



## Green / Red Fluorescent Ratiometric Cell Stress Assay

May 10, 2022

### Product Info & Protocol

Patent Pending: PCT/US2018/041146

[WO2019014072A1]

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## 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).

This version of the assay is designed for two-color measurements using the green fluorescent sensor and a constitutively expressed red fluorescent protein. Red and green fluorescent signals are both localized to the nucleus. Red fluorescence indicates all cells expressing the sensor, and green fluorescence indicates cells undergoing the stress response.

A broad host of both chemical compounds and genetic mutations induce ER-mediated cell stress, making this biosensor a useful tool to study the effects of toxic compounds and stress-inducing mutations that are associated with disease. The sensor is based on splicing of the XBP1 RNA, mediated by the ER protein IRE1. This splicing removes an intron and results in the translation of the mNeonGreen fluorescent protein.

The following protocol is optimized for measuring cell stress responses on a 96-well plate of living cells. It has been validated in live HEK 293T cells as well as in iPSC-derived cardiomyocytes and neurons. Assay fluorescence can be detected on live-cell imaging systems, automated fluorescence plate readers or fluorescence microscopes. 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
#U0901G	<a href="#">Green/Red Ratiometric Cell Stress</a>	CMV	Fluorescence imaging and plate reader assay ( $Z' > 0.78$ )
#U0915G	<a href="#">Green/Red Ratiometric Cell Stress</a>	CAG	Fluorescence imaging and plate reader assay

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*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
	<b>Two-Color Cell Stress Upward Sensor BacMam</b> ≅ 2 × 10 <sup>10</sup> VG/mL in ESF 921 Insect Culture Medium (Expression Systems, product #96-001-01)	Green fluorescent sensor that increases in fluorescence intensity in response to ER and cellular stress. Constitutively expresses a red fluorescent protein for 2-color measurements. VG/mL is the number of viral genes per mL, as distinct from plaque forming units (PFU), that for baculovirus, would be measured in insect cells.	4°C
	<b>SB</b> <b>sodium butyrate</b> (Sigma Aldrich product #B5887) 500 mM in H <sub>2</sub> O	Sodium butyrate is added to the culture to maintain BacMam expression. Other HDAC inhibitors may work as well.	4°C
	<b>TG</b> <b>thapsigargin</b> (Cayman Chemical product # 10522) dissolved in DMSO at 1mM.	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. Thapsigargin is supplied in a 20 µL aliquot in an airtight tube purged with nitrogen. It will arrive on wet ice and should be immediately aliquoted and stored at -20°C. On the day of the experiment an aliquot may be thawed and diluted into H <sub>2</sub> O or PBS buffer and used immediately. Discard any unused thapsigargin aliquot.	-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<sup>2+</sup> and Mg<sup>2+</sup> (PBS) available from VWR [\[4\]](#).
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 pseudo-typed 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 VSFG 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](#).

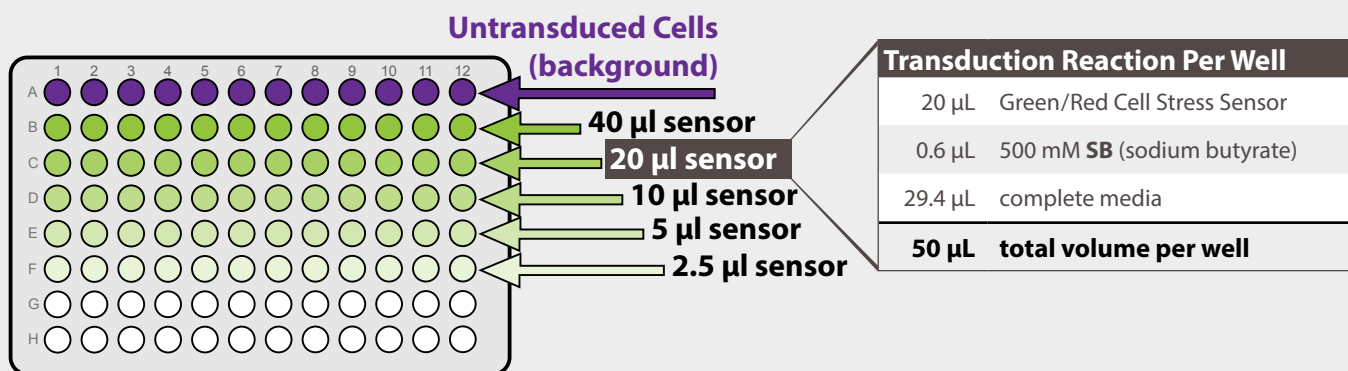
**OPTIMIZE  
SENSOR  
EXPRESSION**

### 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

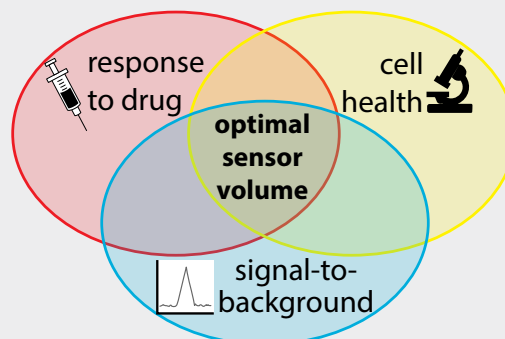
- Set up your plate. Be sure to include control wells (untransduced cells) in order to calculate signal-to-background. **Refer to detailed protocol Day 1 procedure on page 5.**
- Perform titration to determine optimal sensor volume for your cells.



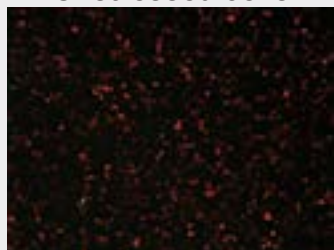
#### Day 2

- Measure red fluorescence to evaluate sensor expression levels.
- Add 1 µM **TG** (thapsigargin, final concentration in well) to activate the set of wells transduced with the Cell Stress Sensor. Monitor the change in green fluorescence.
- Refer to detailed protocol Day 2 procedure on page 8.**

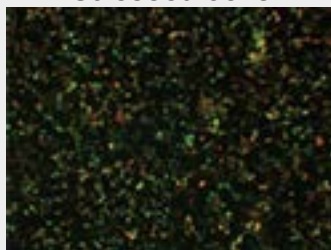
**Check your cells** for these 3 parameters to determine **optimal sensor volume**.



Unstressed cells



Stressed cells





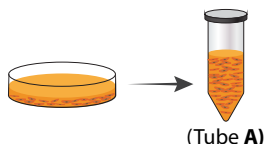
## Day 1 – Detailed Procedure

### Transduce and Plate Cells

#### Step 1)

##### Prepare Cells (Tube A)

- Detach cells using standard trypsinization protocol. Resuspend cells in complete culture media and determine cell count.
- Prepare a dilution of cells at your desired concentration. 100  $\mu\text{L}$  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.



#### Cells (Tube A)

##### Per Well (96-well plate)

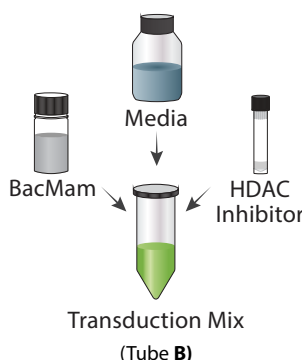
100  $\mu\text{L}$  cells

**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

#### Step 2)

##### Prepare Viral Transduction Reaction (Tube B)

- For each transduction reaction (i.e. one well in a 96-well plate), prepare the transduction solution by mixing 20  $\mu\text{L}$  of the sensor BacMam stock with 0.6  $\mu\text{L}$  of the 500 mM **SB** (stock solution of sodium butyrate), and 29.4  $\mu\text{L}$  of the complete culture media for your cells, for a total volume of 50  $\mu\text{L}$ . Mix gently. Following Step 3, the final concentration of sodium butyrate will be 2 mM.



#### Transduction Reaction (Tube B)

##### Per Well (96-well plate)

20  $\mu\text{L}$  Green/Red Cell Stress sensor  
0.6  $\mu\text{L}$  500 mM **SB** sodium butyrate  
29.4  $\mu\text{L}$  complete media

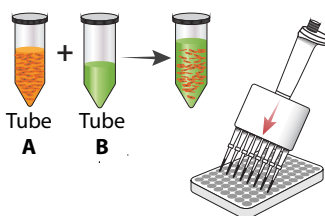
**50  $\mu\text{L}$  total volume**

**Tip:** When preparing a master mix, scale up by 10-15% of the number of wells needed to avoid coming up short.

#### Step 3)

##### Mix Cells and Transduction Mix

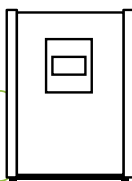
- Mix Tube A and Tube B (100  $\mu\text{L}$  tube A + 50  $\mu\text{L}$  tube B). Mix gently and seed 150  $\mu\text{L}$  of mix per well on the 96-well plate.
- Cover plate to protect from light and let rest at room temperature for 30 minutes.
- Incubate  $\approx$  20-24 hours under normal cell growth conditions (5%  $\text{CO}_2$  and 37°C), protected from light.



Rest 30 minutes at room temperature



Incubate  $\approx$  24 hours  
5%  $\text{CO}_2$  and 37°C



#### (Tube A) + (Tube B)

##### Cells + Transduction Reaction

##### Per Well (96-well plate)

100  $\mu\text{L}$  cell suspension  
50  $\mu\text{L}$  transduction reaction

**150  $\mu\text{L}$  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  $\mu\text{L}$  aliquots and store at  $-20^{\circ}\text{C}$ .

- Detach cells using standard trypsinization protocol. Resuspend cells in complete culture media and perform cell count.
- 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  $\mu\text{L}$**  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.
- Prepare transduction reaction (detailed at right).
- Mix the cells with the transduction reaction (**50  $\mu\text{L}$**  cells + **50  $\mu\text{L}$**  transduction mix). Mix gently, then seed **100  $\mu\text{L}$**  of this mix per well on a 96-well plate.
- Let cells sit at room temperature, protected from light, for 20 minutes.
- 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.
- After spinning the plate, **remove the transduction mix** and replace with fresh media containing **5 mM valproic acid** (100  $\mu\text{L}$ -150  $\mu\text{L}$  per well).
- Return plate to normal growth conditions and incubate for 20-24 hours.

### Transduction Reaction

Per Well (96-well plate)	
15 $\mu\text{L}$	Green/Red Cell Stress sensor
1.7 $\mu\text{L}$	300 mM valproic acid (5.1 mM in well)
0.7 $\mu\text{L}$	1M HEPES
32.6 $\mu\text{L}$	cell culture media

**50  $\mu\text{L}$  total volume**

**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 $\mu\text{L}$	Green/Red Cell Stress sensor
0.34 $\mu\text{L}$	TransDux
17 $\mu\text{L}$	Max Enhancer
1.7 $\mu\text{L}$	300 mM valproic acid (5.1 mM in well)
0.7 $\mu\text{L}$	1M HEPES
15.26 $\mu\text{L}$	cell culture media

**50  $\mu\text{L}$  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

- For each transduction reaction (i.e. one well in a 96-well plate, containing 100  $\mu\text{L}$  culture media per well), prepare a transduction solution by mixing 20  $\mu\text{L}$  of the sensor BacMam stock with 29.4  $\mu\text{L}$  of culture media, and 0.6  $\mu\text{L}$  of the 500 mM stock solution of **SB** sodium butyrate for a total volume of 50  $\mu\text{L}$ . 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.
- 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%  $\text{CO}_2$  and  $37^\circ\text{C}$ ), protected from light, for 20-24 hours.
- Optional step (cell type dependent): After 4-8 hour incubation with sensor BacMam (6 hours is optimal), aspirate transduction solution and add 100  $\mu\text{L}$  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

- 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)

- 50  $\mu\text{L}$  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)

- For each transduction reaction (i.e. one well in a 384-well plate), prepare the transduction solution by mixing 5  $\mu\text{L}$  of the sensor BacMam stock with 0.3  $\mu\text{L}$  of the 500 mM stock solution of **SB** sodium butyrate, and 19.7  $\mu\text{L}$  of the complete culture media for your cells, for a total volume of 25  $\mu\text{L}$ . Mix gently.

#### Step 3) Mix Cells and Transduction Mix

- Mix Tube A and Tube B (50  $\mu\text{L}$  tube A + 25  $\mu\text{L}$  tube B). Mix gently and seed 75  $\mu\text{L}$  of the mix per well on the 384-well plate.

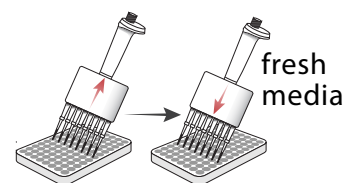
**Tip:** In step 1, the volume of the cell suspension per well can be reduced from 50  $\mu\text{L}$  to 25  $\mu\text{L}$  if desired, for a final plating volume of 50  $\mu\text{L}$  per well after the completion of step 3. Make sure that the well is still receiving 12,500 cells and that you reduce the volume of sodium butyrate to 0.2  $\mu\text{L}$  per well.



## Day 2 – Detailed Procedure

### Measuring Fluorescence

- g. Prior to measuring fluorescence, replace the media with 150  $\mu$ L of fresh media supplemented with 0.6  $\mu$ L sodium butyrate (2mM final concentration). Fluorobrite, a media with low auto-fluorescence, is a good option. The viral transduction mix contains autofluorescence, so changing the media will increase the signal-to-noise in the assay.
- h. Visually inspect cells on microscope to confirm cell health. Check red fluorescence to confirm sensor expression and transduction efficiency.
- i. Measure signal-to-background. Transduced cells should be at least 5 $\times$  brighter compared to untransduced cells.
- j. Add compounds that induce cellular stress. Return the plate to incubator and allow the fluorescence to equilibrate for 1 hour. Capture the change in fluorescence intensity using standard GFP excitation and emission wavelengths. Reserve a few wells to add **TG** (thapsigargin), your positive control compound (final concentration of 1  $\mu$ M).
- k. When monitoring the green fluorescence emitted by the sensor, either the change in fluorescence intensity over time or the absolute fluorescence intensity can be measured.
- l. Refer to **Timing Section** on page 10 for specific information on timing.
- \* **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 $\times$  (in a volume of 50  $\mu$ L PBS) to wells containing 100-150  $\mu$ L 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™
- Biotek Lionheart™ FX
- BMG CLARIOstar®
- Epifluorescence microscopes





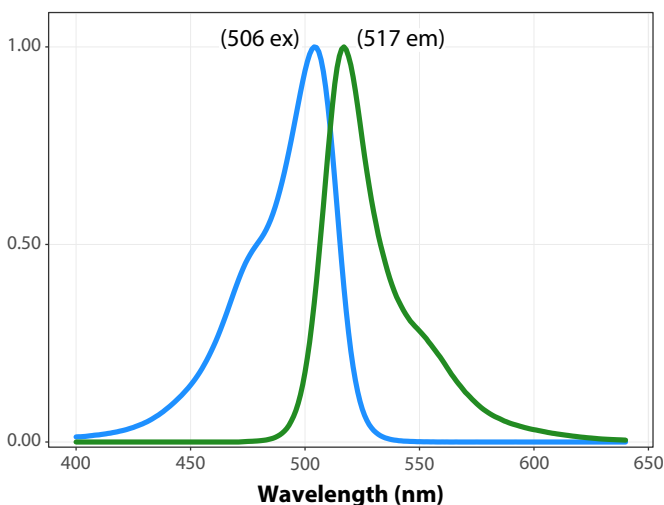
## Fluorescence Properties

The Cell Stress Sensor is constructed with the very bright, mNeonGreen fluorescent protein [6], and the constitutively expressed red fluorescent protein (Santaka Red). While the peak excitation and emission wavelengths for mNeonGreen are 506 nm and 517 nm, respectively, a range of 485-505 nm (excitation) and 515-535 nm (emission) may be used if your instrument does not allow measurement at the peak ex/em. For example, on the BioTek Synergy MX™, the preferred ex/em is 488/525. If using filters, we recommend Chroma's Catalog set #49003 for optimal results. Preferred excitation and emission wavelengths for Santaka RFP are 558/603.

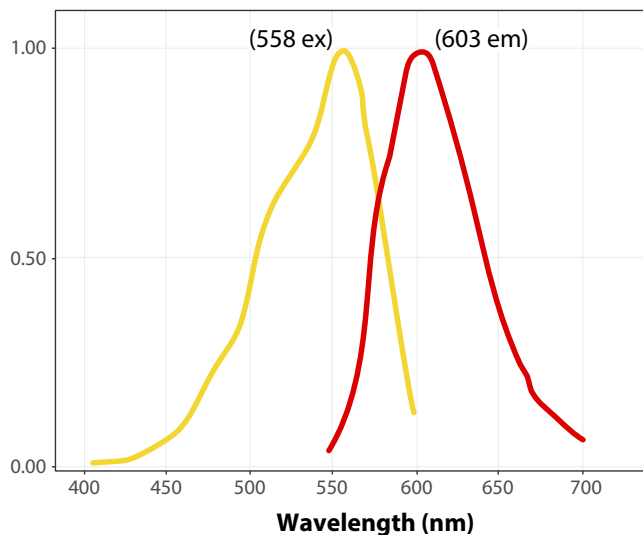
(Figures 1A, 1B)

### 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 1A.** Absorption and emission properties of the mNeonGreen fluorescent protein plotted as a function of wavelength.



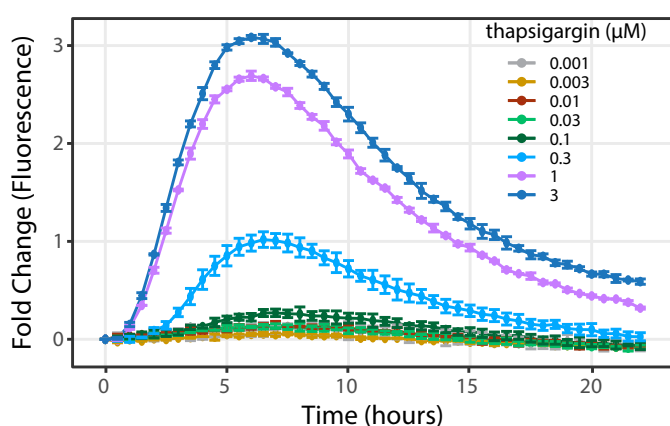
**Figure 1B.** Absorption and emission properties Red Fluorescent Protein plotted as a function of wavelength



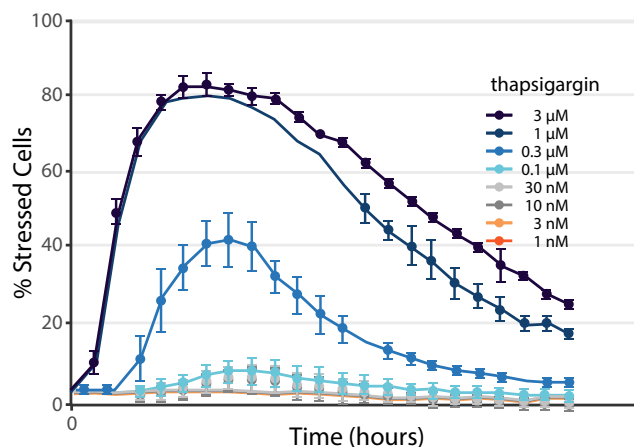
## Timing

The cell stress assay is a live-cell assay, allowing detection over short and long time intervals. For best results, 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 in cellular stress levels. Read or image the fluorescence intensity from the plate at reasonable 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 by ~7 hours after treatment (**Figures 2A, 2B**). Different compounds induce cellular stress at different rates so we suggest taking multiple fluorescence measurements over a 24-48 hour period. Because the fluorescent signal is reversible, the assay can be used to screen for toxic effects of drugs or for drugs that return the cell to a healthy state. This sensor can be combined with a red sensor to study signaling in stressed vs. healthy cells.

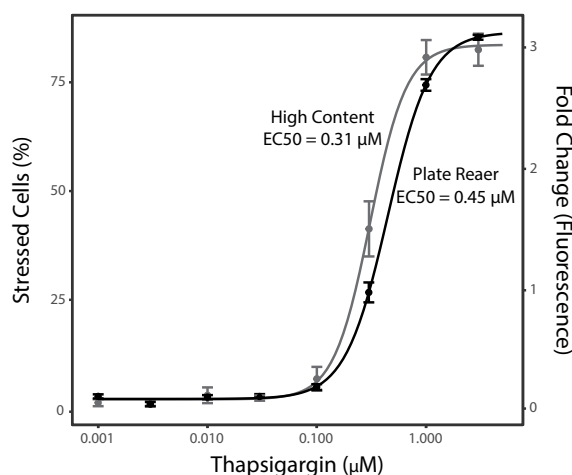
## Monitoring changes in cell stress with the two-color cell stress biosensor.



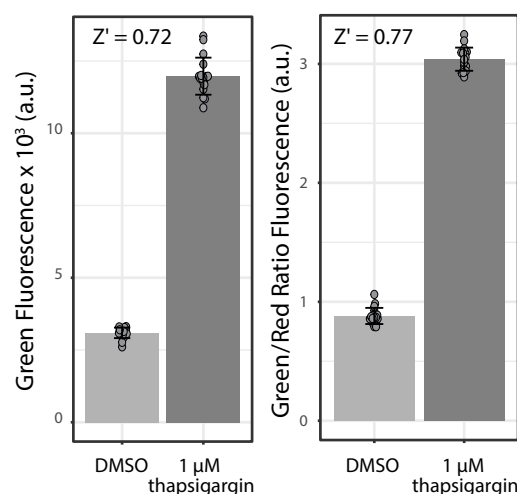
**Figure 2A.** HEK 293T cells transduced with the Green / Red Cell Stress Sensor and treated with increasing amounts of thapsigargin. Green fluorescence was monitored for 24 hours on a BMG CLARIOstar® plate reader with the atmosphere control unit, temperature at 37°C and 5% CO<sub>2</sub>. Note that stress induction, indicated by an increase in green fluorescence, appears within 60 minutes, and begins to decrease by ~7 hours.



**Figure 2B.** HEK 293T cells transduced with the Green / Red Cell Stress Sensor and treated with increasing concentrations of thapsigargin. These data were analyzed in the BioTek Lionheart™ FX high content imaging system. The percentage of stress cells over time was used to quantify cell stress levels.



**Figure 2C.** EC<sub>50</sub> values from A and B comparing analysis of the stress sensor on plate reader or imaging systems.



**Figure 2D.** Z' quantification of the cell stress sensor using either green or green/red fluorescence values.



## 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 [6]. 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  $\mu$ L to 40  $\mu$ L 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.

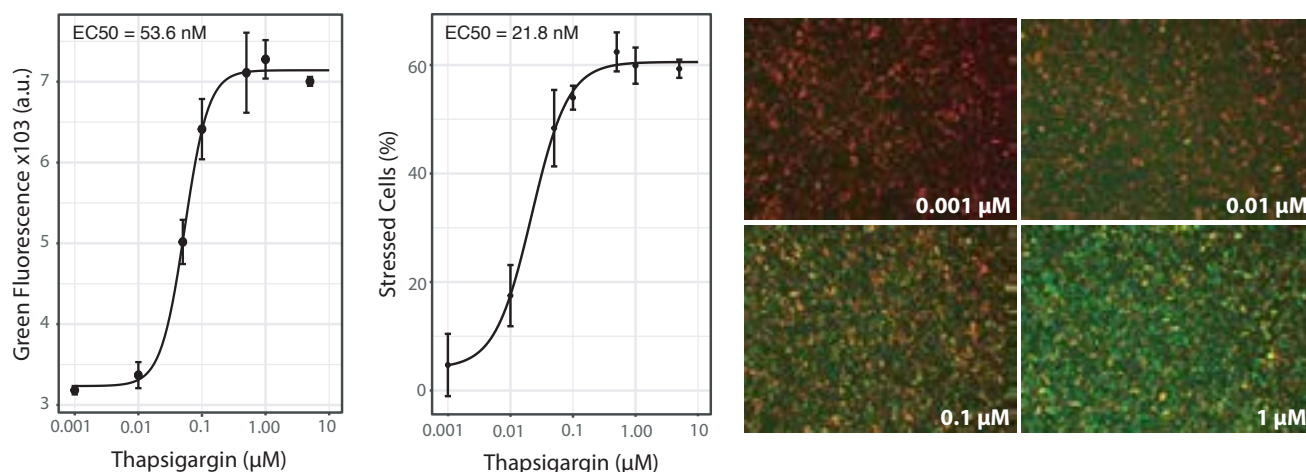
### Use the Positive Control

If the cells are expressing the sensor, and fluorescence is detectable on your instrument, then check the sensor using thapsigargin included in your kit. Add a final concentration of 1  $\mu$ M thapsigargin to control wells ([see Figure 2](#)). In cells that are expressing the sensor, addition of the positive control compound, thapsigargin, will result in increased green fluorescence within 7 hours. Both the green sensor and constitutively active red label are localized to the nucleus to simplify segmentation for high content imaging applications. A serial dilution series of the sensor with a constant amount of thapsigargin positive control can be used to find the optimal expression for your cell type. It is important to determine the kinetics of the response and to set your instrument to measure in the appropriate time frame while avoiding oversampling.



## Two-Color Analysis

The Green/Red Ratiometric Cell Stress Biosensor is a useful tool for determining ER-mediated cell stress on both image-based and plate reader-based systems. Discussed here are important aspects of the two-color stress sensor to be considered when analyzing data generated using this biosensor.



**Figure 3.** Two-color analysis

## Changes in Protein Expression

Expression of mutant genes or treatment of cells with exogenous compounds can alter protein expression. Moreover, activation of cell stress can also alter protein expression. The constitutively expressed, nuclear localized red fluorescent protein can serve as an indicator of changes in overall protein expression. The green stress-induced fluorescence can be compared to the red fluorescence to normalize for changes in overall protein expression. However, as many compounds or mutations can alter cellular expression levels over different time frames it is important to explore changes in protein expression and cell stress activation over a range of time frames.

## Plate Reader Analysis

When analyzing the cell stress sensor on a standard fluorescence plate reader either green fluorescence intensity or green/red ratio fluorescence can be used to determine cellular stress (**Figure 2D**).

## Image Analysis

When using an imaging-based approach to detect cell stress the same fluorescence intensity measurements of green or green/red ratio fluorescence can be used. However, another method that can be used with imaging and high content based analysis is the percent of stressed cells in the region of interest. In this readout, all cells within a region of interest expressing the biosensor are marked in red, while only the stressed cells within the region express green fluorescence. Dividing the number of cells expressing both green and red fluorescence by all cells expressing red fluorescence gives the fraction of stressed cells within the imaged region. This type of measurement does not rely on fluorescent intensities that may be altered by overall protein expression changes and may be useful when analyzing a number of different stress inducing compounds that alter general protein expression. Importantly, both intensity based and percentage based quantifications of cell stress produce highly similar results as shown in **Figure 2**.

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](mailto:info@montanamolecular.com)



## Troubleshooting Guide

1.

### Low sensor expression and/or poor transduction efficiency

Problem	Possible Cause	Solution
	Suboptimal sensor BacMam volume is being used.	<b>Perform titration of the sensor BacMam stock</b> , testing a large range (i.e. 2.5-40 $\mu$ L 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 <a href="#">Suggestions for Assays in Adherent Cells</a> .
	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 $\mu$ M  * 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.	<ul style="list-style-type: none"> <li>• 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).</li> <li>• Perform media exchange after various incubation times with the transduction mix, in addition to leaving the virus on overnight.</li> <li>• Try high-titer, purified BacMam stock.</li> <li>• Validate assay in a different cell type (e.g. HEK 293T cells)</li> <li>• Transduce cells multiple times (e.g. on Day 1, and again on Day 2).</li> <li>• Incubate cells for 48 hours post transduction, before performing assay.</li> <li>• Consider using a different viral vector, such as lentivirus or AAV.</li> </ul>
	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) <b>Low sensor expression and/or poor transduction efficiency</b>	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 <a href="#">guidelines for product storage</a> . 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, <a href="#">consult Montana Molecular</a> for custom production services.
	Cells are contaminated	Monitor cells for bacteria, fungi, etc.
<b>2.</b> <b>Low fluorescence signal on microscope/plate reader.</b>	Low sensor expression, low transduction efficiency.	See solutions for <a href="#">Problem 1</a> .
	Excitation/emission settings are not optimal for the sensor being used.	Refer to protocol for the <a href="#">fluorescence spectra</a> 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. <b>Confirm with visual inspection on a microscope.</b>
<b>3.</b> <b>Signal-to-background is low (i.e. cells/wells with sensor are not much brighter than control cells/wells without sensor).</b>	Low sensor expression, low transduction efficiency.	See solutions for <a href="#">Problem 1</a> .
	Excitation/emission settings are not optimal for the sensor being used.	Refer to protocol for the <a href="#">fluorescence spectra</a> 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. <b>Confirm with visual inspection on a microscope.</b>





Problem	Possible Cause	Solution
<b>4.</b> Signal is noisy.	Low sensor expression, low transduction efficiency.	See solutions for <a href="#">Problem 1</a> .
	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.	Exchange media so that cells are in fresh media at the time of experiment. 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. <b>Confirm with visual inspection on a microscope.</b>
<b>5.</b> 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 <a href="#">storage</a> 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 <a href="#">HEK 293T</a> and <a href="#">CHO cells</a> .
	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 <sub>2</sub> , 60 seconds for cADDiS and cGMP, and 1-2 seconds for GECO Ca <sup>2+</sup> ). Measure long enough to capture max response of sensor.



Problem	Possible Cause	Solution
<b>6. Poor cell health, cells detaching from plate.</b>	Too much BacMam stock was added to cells (e.g. sensor, receptor, Gs mutant).	<b>Titrate lower amounts of BacMam stock</b> 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 $\mu$ M * 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. <b>Confirm with visual inspection on a microscope.</b>
	PBS being used does not contain calcium and magnesium.	Use PBS containing calcium and magnesium.
	Cells are contaminated.	Monitor cells for bacteria, fungi, mycoplasma.
	Cells were not grown under proper growth conditions (i.e. 5% CO <sub>2</sub> , 37°C).	Incubate transduced cells at 37°C, in 5% CO <sub>2</sub> .
	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.



## Cell Stress Sensor - in the Literature

1. K. Harlen, et al. Live-Cell Assays for Cell Stress Responses Reveal New Patterns of Cell Signaling Caused by Mutations in Rhodopsin,  $\alpha$ -Synuclein and TDP-43 Front. Cell. Neurosci., December 2019.

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## Related Products

Product	Sensor Description	Promoter	Recommended Use
#U0900G	Green Up Cell Stress Sensor	CMV	Fluorescence imaging and plate reader assay ( $Z' < 0.74$ )

## 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](mailto:info@montanamolecular.com) or call us at +1 406-200-8321 and we'll respond as quickly as we can.

