Montana Molecular
Fluorescent Biosensors for Live Cell Discovery

3CLglowUP Assay (#C1161G)

May 2, 2024

Product Info & Protocol

About this Assay (Fig. 1, Table 1) 2
- Assay Kit Materials and Storage (Table 2) 3
- Additional Materials Required 3
- Biosafety Considerations 3
- Terms of Sale 3

Protocol for Use 4
- Before Your First Experiment: Optimize Your Assay by Titrating 3CL^{pro} (Fig. 2) 4
- Day 1 - Set up the Assay (Table 3) 5
- Day 2 - Perform Endpoint Assay or Kinetic Assay 5

Fluorescence Properties (Fig. 3) 6

Troubleshooting Guide 7

References 9

Related Products (Table 4) 10

Contact Us 10

+1 406-200-8321 info@montanamolecular.com

About this Assay

This assay is designed to be scalable to high-throughput drug screening, with a quick 24-hour turnaround. The simplicity of screening, fast turnaround, and robust signals should make it possible to search for the next generation of antivirals against SARS-CoV-2 and its variants of concern. The BacMam transduction achieves robust consistency from well-to-well and makes it possible to easily control expression in a wide variety of cell types. The sensor is bright with obvious signal differences between experimental groups thereby making it very easy to take an endpoint fluorescent read using a plate reader in a matter of seconds.

The 3CLglowUP live cell assay is a SARS-CoV-2 3CL^{pro} (also known as M^{pro} or NSP5) inhibitor assay. This new technology was developed by Montana Molecular in response to the COVID-19 pandemic and the rise of mutant proteases that are resistant to Nirmartrelvir. The goal of the assay is to provide a robust, biologically meaningful tool to evaluate therapeutic drugs that target SARS-CoV-2 replication.

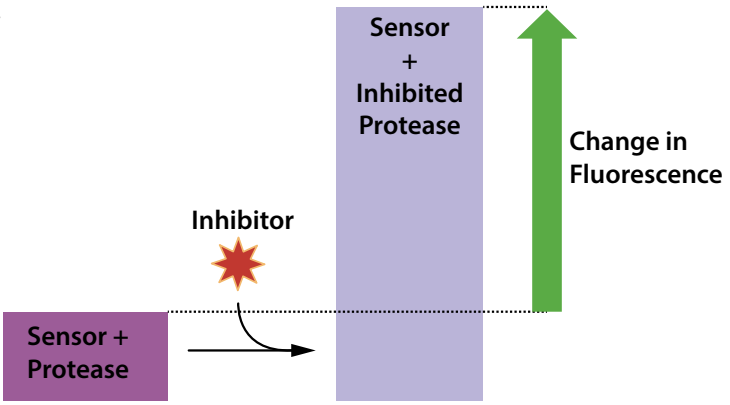

Figure 1.

The SARS-CoV-2 genome consists of positive-sense RNA and encodes two overlapping polyproteins. The polyprotein pp1ab comprises 16 nonstructural proteins (NSP1-16) many of which encode the replicase proteins involved in replication [8]. 3CL^{pro} autocatalytically removes itself from the polyprotein, spontaneously folds, and trans-cleaves the remaining nonstructural proteins (NSPs) from the polyprotein [9]. 3CL^{pro} is essential for viral replication and maturation; therefore, drugs that inhibit the catalytic site of the protease have been the main target for direct-acting antivirals and have reduced the mortality and morbidity of patients infected with SARS-CoV-2 [8].

The 3CLglowUP assay kit includes two BacMam viruses. One virus expresses the 3CL^{pro} enzyme, while the other expresses a green fluorescent biosensor for 3CL^{pro} enzyme activity. The enzyme suppresses the production of fluorescence, and when the enzyme is successfully inhibited the cells become brighter. The 3CL^{pro} enzyme is produced by a DNA sequence encoding the entire 3CL^{pro}, with an additional protein translation start site and methionine positioned at the 5' end. In the presence of an inhibitor, such as Nirmatrelvir or GC376, the fluorescence is rescued. Using reported amino acid substitutions in M^{pro} that confer Nirmatrelvir resistance [10].

While the 3CL^{pro} included in this kit is the wild type from the WIV04 strain, a suite of mutant proteases are separately available. We developed these using reported amino acid substitutions in 3CL^{pro} that confer Nirmatrelvir resistance [11]. These mutant proteases can easily be transduced in place of the included 3CL^{pro} in order to screen inhibitors against a series of mutations in the protease. Please reach out with any questions about these tools, or check our site for more information.

Table 1. Relevant Product

Product	Description	Promoter	Recommended Use
#C1161G	3CLglowUP Assay	CMV	High- content imaging, microscopy, plate reader

Assay Kit Materials and Storage

BacMam stocks should be stored at 4°C protected from light in the original package. Store **sodium butyrate at 4°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
3CLglowUP BacMam ≅ 2 ×10^{10} VG/mL in ESF 921 Insect Culture Medium (Expression Systems, product #96-001-01)	BacMam virus delivers the green fluorescent biosensor for 3CL^{pro} activity. When the protease is inhibited in the cell, this green fluorescence increases. VG/mL is the number of viral genes per milliliter, as distinct from plaque forming units (PFU), that for baculovirus, would be measured in insect cells.	4˚C
3CL^{pro} Enzyme (in BacMam, Product #C1160X) ≅ 2 ×10^{10} VG/mL in ESF 921 Insect Culture Medium (Expression Systems, product #96-001-01)	3CL^{pro} Enzyme under the control of a CMV promoter. VG/mL is the number of viral genes per milliliter, 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) 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. 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 with Ca^{2+} and Mg^{2+} (PBS) available from VWR (16777-257) [6].
3. HEK 293T cells (ATCC CRL-3216) or AK549 cells.
4. EMEM (ATTC 30-2003) with 10% FBS (ThermoFisher 16000044) culture media.
5. TrypLE Express Enzyme (1X), no phenol red (Gibco 12604013).

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 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 [7].

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 assay has been validated in, and optimized for, HEK 293T cells. Other cell types will require re-optimization of the assay. The assay involves only two components, the 3CL^{pro} Enzyme (in BacMam), and the 3CLglowUP BacMam (3CL^{pro} Biosensor). To optimize the amount of BacMam virus to add to the wells, it is important to systematically vary the relative amounts of 3CL^{pro} Enzyme and the 3CL^{pro} biosensor BacMam. The goal is to identify the conditions that produce the greatest fluorescence contrast between wells that do, and do not, have the 3CL^{pro} Enzyme. Increasing the amount of both the 3CLglow BacMam and 3CL^{pro} Enzyme produces brighter cells, but too much expression will become toxic to the cells, increasing well-to-well variability and lowering the Z' of the assay. We have done some optimization in A549 and Caco-2 cells and had similar results as well.

Before your first experiment: Optimize Your Assay by Titrating 3CL^{pro}

We recommend using a titration series to maximize fluorescence depression while maintaining cell health.

Transduce and Plate Cells for Optimization

A. 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).

B. Perform titration to determine optimal 3CL^{pro} BacMam volume for your cells, as detailed in Figure 2.

Measure Parameters to Determine Optimal Conditions

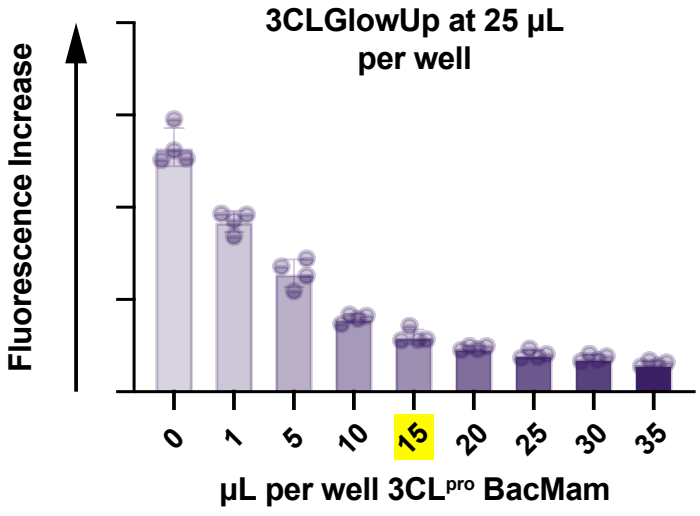

Figure 2.

C. Determine optimal 3CL^{pro} BacMam volume; **the best** conditions for **adding inhibitors** are when the sensor's fluorescence is at it's lowest while still maintaining good cell health.

Tip: *G376 and Nirmatrelvir are good starting compounds to test inhibition of 3CL^{pro}*

Tip: *15 µL is highlighted in Fig. 2 because it is the volume that we determined in our optimization experiemnt*

Green Fluorescence Protocol for HEK 293T Cells:

Day 1 — Set up the Assay

Plate cells with transduction Master mix and add inhibitors

1. Remove the media from a flask of HEK 293T cells. Pipette 2mL of TrypLE directly into the flask and let it incubate for 3 minutes at 37°C. Resuspend the cells with 8 mL of complete media in the flask, bringing to a final volume of 10 mL. After counting the resuspended cells, add the additional media necessary to make a new diluted stock of cells at 400,000 cells/mL.
2. Make up a transduction master mix (Table 1), combining the 3CLGlowUp BacMam, $3CL^{Pro}$ enzyme, sodium butyrate, complete media, and HEK293T cells.
3. Add 150µL from the transduction Master Mix (100µL cell suspension and 50µL transduction reaction for 150µL total) suspension to each of the 96 wells. Gently rock the plate 5 - 10 times to ensure uniform transduction across each well. Let the plate sit covered and protected from light at room temperature for 30 minutes. HEK293T are seeded at 40,000 cells per well.
4. Dilute compounds or known inhibitors such as GC376 in fresh media (EMEM with 10% FBS) with sodium butyrate at 2mM final concentration. After 30 minutes carefully add your compound on top of the transduction mixture, we suggest a maximal final volume of 200 µL per well. Let sit at room temperature for an additional 15 minutes. Incubate the plate overnight at 37°C, 5% CO_2.

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

	Stock	Amount per Well	Final Concentration in Cell Plate	110-Well Master Mix
$3CL^{pro}$ Enzyme in BacMam (or a mutant $3CL^{pro}$ Enzyme BacMam)	2 x 10^{10} VG/mL	15 µL	2 x 10^{9} VG/mL	1.65 mL
3CLglowUP BacMam ($3CL^{pro}$ Biosensor)	2 x 10^{10} VG/mL	25 µL	3.33 x 10^{9} VG/mL	2.75 mL
(SB) sodium butyrate	500 mM	0.6 µL	2mM	66 µL
complete media		9.4 µL		1.034 mL
Cells		100 µL		11 mL
Final Transduction Mix Volume:		**150 µL total volume per well**		

Day 2 — Endpoint Assay

5. Between 22-26 hours after the transduction, remove all of the fluorescent cell culture media from the cells and replace it with 100-150µL PBS. We suggest using a plate washer, or careful pipetting. (Pipetting by hand can be done, but due to the autofluorescence of the media, this can introduce well-to-well variability into the assay). Let the cells sit in the incubator at 37°C for 20-30 minutes after the media is changed to PBS, helping ensure a stable fluorescent read.
6. Using a fluorescent plate reader or imaging system, take a fluorescent endpoint read. Make sure to monitor for cell health through the experiment to ensure overexpression of $3CL^{pro}$ Enzyme has not had cytotoxic effects on the cells. We suggest setting the excitation to 485/20, the emission to 528/20, and the optical read to be taken from the bottom of the 96-well plate.

***A kinetic assay is also a good option if an automated microscope with incubation and environmental (CO_2/O_2) control for optimal long-term live cell imaging is available (such as Lionheart).**

Fluorescence Properties

Fluorescence Properties

The 3CLglow Sensor expresses the very bright, mNeonGreen fluorescent protein[4]. 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.

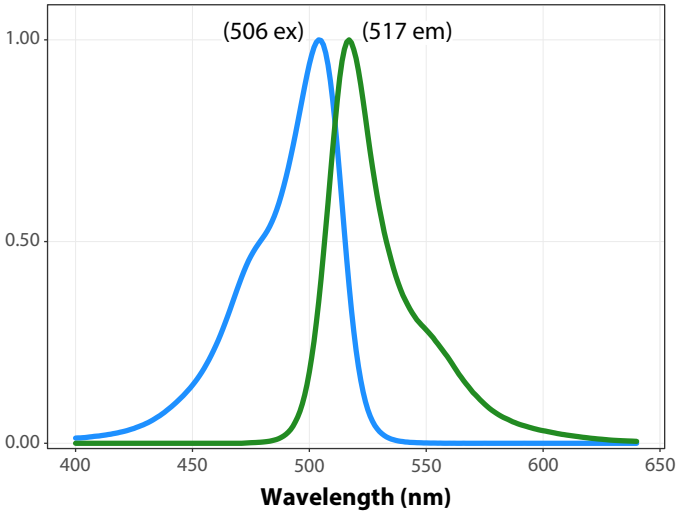

Figure 3. Absorption and emission properties of the mNeonGreen fluorescent protein plotted as a function of wavelength.

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**

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. 2.5-40 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.
	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.
	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 or too high.	Adjust gain setting or exposure time.
		Media exchange was not performed, or plate washing was inadequate causing high well-to-well variability. Cells are not in PBS at the time of experiment.	Exchange media so that cells are in PBS at the time of experiment. If cell culture media must be used, 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.**	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.
		Gain setting on instrument is too high, and signal is saturating. Gain setting is too low, and signal cannot be detected.	Adjust gain setting.
6.	**Poor cell health, cells detaching from plate.**	Too much BacMam stock was added to cells..	Titrate lower amounts of BacMam stock to identify the optimal volume for your cells.
		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.

`

Our goal is to make your workflow easy and reproducible.

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

We'd love to hear about your research.

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

References

1. Muramatsu, T. et al. *SARS-CoV 3CL protease cleaves its C-terminal autoprocessing site by novel subsite cooperativity.* Proc. Natl. Acad. Sci. U. S. A. 113, 12997–13002 (2016).
2. Zhu, W. et al. *Identification of SARS-CoV-2 3CL protease inhibitors by a quantitative high-throughput screening.* bioRxiv (2020) doi:10.1101/2020.07.17.207019.
3. Froggatt, H. M., Heaton, B. E. & Heaton, N. S. *Development of a fluorescence based, high-throughput SARS-CoV-2 3CLpro reporter assay.* bioRxiv (2020) doi:10.1101/2020.06.24.169565.
4. Shaner, N. C. et al. *A bright monomeric green fluorescent protein derived from Branchiostoma lanceolatum.* Nat. Methods 10, 407–409 (2013)..
5. Vuong, W. et al. *Feline coronavirus drug inhibits the main protease of SARS-CoV-and blocks virus replication.* doi:10.1101/2020.05.03.073080.
6. Dulbecco R and Vogt M: *Plaque formation and isolation of pure lines with poliomyelitis viruses.* The Journal of experimental medicine 1954.
7. Pidre, M. L., Arrías, P. N., Amorós Morales, L. C., & Romanowski, V. (2022). *The Magic Staff: A Comprehensive Overview of Baculovirus-Based Technologies Applied to Human and Animal Health*. Viruses, 15(1). https://doi.org/10.3390/v15010080
8. Zhao Y. et al. *Structural basis for replicase polyprotein cleavage and substrate specificity of main protease from SARS-CoV-2.* PNAS, April 5, 2022.
9. Fu, L. et al. *Both Boceprevir and GC376 efficaciously inhibit SARS-CoV-2 by targeting its main protease.* Nat. Commun. 2020; 11: 4417. Published online 2020 Sep 4. doi: 10.1038/s41467-020-18233-x.
10. Hu et al 2023: *Naturally Occurring Mutations of SARS-CoV-2 Main Protease Confer Drug Resistance to Nirmatrelvir* - PubMed (nih.gov)
11. Huang et al, 2023: *A new generation Mpro inhibitor with potent activity against SARS-CoV-2 Omicron variants* - PubMed (nih.gov)

Table 4. Related Products

Product	Description	Promoter	Recommended Use
C1200N-C1215N	3CL Mutant Proteases	CMV	High- content imaging, microscopy, plate reader
#C1100G	mNeonGreen Fluorescent ACE2	CMV	High- content imaging, microscopy, plate reader
#C1100R	Red Fluorescent ACE2	CMV	High- content imaging, microscopy, plate reader
#C1100N	Unlabeled **ACE**2	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
#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
#C1123G	Pseudo Sars-CoV-2 Green Reporter with Spike Delta Variant (nuclear targeted green fluorescence)	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

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.

certified WBENC WOMEN'S BUSINESS ENTERPRISE

WOMEN OWNED™