Montana Molecular

Fluorescent Biosensors for Live Cell Discovery

Pseudo SARS-CoV-2 Reporter

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Introduction

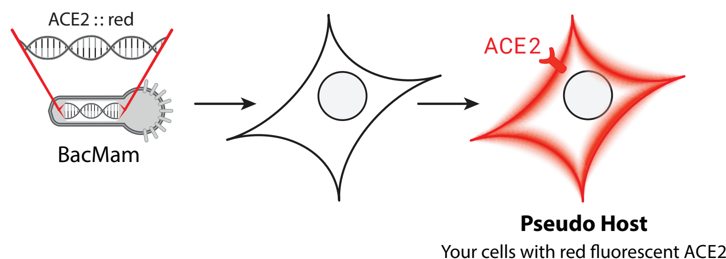
Spike protein on the surface of SARS-CoV-2 interacts with the ACE2 protein expressed on the surface of human cells to mediate viral entry into the host cell. The tight binding of spike to ACE2 is the first step in the infection process. If this interaction could be blocked, with antibodies or compounds, a therapeutic drug for COVID-19 could be developed **[6], [7]**. The viral entry process for SARS-CoV-2 appears to be triggered by host cell proteases that cut the spike protein in two places, exposing a new multibasic site adjacent to one of the cleavage sites. There is new evidence that this multibasic site interacts with neuropilin-1 (NRP1), a host receptor, expressed in vascular epithelial cells **[8]**. There are three different proteases that appear to be involved in the processing and activation of spike protein. One is furin, thought to be involved during viral replication **[9]**. Another is TMPRSS2, a host protease on the cell surface **[10], [11]**, and finally, cathepsin-L, a pH dependent lysosomal protease **[12], [13]**. Different cell types may require addition of different host factors. Host factor products can be ordered separately: **TMPRSS2 (#C1130N)**, **Neuropilin-1 (#C1140R)**, and **Cathepsin-L (#C1150N)**.

About Fluorescent Pseudo SARS-CoV-2 Reporters

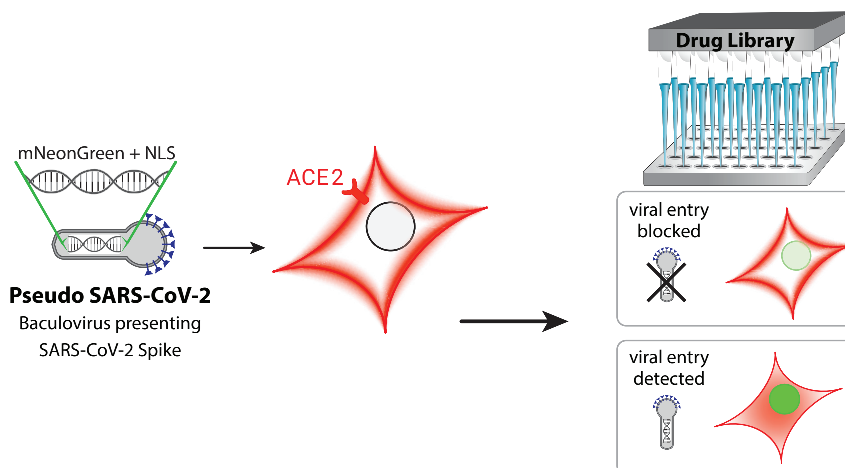
The pseudovirus tools described here **(see Table 1)**, combined with our pseudo host tools **(see Table 5)** provide biologists and drug discovery teams with robust, safe tools to study SARS-CoV-2 entry and the potential to discover compounds or antibodies that can stop this process. The Pseudo SARS- CoV-2 Reporters are safe facsimiles of the virus that do not replicate in human cells and can be used in pseudo host cells that express ACE2 and possibly other host factors. Pseudo SARS-CoV-2 Reporters are baculoviruses that are decorated with the spike protein just like the real SARS-CoV-2 virus.

Pseudo SARS-CoV-2 enters host cells through interactions with ACE2 and other host factors, but once inside the cell, baculovirus does not replicate or integrate into the host cell genome. Twenty-four hours after entry, the host cell expresses either a green or red bright fluorescent protein in the nucleus indicating pseudovirus entry. If viral entry is blocked, the cell nucleus will decrease in fluorescence. By combining Pseudo SARS-CoV-2 and pseudo host cells, safety requirements are minimal and screening is cost effective. This means that most laboratories can be screening for blocking agents within a few days, with a minimum of changes to work flow or safety/regulatory procedures. Pseudo-SARS-CoV-2 titer is 2×10^{10} VG/mL. A 5 mL kit will generously cover four 96-well plates depending upon cell density and cell type.

Step 1: Make Pseudo Host Cell



Step 2: Pseudovirus Assay



Validation and Controls

Pseudovirus affinity for ACE2 was confirmed using absorption experiments in A549 cells expressing different amounts of ACE2 **(Figure 1)** and in the antibody blocking experiment shown in **Figure 2**. Further validation in other cell types is ongoing.

Figure 1. Pseudovirus Absorption Experiment: A549 cells in a 96-well plate received a constant amount of virus with increasing amounts of ACE2 that were adjusted with decreasing amounts of a red fluorescent protein control to balance the viral load at 6.6 x 10^8 VG/mL for each condition. The following day, Pseudo SARS-CoV-2 pseudovirus was added to the plate and incubated for 2 hours. The media from the plate was transferred to a second plate of fresh A549 cells expressing a constant amount of ACE2. Twenty-four hours later, the cells were washed with PBS and the green fluorescence produced by the pseudovirus from the media was measured on a BioTek Synergy™ plate reader. The two hour incubation on ACE2 expressing cells clearly reduces the pseudovirus concentration in an ACE2 dependent manner.

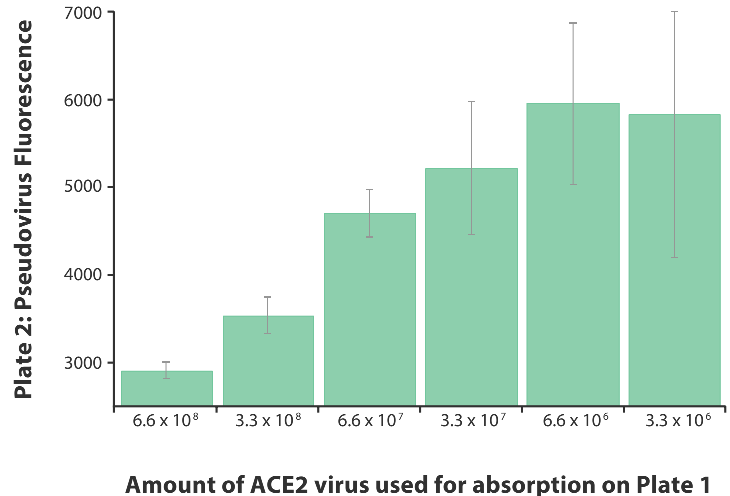
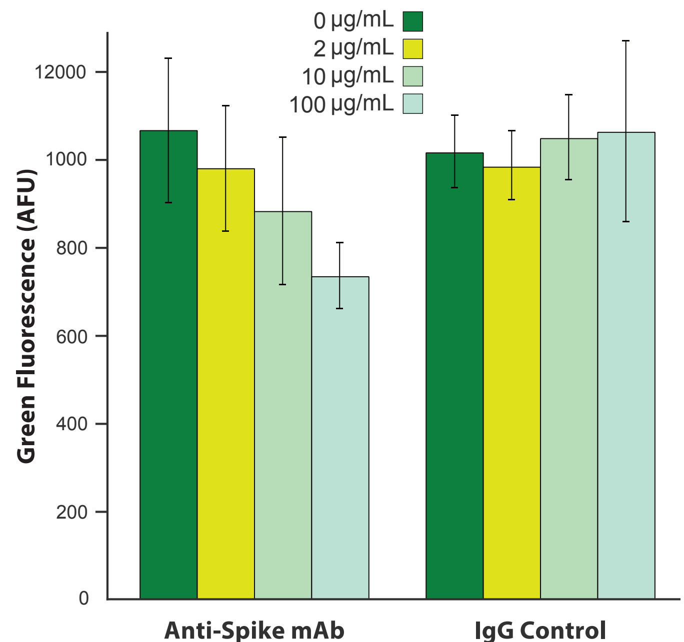


Figure 2. SARS-CoV-2 pseudovirus is blocked by anti-Spike antibody. A549 cells were transduced with 6.6 x 10^8 VG/mL Fluorescent ACE2 **(#C1100R)**. The following day, cells were treated with a monoclonal anti-Spike antibody or an equivalent amount of IgG isotype control, then challenged with 3.3 x 10^8 VG/mL SARS-CoV-2 pseudovirus **(#C1110G)**. Green fluorescence was quantified ~20 hours later. Data represent the average of 3 replicates (error bars = SD).

1. Mouse anti-Spike mAb **(Sino Biological, catalog # 40591-MM43)**
2. Human anti-Spike mAb **(Acro Biosystems, catalog # SAD-S35)**



Our goal is to make your workflow easy and reproducible.

Questions?
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We'd love to hear about your research.

We also have a **Troubleshooting Guide** *at the end of this document*

This protocol applies to the products in Table 1:

Table 1. Pseudovirus Reporters

Product	Description	Promoter	Recommended Use
#C1110G	**Pseudo SARS-CoV-2 - Green Reporter (nuclear targeted green fluorescence)**	CMV	High-content imaging, microscopy, plate reader
#C1120G	**Pseudo SARS-CoV-2 -D614G mutant - Green Reporter (nuclear targeted green fluorescence)**	CMV	High-content imaging, microscopy, plate reader
#C1110R	**Pseudo SARS-CoV-2 - Red Reporter (nuclear targeted red fluorescence)**	CMV	High-content imaging, microscopy, plate reader
#C1120R	**Pseudo SARS-CoV-2 -D614G mutant - Red Reporter (nuclear targeted red fluorescence)**	CMV	High-content imaging, microscopy, plate reader

Pseudovirus Kit Materials and Storage

Baculovirus should be stored at 4°C protected from light in the original package. Store **HDAC inhibitor at 4°C. Avoid repeated freeze/thaw cycles.** The Pseudo SARS-CoV-2 Reporter is a new product and we do not yet know the shelf life of this virus. It should be used as soon as possible. Montana Molecular is currently testing the virus for functionality on a monthly basis and will update customers on the shelf life over time.

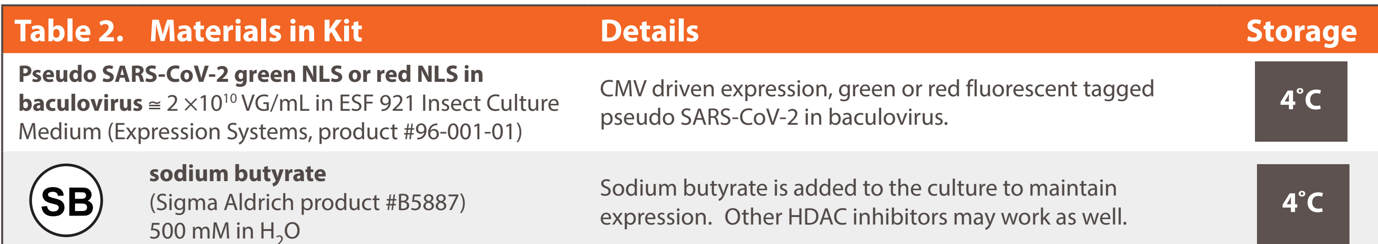


Table 2. Materials in Kit	Details	Storage
Pseudo SARS-CoV-2 green NLS or red NLS in baculovirus ≅ 2×10^{10} VG/mL in ESF 921 Insect Culture Medium (Expression Systems, product #96-001-01)	CMV driven expression, green or red fluorescent tagged pseudo SARS-CoV-2 in baculovirus.	4°C
SB **sodium butyrate** (Sigma Aldrich product #B5887) 500 mM in H_2O	Sodium butyrate is added to the culture to maintain expression. Other HDAC inhibitors may work as well.	4°C

Additional Materials Required (not included in kit)

1. Fluorescent ACE2 (**#C1100R** or **#C1100G**). Depending on the cell type, other host factors may be needed.
2. Black, clear bottom microplate coated with a cell attachment factor. **Greiner Cell Coat (#655946)** is our preferred 96-well plate available from VWR.
3. Dulbecco's Phosphate Buffered Saline with Ca^{2+} and Mg^{2+} (PBS) available from VWR **[2]**.
4. Cells and cell media of your choice. For A549 cells, we recommend F-12K media **(ATCC cat# 30-2004)** supplemented with 10% FBS.

Biosafety Considerations

Pseudo SARS-CoV-2 is a modified baculovirus, *Autographa californica*, AcMNPV. The natural host of baculovirus is larvae of the order *Lepidoptera*. The baculovirus is produced using Sf9 insect cells and is pseudotyped with spike protein from SARS-CoV-2. 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 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 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. Under no circumstances should these products be administered to, nor consumed by humans.

Protocol for Use

This protocol is optimized for detection on a fluorescence plate reader in **adherent A549 cells**. The protocol can be adapted to other cell types, but additional host factors may be necessary such as **TMPRSS2 (#C1130N)**, **Neuropilin-1 (#C1140R),** or **Cathepsin-L (#C1150N).** See section on **Optimizing Host Factors for Other Cell Types** on page 7.

Day 1 – Set up your plate

a. Use ~15,000 A549 cells per well on a 96-well plate in complete media. One row of wells are reserved for control cells that are not transduced with ACE2. Incubate under normal growth conditions (5% CO_2 and 37°C, protected from light), for 12-24 hours.

Day 2 – Transduce Cells with ACE2

b. Prepare a transduction mix of ACE2, complete media, and sodium butyrate. See Table 3.

Table 3. ACE2 Transduction Mix, Per Well (96-well plate)

	Stock	Amount per Well	Final Concentration	100-Well Master Mix
ACE2 BacMam	2 x 10^{10} VG/mL	5 µL	6.6 x 10^{8} VG/mL	500 µL
(SB) sodium butyrate	500 nM	0.6 µL	2 mM	60 µL
complete media		44.4 µL		4.440 mL
Final Transduction Mix Volume:		**50 µL total volume per well**		

c. Pipet transfer 50 µL volumes of the transduction mix into each well of the cells plated on Day 1. Rock the plate gently 5 - 10 times to ensure uniform transduction across each well. Incubate at 37°C for 12-24 hours.

Day 3 – Add the Pseudo SARS-CoV-2 Pseudovirus

d. Prepare a transduction mix that includes pseudovirus, fresh complete media, sodium butyrate, and blocking compounds (if used). See Table 4.

Table 4. Pseudo SARS-CoV-2 Pseudovirus Transduction Mix, Per Well (96-well plate)

	Stock	Amount per Well	Final Concentration
SARS-CoV-2 Pseudovirus	2 x 10^{10} VG/mL	2.5 µL	3.3 x 10^{8} VG/mL
(SB) sodium butyrate	500 nM	0.6 µL	2 mM
antibodies or compounds		if using	
complete media		adjust to reach total	
Final Transduction Mix Volume:		**50 µL total volume per well**	

e. Remove the media from the cells and wash once with pre-warmed PBS (100 µL per well).

f. Add 100 µL of fresh media to each well.

g. Pipet transfer 50 µL volumes of the pseudovirus transfection mix into each well of the A549 cells. Rock the plate gently 5 - 10 times to ensure uniform transduction across each well. Incubate at 37°C for 12-24 hours.

TIP: *3.3 x 10^{8} VG/mL is a good starting point for detection on a plate reader. For imaging applications try adding a smaller amount of pseudovirus such as 3.3 x 10^{7} VG/mL*

Day 4 – Measure Fluorescence

h. Just before reading the plate, replace the media with PBS (1X, containing Ca^{2+} and Mg^{2+}.)

i. Read fluorescence on either a plate reader or imaging system.

Fluorescence Properties

The green fluorescent reporters express the very bright, mNeonGreen fluorescent protein **[5]**. While the peak excitation and emission wavelengths 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.

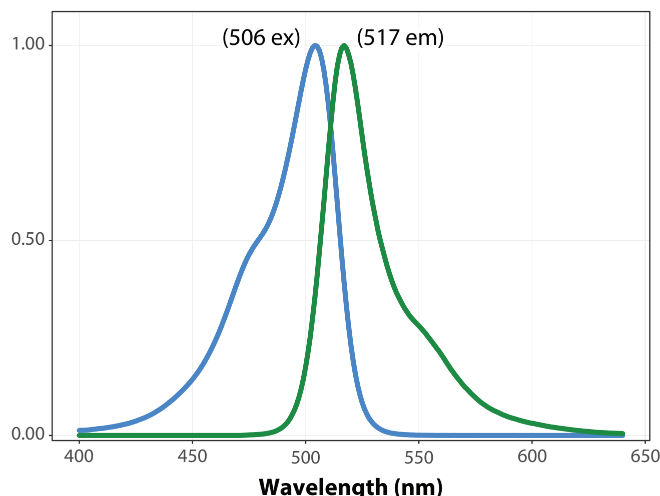


Figure3A. Absorption and emission properties of the mNeonGreen fluorescent protein plotted as a function of wavelength.

For the red fluorescent reporters, the optimal excitation wavelength is 558 nm, but the absorption band of this protein is quite broad. Broad bandpass filters that pass 540 to 580 nm light can be used quite effectively. On the emission side, the red light spans 600 to 700 nm (peak excitation at 603 nm), so broad bandpass emission filters can also help to collect much of the emission.

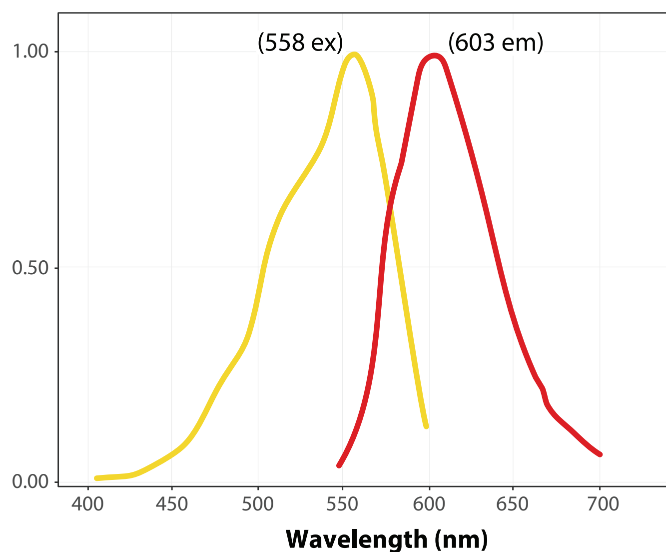


Figure 3B. Absorption and emission properties of the red fluorescent protein plotted as a function of wavelength.

Optimization

Optimizing Fluorescence

Twenty-four hours after transduction, check your cells for fluorescence. Wells that were transduced with the fluorescent ACE2 should be 5-10 times brighter than control wells that were not treated with the BacMam.

HDAC inhibitors may be used to maintain expression. While BacMam transduction alone will result in protein 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. 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 Host Factors for Other Cell Types

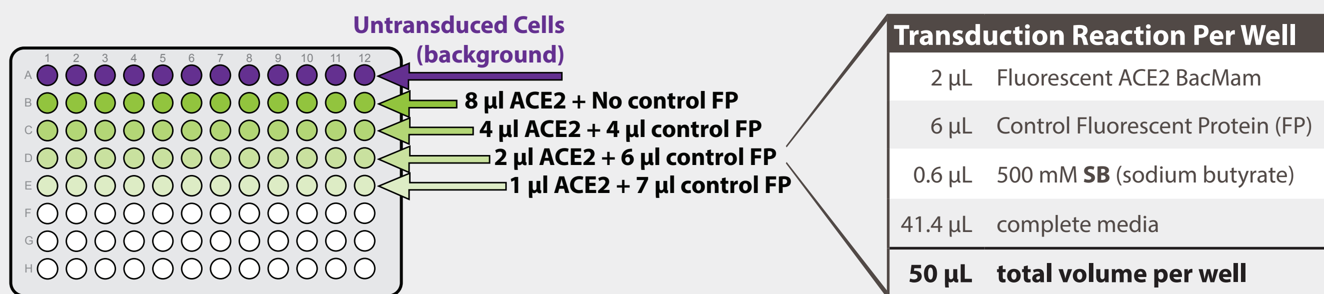
Pseudovirus assays often involve multiple BacMam transductions over two days, to first introduce host factors and then to challenge the cells with Spike-decorated pseudovirus. The first BacMam transduction places a metabolic load on the cells such that the expression of the pseudovirus reporter is less than normal. To account for this metabolic load during host factor optimization, we recommend using the red fluorescent protein control provided in the Fluorescent ACE2 kit, so that every condition has the same total amount of BacMam virus. An example of setting up such an optimization with ACE2 is included below.

ACE2 Example

For cell types other than A549, other host factors may be necessary. We recommend using a titration series to optimize viral entry.

a. Set up your plate with cells in complete media and 2mM sodium butyrate. One row of wells are reserved for cells that are not transduced with ACE2.

b. Titrate ACE2 + Control Fluorescent Protein (FP) in a series of wells to optimize fluorescent signal. Refer to **Fluorescent ACE2 Detailed Protocol.**



Transduction Reaction Per Well	
2 µL	Fluorescent ACE2 BacMam
6 µL	Control Fluorescent Protein (FP)
0.6 µL	500 mM **SB** (sodium butyrate)
41.4 µL	complete media
50 µL	**total volume per well**

Note:

These assays and protocols are rapidly evolving as we strive to develop enhancements to improve spike presentation, better controls and shorter assay time frames. We sincerely appreciate your feedback and look forward to working with you over the coming weeks. Write to us at **info@montanamolecular.com**. New assay developments will be posted on our webpage **Reagents for SARS-CoV-2 Research**

Troubleshooting Guide

Problem	Possible Cause	Solution
1. **Low expression and/ or poor transduction efficiency**	Suboptimal BacMam volume is being used.	Perform titration of the BacMam stock, testing a large range (i.e. 3-15 µL 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.
	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 trans-duction mix, or the concentration was wrong.	Add HDAC inhibitor at the proper concentration: sodium butyrate - 2mM valproic acid - 5mM trichostatin A - 0.25µM * 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 culture media is inhibiting transduction.	Remove media during transduction, preparing the transduction mix in DPBS and adding to cells. Replace transduction mix with media after 2-4 hours.
	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.
	BacMam stock was not mixed adequately before transducing cells.	Mix BacMam stock thoroughly before transduction, especially after being stored for long periods.
	Cells are contaminated	Monitor cells for bacteria, fungi, etc

Problem	Possible Cause	Solution
2. **Low fluorescence signal on microscope/plate reader.**	Low expression, low transduction efficiency.	See solutions for **Problem 1**.
	Excitation/emission settings are not optimal for mNeonGreen or Red FP.	Refer to protocol for the **fluorescence spectra**. Make sure that filter sets or monochromators are aligned with the peak excitation and emission wavelengths.
	Cells are in cell culture media, and the media is producing a large fluorescent signal (autofluorescence).	Exchange media so that cells are in DPBS 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 fluorescence are not much brighter than control cells/wells without the fluorescent protein.**	Low expression, low transduction efficiency.	See solutions for **Problem 1**.
	Excitation/emission settings are not optimal.	Refer to protocol for the **fluorescence spectra**. Make sure that filter sets or monochromators are aligned with the peak excitation and emission wavelength.
	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 DPBS. Cell culture media being used has high autofluorescence.	Perform media exchange so that cells are in DPBS 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**.

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Table 5. Related Products

Product	Description	Promoter	Recommended Use
#C1100G	**mNeonGreen Fluorescent ACE2**	CMV	High- content imaging, microscopy, plate reader
#C1100R	**Red Fluorescent ACE2**	CMV	High- content imaging, microscopy, plate reader
#C1130N	**Protease BacMam TMPRSS2**	CMV	High- content imaging, microscopy, plate reader
#C1140R	**Neuropilin 1- Red**	CMV	High- content imaging, microscopy, plate reader
#C1140N	Neuropilin 1- Untagged	CMV	High- content imaging, microscopy, plate reader
#C1150N	**Cathepsin-L**	CMV	High- content imaging, microscopy, plate reader
#C1160N	**3CLpro Inhibitor Assay**	CMV	High- content imaging, microscopy, plate reader

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.