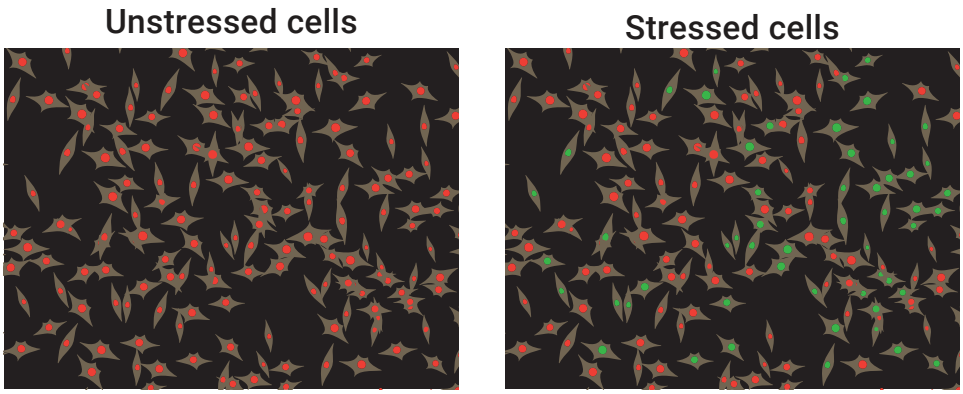
XBP1-IRE1 Ratiometric Cell Stress Assay

August 22, 2024

Product Info & Protocol

About these Assays

The XBP1- IRE1 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.



The XBP1- IRE1 ratiometric cell stress sensor (#U0921G) is an updated version of the Ratiometric Cell Stress sensor (#U0901G). It utilizes the bright, rapidly folding Montana Paintbrush red fluorescent protein for improved compatibility with plate readers.

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 HEK293T cells. 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.

Table 1. Relevant Product

Product	Description	Promoter	Recommended Use
#U0921G	XBP1- IRE1 Ratiometric Cell Stress	CMV	Fluorescence imaging and plate reader assay (Z' > 0.9)

Our goal is to make your workflow easy and reproducible.

We'd love to hear about your research.

Questions?
Call us, we can help!
+1 406-200-8321
info@montanamolecular.com

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 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.
Store **sodium butyrate at 4°C.**

Table 2 Materials in Kit	Details	Storage
XBP1- IRE1 Cell Stress Sensor BacMam ≅ 2×10^{10} 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
SB **sodium butyrate** (Sigma Aldrich product #B5887) 500mM in H_2O	Sodium butyrate is added to the culture to maintain BacMam expression. Other HDAC inhibitors may work as well.	4˚C
TG **thapsigargin** (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 20µL 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_2O 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. We recommend the following plates; 96-well Greiner Bio-One (#655946), 96-well Thermo Fisher Scientific (#152037), 384-well Greiner Bio-One: (#781946), 384-well Corning: (#354663).
2. Dulbecco's Phosphate Buffered Saline 1× with Ca^{2+} and Mg^{2+} (DPBS 10× solution from Gibco #14080055) [4].
3. Cells and cell media. We recommend media with low autofluorescence such as EMEM, McCoy's 5A, and F12K culture media.

Biosafety Considerations

The BacMam vector carrying the fluorescent biosensor in this construct 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 pseudotyped to infect mammalian cells. In mammalian cells, the baculovirus genome is silent, and it cannot replicate to produce new virus. 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 VSVG 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 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, Scaling for 384-well Plates, and Alternative Spinoculation Protocol.

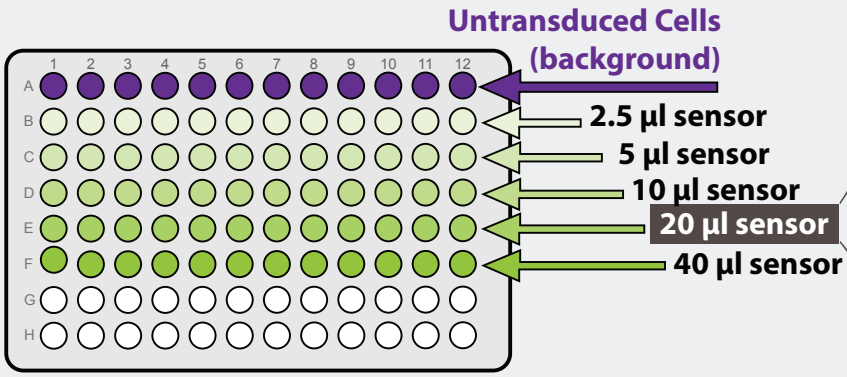
First Experiment - Optimize by Titrating the Sensor

We recommend using a titration series to determine the best combination of signal above background, cell health, and response. Ideally, the the red fluorescent signal in each well before drug addition should be at least 5 times above background.

Transduce and Plate Cells for Optimization

i. Set up your plate. Be sure to include control wells (untransduced cells) in order to calculate signal-to-background. (For details on preparing cells and transduction mix, refer to Day 1 procedure on page 5).

ii. Perform titration to determine optimal sensor volume for your cells, as detailed in plate diagram and tables below.



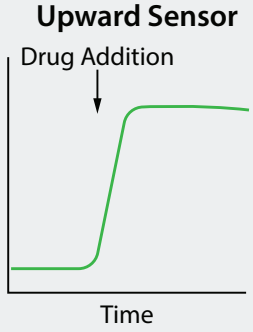
Transduction Reaction Per Well	
XBP1- IRE1 Cell Stress Sensor	20µL
500mM **SB** (sodium butyrate)	0.6µL
complete media	29.4µL
total volume per well	**50µL**

Measure Parameters to Determine Optimal Conditions

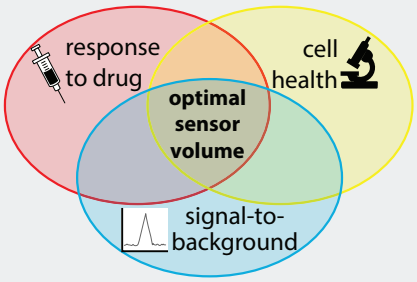
iii. Measure red fluorescence to evaluate sensor expression levels.

iv. Add 1µM **TG** (thapsigargin, final concentration in well) to activate the set of wells transduced with the Cell Stress Sensor. Monitor the change in green fluorescence.

v. Refer to detailed protocol Day 2 procedure on page 8.

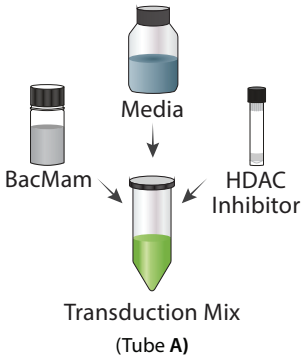


Check your cells for these 3 parameters to determine **optimal sensor volume.**



Day 1 – Transduce and Plate Cells for your Experiment

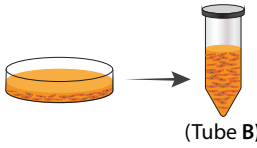
A. **Prepare Viral Transduction Mix (Tube A):** For each transduction reaction (i.e. one well in a 96-well plate), prepare the transduction mix as detailed in table at right (using the optimal volume of sensor that was determined in your optimization experiment). Mix gently.



Transduction Mix (Tube A)	
XBP1- IRE1 Cell Stress Sensor	Variable
SB 500mM sodium butyrate	0.6µL
complete media	(To 50µL)
total volume per well	**50µL**

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

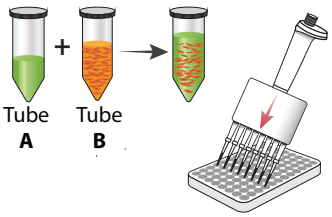
B. **Prepare Cells (Tube B):** Detach cells using standard trypsinization protocol. Resuspend cells in complete culture media and determine cell count.



Cells (Tube B)	
cells per well	100µL

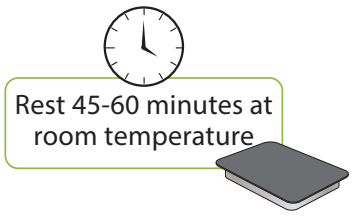
C. **Prepare a dilution of cells at your desired concentration.** (100µL of this cell resuspension will be required for a single well in a 96-well plate, so prepare enough of the dilution to seed the desired number of wells in the plate).

D. **Combine Transduction Mix and Cells**: Combine Tube A and Tube B (50µL Tube A + 100µL Tube B). Mix by pipetting up and down gently, and seed 150µL per well on the 96-well plate



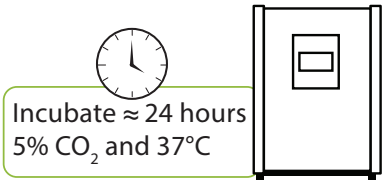
Transduction Mix + Cells (Tube A) + (Tube B)	
transduction mix	50µL
cell suspension	100µL
total volume per well	**150µL**

E. **Cover plate** to protect from light and let rest at room temperature for 45-60 minutes.



Tip: *90% cell confluence after 24 hours is ideal. For HEK 293T cells 50,000 cells per well works well but plating density is cell type dependent*

F. **Incubate ≈ 20-24 hours** under normal cell growth conditions (5% CO_2 and 37°C), protected from light.



Tip: *Boost expression in CHOs and other cell types with a 40-48 hour incubation.*

Suggestions for Assays in Adherent Cells

This protocol is optimized for rapidly dividing immortalized cells.

However, this Cell Stress biosensor can be used with primary cultures of cells as well as iPSC-derived cells. In the case of non-dividing, or differentiated cells, the transduction should be done with adherent cells, and the media should be exchanged the following day. 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.

***When working with adherent cell cultures, add an extra day to seed the cells prior to transduction.**

Adherent Cells Day 1:

- Seed the cells the day before you add transduction mix, incubate 24 hours

Adherent Cells Day 2:

- Perform transduction as directed on page 5, but 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 20-24 hours.
- Optional step (cell type dependent): After 4-8 hour incubation with sensor BacMam (6 hours is optimal), very gently aspirate transduction solution (we recommend using a plate washer). Add 100µL complete growth media with sodium butyrate at a concentration of 2mM. If cells will not tolerate a full media exchange, partial media exchanges can be done.

Adherent Cells Day 3:

- Measure Fluorescence as as detailed in Day 2 procedure.

Scaling for 384 Wells (1 plate)

To set up the assay in a 384-well plate, follow all of the protocol steps, adjusting reagent volumes as follows:

Transduction Mix (Tube A) (384-well plate)	
XBP1- IRE1 Cell Stress Sensor	2µL
500mM **SB** sodium butyrate	0.1µL
complete media	10.4µL
total volume per well	**12.5µL**

Cells (Tube B) (384-well plate)	
cells per well	12.5µL

12.5µL of the cell resuspension will be required for a single well in a 384-well plate. A plating density of 7,500 cells per well is a good starting point, so prepare the cell suspension at 600,000 cells/mL. Depending on the cell type and plate type, 5,000-15,000 cells per well may be optimal.

Transduction Mix + Cells (Tube A) + (Tube B) (384-well plate)	
transduction mix	12.5µL
cell suspension	12.5µL
total volume per well	**25µL**

Tip: *When scaling for 384-well plates, the volume of the cell suspension per well can be increased to 50µL to improve cell health. Make sure that the well is still receiving 7,500 cells and that you increase the volume of sodium butryate to 0.2µL per well.*

Alternative Spinoculation Protocol

This alternative protocol may be useful for particularly difficult-to-transduce or low expressing cell types:

1. Prepare transduction mix (detailed in table at right).

2. Detach cells using standard trypsinization protocol. Resuspend cells in complete culture media and perform cell count.

3. Prepare a dilution of cells at your desired concentration (22,500 cells/well in a 96-well plate is a good starting point, but will ultimately depend on the cell type being used). 50µL 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.

4. Combine the transduction mix with the cell suspension (**50µL** transduction mix + **50µL** cells). Mix gently, then seed **100**µL of this mix per well on a 96-well plate.

5. Let cells sit at room temperature, protected from light, for 20 minutes.

6. **Spin the plate at 1,500 x g for 1.5-2 hours at room temperature.**

7. * We recommend sealing the plate with Breathe-Easy® (Cat. No. 70536-10) during this step to avoid contamination.

8. After spinning the plate, **remove the transduction mix** and replace with fresh media containing **0.6µL sodium butyrate** (2mM per well).

9. Return plate to normal growth conditions and incubate for 48 hours.

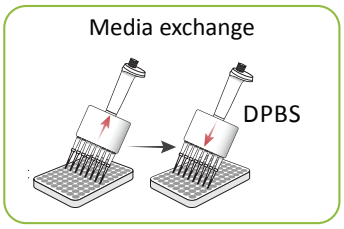
Alternative Transduction Mix	
XBP1- IRE1 Cell Stress Sensor	Variable
500mM **SB** sodium butyrate	0.6µL
1M HEPES (pH 7.4)	0.7µL
cell culture media	(To 50µL)
total volume per well	**50µL**

Tip: *Titrate the sensor to determine optimal volume;* *see optimization page*

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 Mix + Enhancer Reagents	
XBP1- IRE1 Cell Stress Sensor	Variable
TransDux	0.34µL
Max Enhancer	17µL
500mM **SB** (sodium butyrate)	0.6µL
1M HEPES (pH 7.4)	0.7µL
cell culture media	(To 50µL)
total volume per well	**50µL**

Day 2 – Measure Stress Response



G. Prior to measuring fluorescence, replace the media with 150µL of fresh media supplemented with 0.6µL 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× 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 1µM).

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× (in a volume of 50µL PBS) to wells containing 100-150µL 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 XPB1- IRE1 Cell Stress Sensor is constructed with the very bright, mNeonGreen fluorescent protein [6], and a constitutively expressed red fluorescent protein (Montana Paintbrush RFP). While the peak excitation and emission wavelengths for mNeonGreen are 506nm and 517nm, respectively, a range of 485-505nm (excitation) and 515-535nm (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. For detecting the Montana Paintbrush RFP, our preferred ex/em settings are 565/620. If using filters, we recommend Chroma's Catalog set #49003 for optimal results. **(Figures 1A, 1B)**

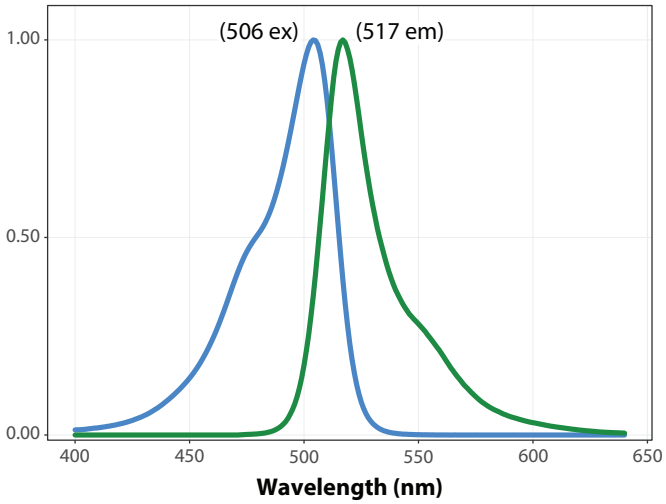


Figure 1A. Absorption and emission properties of the mNeonGreen fluorescent protein plotted as a function of wavelength.

Tip: *We offer mNeonGreen and Montana Paintbrush BacMam Kits (products #P1000G and #P1000R). These test kits are a good way to determine BacMam transduction efficiency, refine fluorescence detection settings, and optimize expression in your cells of choice.*

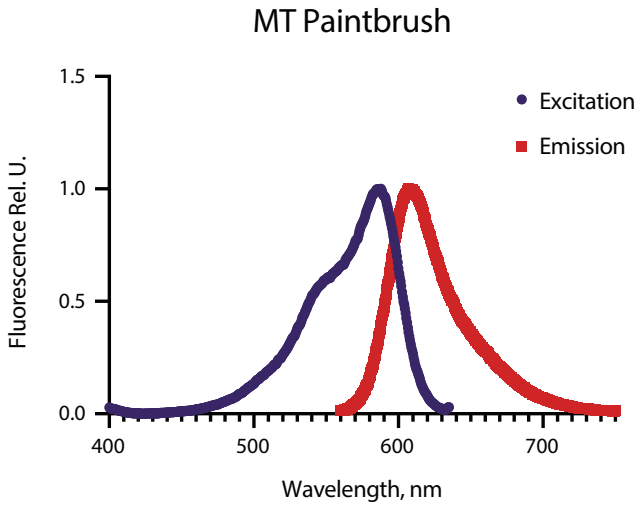


Figure 1B. Absorption and emission properties of the Montana Paintbrush Red Fluorescent Protein plotted as a function of wavelength

Timing

The XBP1- IRE1 Cell Stress Sensor 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. Different compounds induce cellular stress at different rates so we suggest taking multiple fluorescence measurements over a 24-48 hour period.

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 2.5µL to 40µL 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 1µM 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.

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/red ratio fluorescence, or green-only fluorescence intensity can be used to determine cellular stress (Fig. 2).

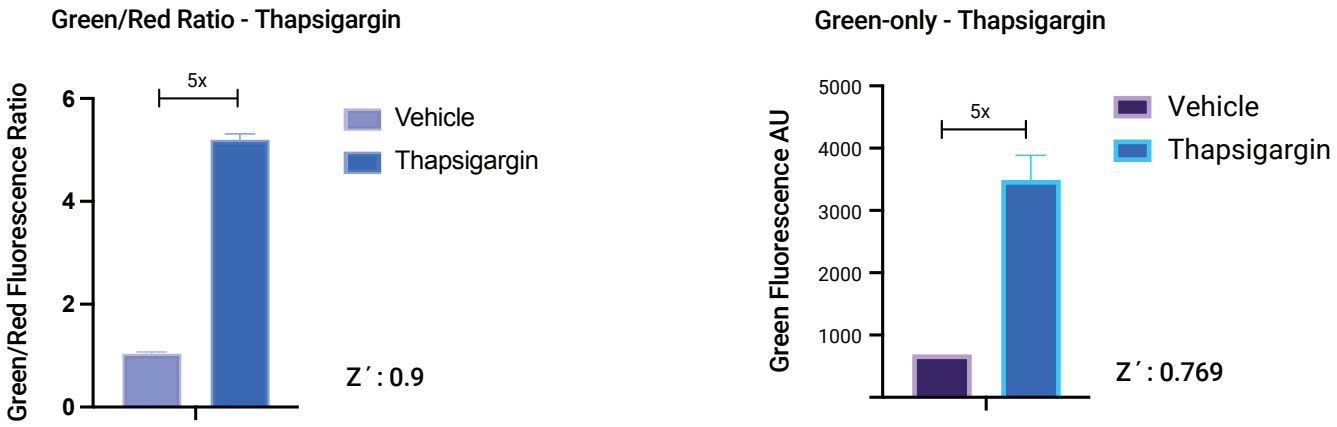


Figure 2. Z' quantification of the cell stress sensor using either green/red fluorescence values, or green only fluorescence values.

Image Analysis

When using an imaging-based approach to detect cell stress the same fluorescence intensity measurements of green/red ratio fluorescence, or green-only 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 fluorescence 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.

Troubleshooting Guide

	Problem	Possible Cause	Solution
1.	**Low sensor expression and/or poor transduction efficiency**	Suboptimal sensor BacMam volume is being used.	Perform titration of the sensor BacMam stock, testing a large range (i.e. 2.5-40µL 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 Suggestions for Assays in Adherent Cells.
		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 - 2mM final concentration valproic acid - 5mM final concentration trichostatin A - 0.25 µM final concentration * 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.	• 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). • Perform media exchange after various incubation times with the transduction mix, in addition to leaving the virus on overnight. • Try high-titer, purified BacMam stock. • Validate assay in a different cell type (e.g. HEK 293T cells) • Transduce cells multiple times (e.g. on Day 1, and again on Day 2). • Incubate cells for 48 hours post transduction, before performing assay. • Consider using a different viral vector, such as lentivirus or AAV.
		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) **Low sensor expression and/or poor transduction efficiency**	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 guidelines for product storage. 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, consult Montana Molecular for custom production services.
	Cells are contaminated	Monitor cells for bacteria, fungi, etc.
2. Low fluorescence signal on microscope/plate reader.	Low sensor expression, low transduction efficiency.	See solutions for Problem 1.
	Excitation/emission settings are not optimal for the sensor being used.	Refer to protocol for the fluorescence spectra 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. **Confirm with visual inspection on a microscope.**
3. Signal-to-background is low (i.e. cells/wells with sensor are not much brighter than control cells/wells without sensor).	Low sensor expression, low transduction efficiency.	See solutions for Problem 1.
	Excitation/emission settings are not optimal for the sensor being used.	Refer to protocol for the fluorescence spectra 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. **Confirm with visual inspection on a microscope.**

	Problem	Possible Cause	Solution
4.	**Signal is noisy.**	Low sensor expression, low transduction efficiency.	See solutions for Problem 1.
		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. **Confirm with visual inspection on a microscope**.
5.	**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 storage 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 HEK 293T and difficult to transduce cells.
		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_2, 60 seconds for cADDis and cGMP, and 1-2 seconds for GECO Ca^{2+}). Measure long enough to capture max response of sensor.

Problem	Possible Cause	Solution
6. Poor cell health, cells detaching from plate.	Too much BacMam stock was added to cells (e.g. sensor, receptor, Gs mutant).	**Titrate lower amounts of BacMam stock** 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 - 2mM valproic acid - 5mM trichostatin A - 0.25 µM * 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. **Confirm with visual inspection on a microscope**.
	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_2, 37°C).	Incubate transduced cells at 37°C, in 5% CO_2.
	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, α-Synuclein and TDP-43 Front. Cell. Neurosci.,December 2019.

References

1. Iwawaki, Takao, Ryoko Akai, Kenji Kohno, and Masayuki Miura. 2003. "A Transgenic Mouse Model for Monitoring Endoplasmic Reticulum Stress." Nature Medicine 10 (1). Nature Publishing Group: 98–102.

2. Rong, Juan, Ian Pass, Paul W. Diaz, Tram A. Ngo, Michelle Sauer, Gavin Magnuson, Fu-Yue Zeng, et al. 2015. "Cell-Based High-Throughput Luciferase Reporter Gene Assays for Identifying and Profiling Chemical Modulators of Endoplasmic Reticulum Signaling Protein, IRE1." Journal of Biomolecular Screening 20 (10). SAGE Publications: 1232–45..

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

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

7. Tewson PH, Martinka S, Shaner N, Hughes TE, Quinn AM: New DAG and cAMP sensors optimized for live cell assays in automated laboratories. Journal of Biomolecular Screening 2015.

8. Shaner, N.C., Lambert, G.G., Chammas, A., Ni, Y., Cranfill, P.J., Baird, M.A., Sell, B.R., Allen, J.R., Day, R.N., Israelsson, M., Davidson, M.W., & Wang, J. (2013) "A bright monomeric green fluorescent protein derived from Branchiostoma lanceolatum." Nature Methods, May;10(5):407-9. doi: 10.1038/nmeth.2413.

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 or call us at **+1 406-200-8321** and we'll respond as quickly as we can.

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