Fluorescent ACE2

June 24, 2021

Product Info & Protocol

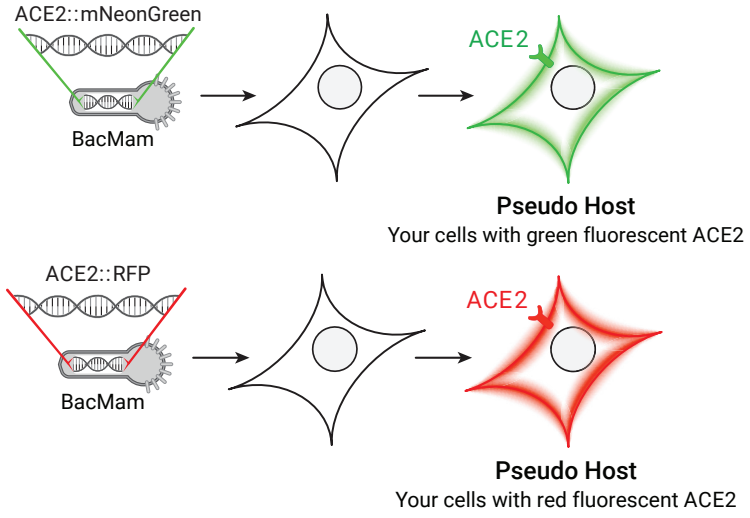
Introduction

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 ACE2

The viral entry process may also involve proteases. Theses enzymes, such as TMPRSS2 (product #C1130N) are thought to cleave the spike protein such that a membrane fusion complex springs into action, fusing the envelope membrane with the host cell membrane, ensuring that the virus enters the cell **[8], [10], [14]**.

The fluorescent ACE2 described here, combined with our pseudovirus reporters (products #C1110G, #C1110R, #C1120G, #C1120R), provides biologists and drug discovery teams with robust, safe tools to study SARS-CoV-2 entry and the potential to discover compounds or neutralizing antibodies that can stop the viral entry process. Pseudo SARS- CoV-2 and fluorescent ACE2 are based on a BSL-1 BacMam vector that does not replicate in mammalian cells. BacMam vectors make it possible to generate pseudo host cells that express a fluorescent ACE2 (products #C1100G, and #C1100R) and other host factors **(See Table 4)**. A significant advantage of pseudo host cells is that cell lines which are easy to culture and well suited for use in high throughput screening can be used. 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.



This protocol applies to the products in Table 1:

Table 1. Fluorescent ACE2 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

BacMam 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. Avoid repeated freeze/thaw cycles.** We recommend re-testing BacMam stock after storing for more than 12 months.

Table 2. Materials in Kit	Details	Storage
Green or Red fluorescent ACE2 in BacMam ≅ 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 Angiotensin Converting Enzyme 2 (ACE2) protein in BacMam.	4˚C
Control FP- Green or Red Fluorescent Protein	mNeonGreen BacMam is included for the green ACE2 kit and Red Fluorescent BacMam is included for the red ACE2 kit	4˚C
SB **sodium butyrate** (Sigma Aldrich product #B5887) 500 mM in H_2O	Sodium butyrate is added to the culture to maintain BacMam expression. Other HDAC inhibitors may work as well.	4˚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^{2+} and Mg^{2+} (PBS) available from VWR **[2]**.
3. 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

The BacMam vector carrying the fluorescent protein 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 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.

Terms of Sale

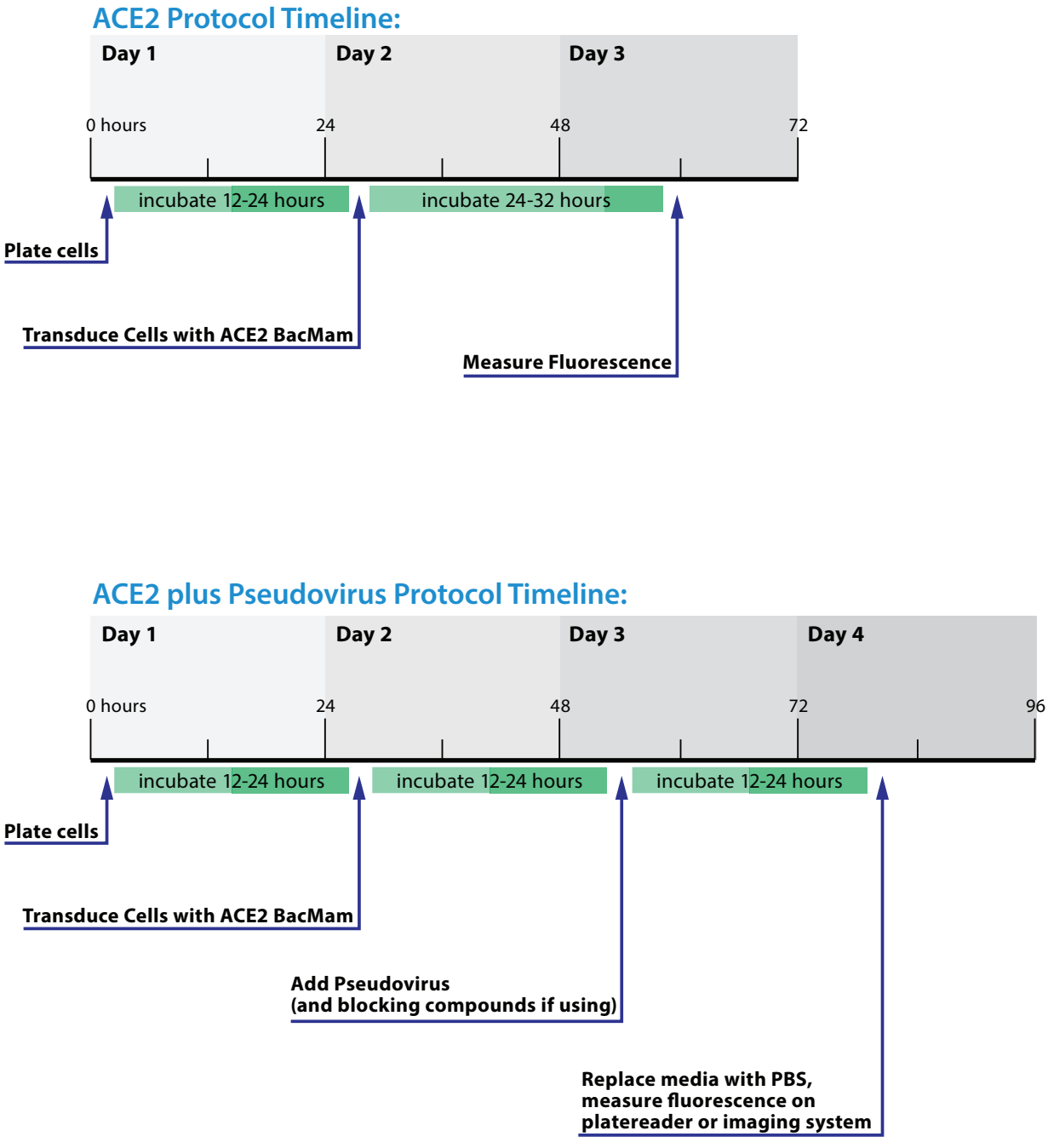
All materials in this kit are provided without warranty, express or implied. User is responsible for making sure product use complies with applicable regulations. No right to resell products or any components of these products is conveyed. Use of materials is restricted to the intended purpose described 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 detection on a fluorescence plate reader in **adherent A549 cells**. The protocol can be adapted for suspension cultures and other cell types, but additional host factors may be necessary such as **TMPRSS2 (#C1130N)**, **Neuropilin-1 (#C1140R),** or **Cathepsin-L (#C1150N). See Table 4 Related Products.**

Products #C1100G mNeonGreen Fluorescent ACE2 BacMam and #C110R Red Fluorescent ACE2 BacMam can be used on their own or in conjunction with one of our SARS-CoV-2 Pseudovirus Products. Figure 1 gives an overview of the protocol timelines. (Detailed protocol steps are on page 5.)

Figure 1. Protocol TImelines



Detailed Protocol for ACE2 BacMam is below. (For detailed protocol for our SARS-Cov-2 Pseudovirus products, see **Fluorescent Pseudo SARS-CoV-2 Reporters Protocol**.)

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 BacMam. Incubate under normal growth conditions (5% CO_2 and 37°C, protected from light), for 12-24 hours.

Day 2 – Transduce Cells with ACE2 BacMam

b. Prepare a transduction mix of ACE2 BacMam, 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 mM	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. Pipette 50 µL of the transduction mix into each well of the cell plate. Incubate under normal growth conditions (5% CO_2 and 37°C, protected from light), for at least 24 hours (for Red ACE2 BacMam, we recommend incubating the cell plate for 24-32 hours).

Day 3 – Measure Fluorescence

d. **For measuring on a plate reader:** Replace cell culture media with PBS (1X, with Ca^{2+} and Mg^{2+}). Wash very gently, to avoid dislodging the cells. Experiments are performed at room temperature.

e. If possible, visually inspect cells on microscope to confirm cell health, fluorescent ACE2 expression, and transduction efficiency.

f. Measure signal-to-background. Transduced cells should be at least 5× brighter compared to untransduced cells.

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.

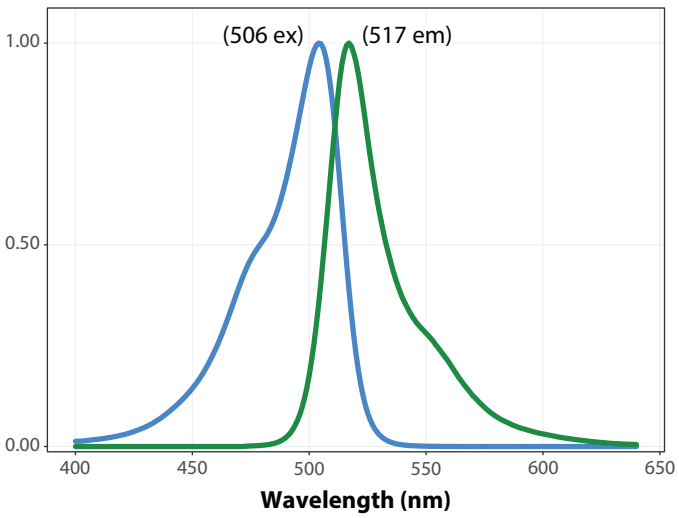


Figure 2A. Absorption and emission properties of the mNeonGreen fluorescent protein plotted as a function of wavelength.

For the red fluorescent protein, 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.

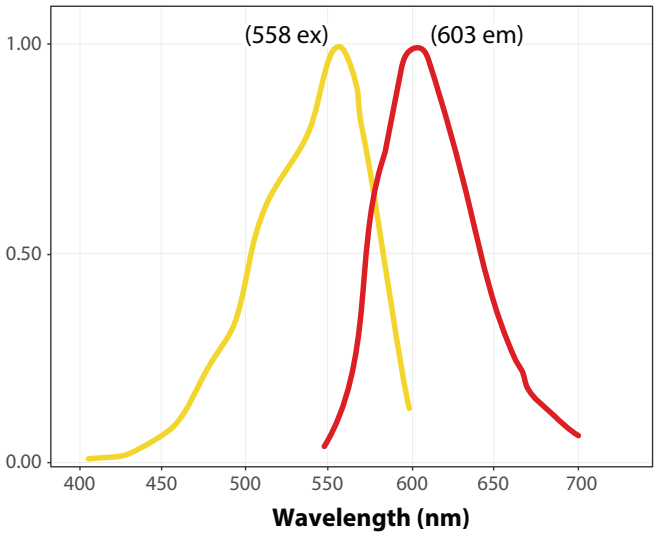


Figure 2B. Absorption and emission properties of the red fluorescent protein plotted as a function of wavelength.

Optimization

Optimizing Fluorescence

Check your cells for fluorescence 24-48 hours after transuction. Wells that were transduced with the fluorescent ACE2 should be 5-10 times brighter than control wells that were not treated with ACE2 BacMam.

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. For A549 cells, we recommend F-12K media (**ATCC catalog #30-2004**) +10% fetal bovine serum (10% FBS).

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 1 µL to 8 µL per well is a good starting range in a 96-well plate. Please see **Optimizing Host Factors for Other Cell Types** in our **Pseudo SARS-CoV-2 Reporter Protocol** (page 7).

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 transduction 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 PBS 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 green or red fluorescent protein.	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 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 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 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**.

Questions?
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Our goal is to make your workflow easy and reproducible.

We'd love to hear about your research.

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Table 4. Related Products

Product	Description	Promoter	Recommended Use
#C1100N	Unlabeled ACE2	CMV	High- content imaging, microscopy, plate reader
#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
#C1121G	Pseudo SARS-CoV-2 Spike M1 - Green Reporter (nuclear targeted green fluorescence D614G, E484K, N501Y, K417T mutations)	CMV	High- content imaging, microscopy, plate reader
#C1122G	Pseudo SARS-CoV-2 Spike M2 - Green Reporter (nuclear targeted green fluorescence D614G, E484K, N501Y, K417N mutations)	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
#C1121R	Pseudo SARS-CoV-2 Spike M1 - Red Reporter (nuclear targeted green fluorescence D614G, E484K, N501Y, K417T mutations)	CMV	High- content imaging, microscopy, plate reader
#C1122R	Pseudo SARS-CoV-2 Spike M2 - Red Reporter (nuclear targeted green fluorescence D614G, E484K, N501Y, K417N mutations)	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
#C1160G	3CLglow SARS-CoV-2 Protease 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.

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