensor to detect a wide range of stress inducing stimuli, some whose primary target is not the ER. This sensor is based on the XBP1 mRNA which is spliced by the IRE1 protein during ER stress. Briefly, the model for this response involves ER stress activating the RNase activity of the IRE1 protein, which in turn splices the immature, cytosolic mRNA for XBP1, generating an active transcription factor that alters gene expression. This unique cytosolic splicing of XBP1 has been used to generate reporter systems in the past [Iwawaki et al. 2003], many of which were PCR-based splicing assays or reporters of XBP1 transcriptional activity [Rong et al. 2015]. The cell stress sensor is unique in that the assay does not rely on changes in gene expression or transcription activation. Rather, the splicing of the XBP1 intron results in translation of the bright fluorescent protein, mNeon Green. Increased translation of mNeon Green results in increased fluorescence output, indicating the occurrence of cellular stress.

Overview

The following protocol is optimized for measuring stress responses in rapidly dividing, immortalized cell lines on a 96-well plate, and has been validated in live HEK 293 cells [Graham FL, 1977] and cardiomyocytes. 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.

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	Stress Pathway Detected	Promoter	Recommended Use
U0900G	Green Upward Cell Stress Sensor	Endoplasmic Reticulum (ER) and ER Unfolded Protein Response	CMV	Fluorescence imaging and plate reader assay (Z' > 0.74)
U0901G	Nuclear localized Green Upward Cell Stress Sensor with constitutively expressed nuclear localized red fluorescent protein	Endoplasmic Reticulum (ER) and ER Unfolded Protein Response	CMV	Fluorescence imaging and plate reader assay

Materials in the Kit

- Cell stress sensor BacMam ≅ 1 x10^{10} VG/mL in TNM-FH Insect Culture Medium (Allele Biotech product #ABP-MED-10001).

  Green fluorescent sensors that increase in fluorescence intensity in response to ER and cellular stress. VG/mL is the number of viral genes per milliliter.

- 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.

- Thapsigargin (Caymen Chemical product # 10522) dissolved in DMSO, diluted to 100 µM in in H_2O.

    Thapsigargin is a SERCA pump inhibitor that induces high levels of ER stress. It is used as the positive control when conducting the cell stress assay.

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].
- Optional: FluoroBrite DMEM media (ThermoFisher Scientific product # A1896701) supplemented with 10% FBS and 4 mM GlutaMAX.

BioSafety Considerations

BacMam is a modified baculovirus, Autographa californica, AcMNPV. The virus 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 Biosafety Level 1 (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

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 following this protocol.

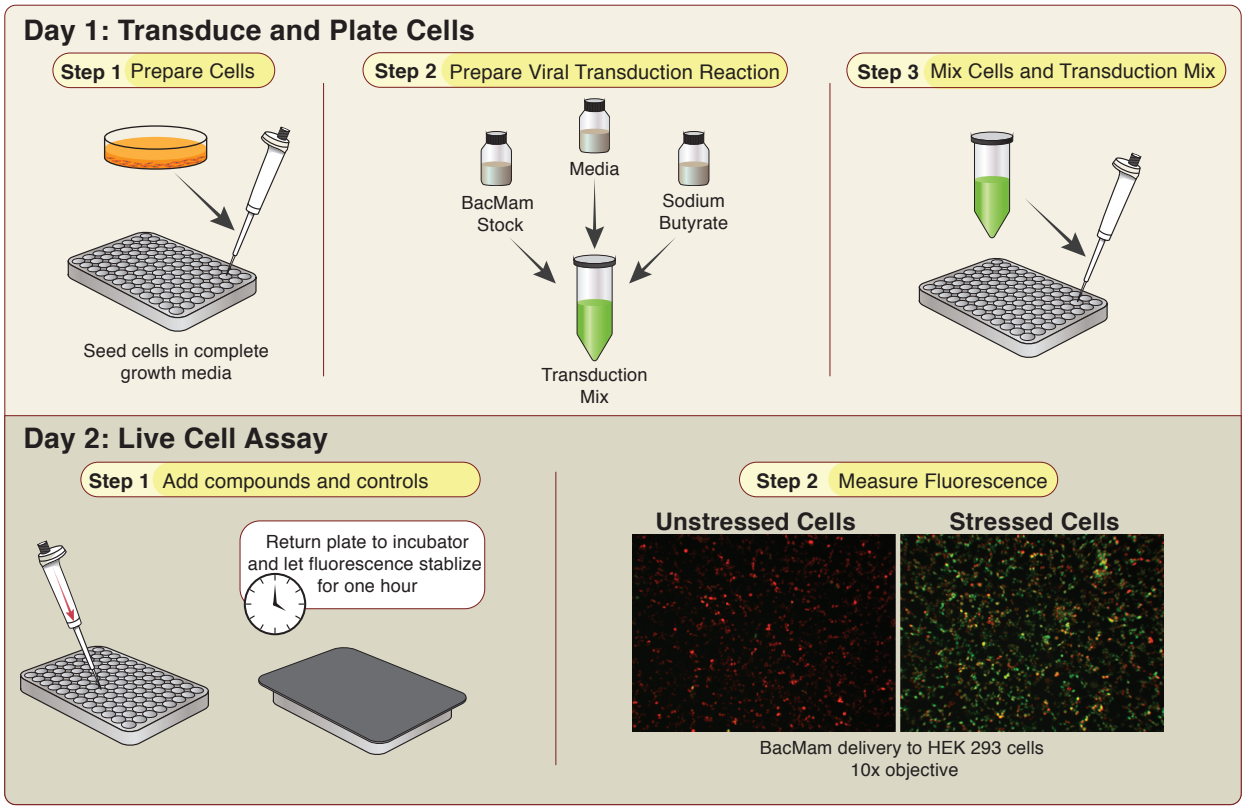
DAY 1 TRANSDUCE AND PLATE CELLS

Step 1) Prepare cells (Tube A)

- Detach cells from flask using standard trypsinization protocol. Resuspend cells in complete culture media or FluoroBrite media and determine cell count.
    - Note: The assay will work in either standard media or FluorBrite media, however as FluoroBrite media is formulated to reduce background fluorescence signals and signal to noise ratios are generally improved using FluoroBrite media. Alternatively, if only a single time point the media can be exchanged for DPBS before analysis.

- 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.

* 480,000 cells/mL works well for HEK293 cells.



Example:

For **96** wells (1 plate)

100 µL cell suspension (480,000 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 25 µL of the Sensor BacMam stock with 0.6 µL of the 500 mM stock solution of sodium butyrate*, and 24.4 µL of the complete culture media for your cells, for a total volume of 50 µL. Mix gently.

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

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*
25 µL Sensor	x **110** = 2750 µL
0.6 µL 500 mM Sodium Butyrate	x 110 = 550 µL
24.4 µL Complete Media	x **110** = 2200 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 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. This step is important to maintain and even distribution of cells throughout the well and minimize edge effects.
- Incubate ≈ 16-24 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 MEASURING FLUORESCENCE

- Add desired compounds to each well, return plate to incubator and allow the fluorescence to equilibrate for 1 hour.
- After 1 hour take an initial fluorescent reading or image. Experiments are performed using standard GFP excitation and emission wavelengths.
- Use positive controls. Add 50 µL of 4 µM thapsigargin diluted in buffer of choice from the 100 µM stock to a set of wells. This creates a 1 µM concentration of thapsigargin in 200 µL of media/buffer.
- Read or image the fluorescence intensity from the plate at time points after the addition of compounds. Cells treated with thapsigargin will begin to increase in fluorescence as soon as 3 hours after initial treatment and will reach a peak intensity 7 hours after treatment (See Figure 2).
- When monitoring the green fluorescence emitted by the sensor, either the change in fluorescence intensity over time or the absolute fluorescent intensity can be measured.
- Different compounds induce cellular stress at different rates. Thus, we suggest taking multiple fluorescent readings or images over a 24-48 hour period.
- If a single endpoint measurement is required the media can be exchanged for DPBS after the desired incubation time followed by either image or plate reader analysis.

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.

- 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 100 µL culture media per well), prepare a transduction solution by mixing 25 µL of the Sensor BacMam stock with 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.
- Prepare a dilution series of transduction reactions by varying the amount of BacMam. For example, a range of 10 µL to 80 µL , 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 at least 4 hours, overnight is preferable.
- Aspirate transduction solution and add 100 µL complete growth medium with sodium butyrate at a concentration of 3-6 mM. Return cells to normal growth conditions for approximately 18-22 hrs before measuring fluorescence as described above. If cells will not tolerate a full media exchange, partial media exchanges can be done.

Fluorescence Detection

Our assays are compatible with automated fluorescent plate readers and have been validated on the Biotek Synergy MX and Epifluorescence microscopes. Our customers have reported good results for other fluorescent biosensors from Montana Molecular on:

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

Fluorescence Properties

The stress sensor is constructed with the very bright, mNeon green fluorescent protein [6]. We recommend Chroma's Catalog set #49003 for optimal results.

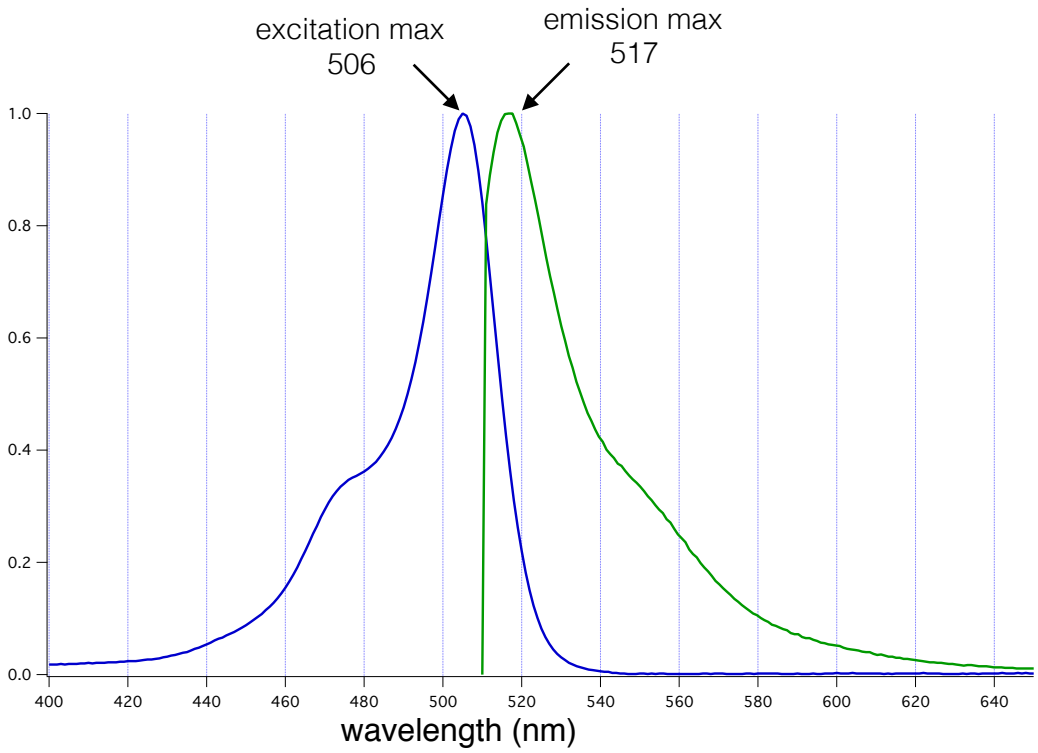


Figure 1. Absorption and emission properties of the mNeon green fluorescent protein plotted as a function of wavelength.

Timing

The cell stress assay is a live cell assay, allowing detection over short and long time intervals. For best results, be sure to analyze fluorescence between 0 and 1 hour after addition of stress inducing compounds and at multiple time points up to 48 hours after to capture changes cellular stress levels. In Figure 2, fluorescence was captured from cells at 1 hour after compound addition then sampled at regular intervals. The maximal response for the positive control, thapsigargin is reached at 7 hours after the addition of the drug.

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 viral genes (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.

Level of sensor expression

To optimize the assay in your particular cell type, it is important to optimize 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. In the case of HEK 293 cells, the baseline fluorescence goes up as you add more virus, and when a particular threshold is reached the absolute change in sensor fluorescence, as well as the Z' for the assay, becomes constant.

Trouble Shooting

The cell stress assays are robust and easy to use. There 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. Twenty four hours after transduction, you should see bright green 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 valproic acid 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.

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 addition of the thapsigargin positive control should result in increased fluorescence within 7 hours after addition. If only the green stress sensor is being used then green fluorescence should be seen throughout the cell. If the green stress sensor with the constitutively expressed red fluorescent protein is being used then green fluorescence should be observed throughout the cell and red nuclear fluorescence should be observed in all cells expressing the sensor. Importantly, prior to addition of test compounds or the positive control cells may express the red nuclear fluorescent in the absence of green fluorescence. Addition of the positive control will result in green fluorescence upon stressing the cells.

Addition of thapsigargin will result in an increase in green fluorescence, as shown in Figure 2. If it does not, then it is important to use this positive control to optimize three aspects of your

assay, such as viral transduction volume, HDAC inhibitor concentration, or compound concentrations. First, a serial dilution series of the sensor with a constant amount of thapsigargin positive control can be used to find the optimal sensor expression for your instrument and cell line. Second, it is important that you 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 instrument can measure in the appropriate time frame.

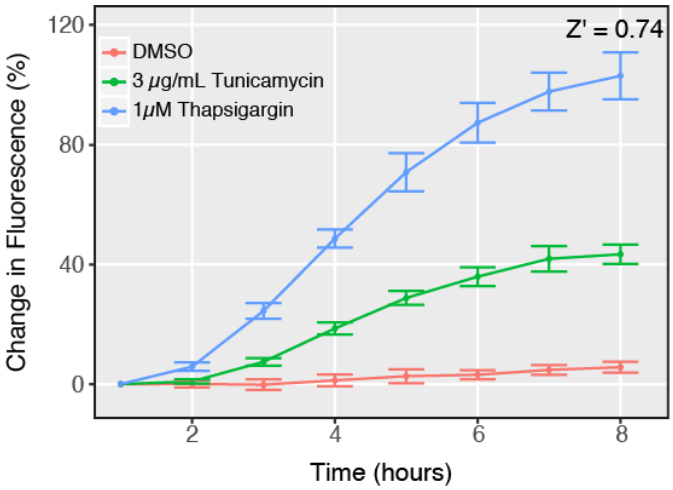


Figure 2. HEK 293 cells transduced with 25 µL of BacMam carrying green cell stress sensor activated with either thapsigargin or tunicamycin. The plot displays the percent change in fluorescence over time compared to 1 hour after addition of either drug or DMSO control. Data points are plotted as the mean ± standard deviation from 6 wells for each sample on a 96-well plate. The Z' for thapsigargin after 8 hours is 0.74.

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, and we'll respond as quickly as we can.

References

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