Montana Molecular
Fluorescent Biosensors for Live Cell Discovery

3CLglow Assay (Patent Pending, US 63/077,096)

May 10, 2022

Product Info & Protocol

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

About this Assay

The 3CLglow Assay is a SARS-CoV-2 3CLpro (also called Mpro) inhibitor assay, a new technology developed by Montana Molecular in response to the COVID-19 pandemic. The goal is to provide a robust, biologically meaningful tool to evaluate therapeutic drugs that target SARS-CoV-2 replication.

SARS-CoV-2 viral replication depends on the 3CL protease (3CLpro). This protease is responsible for generating many of the Non Structural Proteins (NSPs) involved in viral replication by cleaving the SARS-CoV-2 1ab propeptide at specific sites. If the 3CLpro enzyme can be blocked, viral replication can be blocked. The 3CLpro enzyme is itself one of the Non Structural Proteins (NSP5), and it autocatalytically removes itself from the long 1ab propeptide, at least in the case of SARS-CoV-1 [1].

The 3CLglow Assay kit includes 2 BacMam viruses. One expresses the 3CLpro enzyme, the other expresses a green fluorescent biosensor for 3CLpro enzyme activity as well as a red fluorescent protein to control for compound toxicity. This live cell assay bridges the gap between *in vitro* biochemical assays [2], [3] and living cells and organisms. To produce the 3CLpro Enzyme, we synthesized a cDNA encoding the enzyme with an optimal protein translation start site. To create a robust, fluorescent biosensor for 3CLpro activity, we started with a bright mNeonGreen [4] fluorescent protein. We added a domain that inhibits formation of a functional, fluorescent protein. In the presence of the active 3CLpro Enzyme, the inhibitor tag is cleaved off and a functional fluorescent protein can fold, increasing fluorescence in cells.

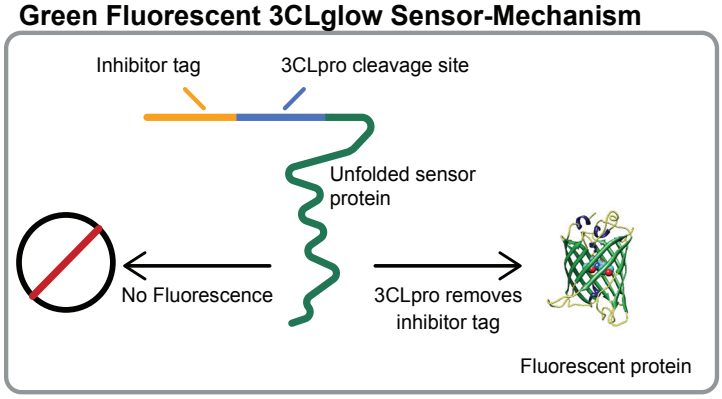
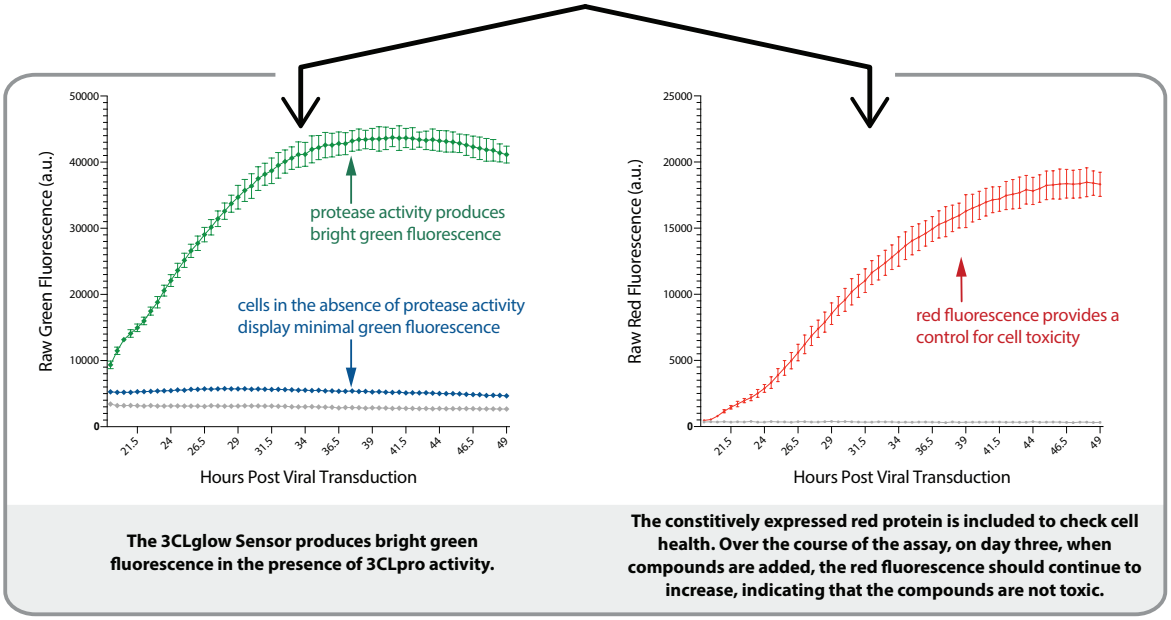


Figure 1. To create a robust assay for 3CLpro activity in living cells, we added an inhibitor tag to the bright mNeonGreen fluorescent protein. In the absence of 3CLpro, the protein displays minimal green fluorescence. A 3CLpro cleavage site is included that separates the inhibitor tag from the fluorescent protein. The result is a biosensor that lights up when 3CLpro activity is present in the cell.

Figure 2. A single BacMam delivers a gene encoding the green fluorescent 3CLglow Sensor as well as constitutively expressed red protein to check cell health.



The 3CLglow Sensor produces bright green fluorescence in the presence of 3CLpro activity.

The constitutively expressed red protein is included to check cell health. Over the course of the assay, on day three, when compounds are added, the red fluorescence should continue to increase, indicating that the compounds are not toxic.

This protocol is optimized for HEK 293T cells and applies to the product in Table 1.

Table 1. Relevant Product

Product	Description	Promoter	Recommended Use
#C1160G	3CLglow 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 **HDAC inhibitor 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
3CLglow BacMam ≅ 2×10^{10} VG/mL in ESF 921 Insect Culture Medium (Expression Systems, product #96-001-01)	A single BacMam virus delivers both the green fluorescent biosensor for 3CLpro activity and a constitutively-expressed red fluorescent protein. When the protease is active in the cell, this green biosensor becomes fluorescent. Red fluorescence will be continually expressed in healthy cells. 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
3CLpro Enzyme (in BacMam) ≅ 2×10^{10} VG/mL in ESF 921 Insect Culture Medium (Expression Systems, product #96-001-01)	3CLpro 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. 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 (16777-257) **[6]**.
3. HEK 293T cells (ATCC CRL-3216).
4. EMEM (**ATTC 30-2003**) with 10% FBS (ThermoFisher 16000044) culture media.
5. For the **kinetic assay protocol**, you will also need complete low fluorescent media. Please note that media without phenol red does not remove auto-fluorescence so we recommend FluoroBrite DMEM (ThermoFisher A1896701**)**, 10% FBS, 4 mM GlutaMax (ThermoFisher 35050079), 25 mM HEPES buffer (ThermoFisher 15630080).
6. 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.

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 use in HEK 293T cells.

This assay has only 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 3CLpro Enzyme (in BacMam), and the 3CLglow BacMam (3CLpro Biosensor). To optimize the amount of BacMam virus to add to the wells, it is important to systematically vary the relative amounts of 3CLpro Enzyme and the 3CLglow BacMam. The goal is to identify the conditions that produce the greatest fluorescence contrast between wells that do, and do not, have the 3CLpro Enzyme. Increasing the amount of both the 3CLglow BacMam and 3CLpro 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. If using a different cell type, kinetics of the assay may vary, requiring optimization of incubation and read times.

Day 1

Set up the Assay

– Plate the Cells

a. Remove the media from a flask of HEK 293T cells, and pipette 2 mL of a gentle dissociation reagent TrypLE (Gibco 12604013) directly into the flask and let it incubate on the cells for 8 minutes, at 37°C. Resuspend the cells with 12 mL of complete media in the flask, bringing it to a final volume of 14 mL. After counting the resuspended cells, add the additional media necessary to make a new diluted stock of cells at the 350,000 cells/mL that is recommended for this assay.

b. Add 100 µL of this suspension to each well of a 96-well plate, seeding the plate with 35,000 HEK 293T cells per well. It is important to use a poly D lysine (PDL) coated plate to ensure cell adherence. The cells are grown in EMEM with 10% FBS. Let the plate sit covered at room temperature, for 30 minutes. Incubate overnight at 37°C, 5% CO_2.

Day 2

Make master mix of BacMam viruses to transduce the cells plated on Day 1

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

	Stock	Amount per Well	Final Concentration in Cell Plate	110-Well Master Mix
3CLpro Enzyme (in BacMam)	2 x 10^{10} VG/mL	15 µL	2 x 10^{9} VG/mL	1.65 mL
3CLglow BacMam (3CLpro 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	2 mM	66 µL
complete media		9.4 µL		1.034 mL
Final Transduction Mix Volume: 50 µL total volume per well				

– Make Transduction Master Mix and Add to Cells

c. Make up the following transduction mix (Table 3), combining the 3CLpro Enzyme, the 3CLglow BacMam, sodium butyrate, and complete media.

Table 4. Per Well (96-well plate)

100 µL	cells from day 1 preparation (35,000 cells/well)
50 µL	transduction mix

150 µL total volume per well

d. Pipette 50 µL of the transduction mix into each well of the cell plate. This will increase the total volume in each well of the cell plate to 150 µL. Gently rock the plate 5 - 10 times to ensure uniform transduction across the each well. Let the plate sit covered at room temperature, for 30 minutes. Incubate overnight at 37°C, 5% CO_2

Day 3 — Add Inhibitors and Measure Green and Red Fluorescence

Choose which of the two different protocols to follow:

Endpoint Assay Protocol is on page 5 (labeled "Day 3A"): To maximize the throughput of this assay, single endpoint measurements make it possible to read many plates in a single day.

Kinetic Assay Protocol is on page 6 (labeled "Day 3B"): Capturing green and red fluorescence over time lowers throughput but provides real-time measurements of inhibitor action in living cells.

Day 3A — Endpoint Assay

– **Add Inhibitors** (see Fig. 4 for Endpoint Assay timeline)

e. Dilute compounds of known inhibitors such as GC376 to 4x desired concentration in fresh media with 2 mM **SB** (sodium butyrate). Eighteen hours post-transduction, add 50 µL of 4x drug to each well bringing the total volume in the well to 200 µL.

f. Let the inhibitors incubate on your cells for 10 -18 hours before taking a fluorescence read 28-36 hours after the initial transduction.

– **Measure Green and Red Fluorescence at Endpoint**

g. Between 28-36 hours after the transduction, remove all autofluorescent cell culture media from the cells and replace with PBS. This is best done with a plate washer, or careful hand pipetting. Read the plate on a fluorescence plate reader or imaging system. Bright green fluorescence means that the 3CLpro Enzyme activity is present in the cells, and decreases in fluorescence mean that the enzyme is inhibited. Taking your read at the right time is very important for optimizing signal - **see Fig. 4 timeline below**. However, monitioring cell health thoughout the experiment is also necessary to ensure over expression of the 3CLpro Enzyme has not had cytotoxic effects.

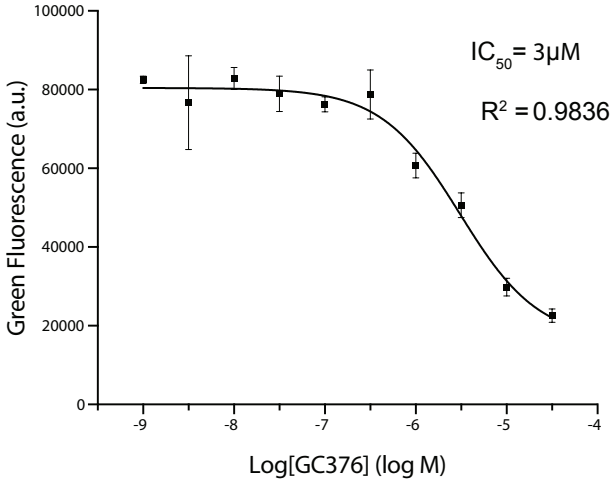
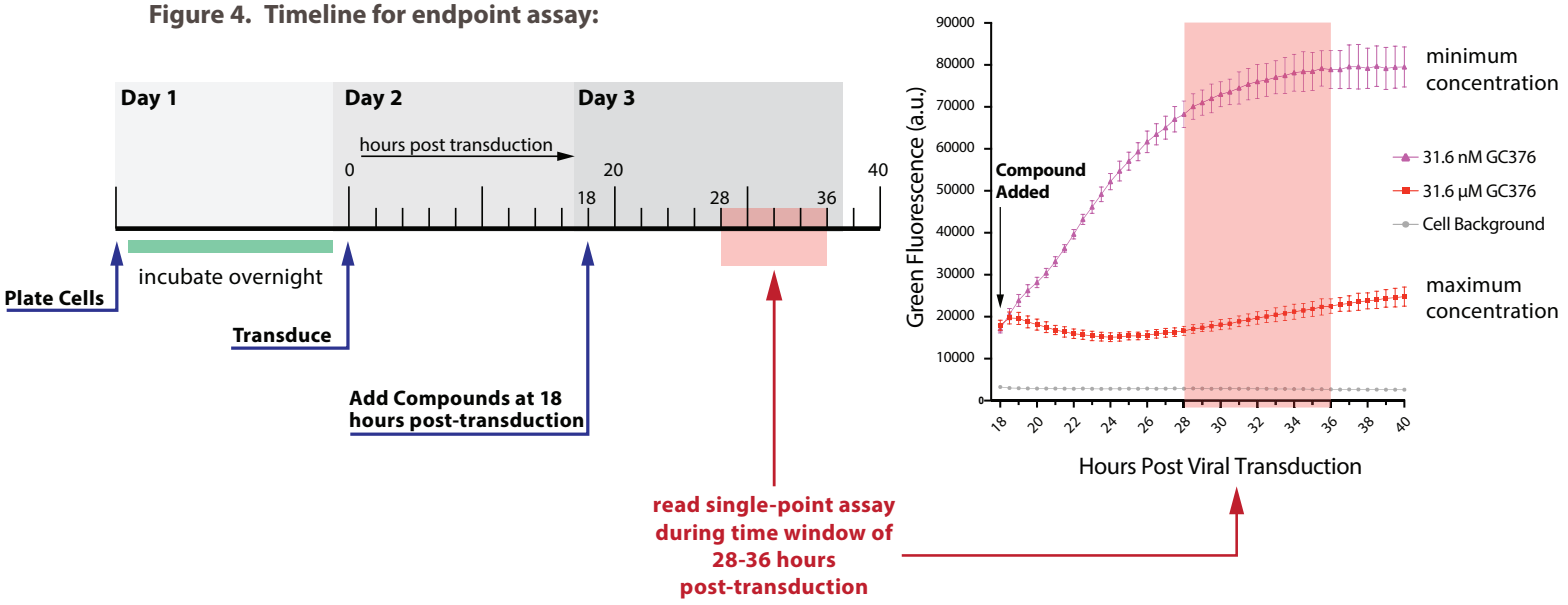


Figure 3. HEK 293T cells were used to generate this dose response curve with 3CLglow Assay at 36 hours post-transduction. Measured with a BioTek Synergy™ plate reader , the IC_{50} values for GC376 were 3 µM, which is consistent with 0.9 µM EC_{50} values recently reported for inhibition of SARS-CoV-2 replication in culture [5].

Figure 4. Timeline for endpoint assay:



Day 3B — Kinetic Assay

– Add Inhibitors

d. Make up complete low background media. Cell culture media in your assay plate and in your inhibitor dilutions needs to be changed to a complete non-autofluorescent alternative with 2 mM **SB** (sodium butyrate). We recommend FluoroBrite DMEM (ThermoFisher A1896701), 10% FBS, 4 mM GlutaMax (ThermoFisher (GlutaMAX) 35050079), 25 mM HEPES buffer (ThermoFisher 15630080)

e. Change the media on your cells, leaving 150 µL per well.

f. Dilute compounds of known inhibitors such as GC376 to 4x desired final concentration in your complete low background media with 2 mM **SB**.

g. Add 50 µL of 4x drug to each well bringing the total volume in the well to 200 µL.

– Measure Green and Red Fluorescence over Time

h. Put your plate in the plate reader at 37°C and take green and red measurements every 30 minutes to see full kinetic readout.

i. Watch the compound inhibit green fluorescence in real time in the green channel, while simultaneously monitoring cell health in the red channel. (Figure 5)

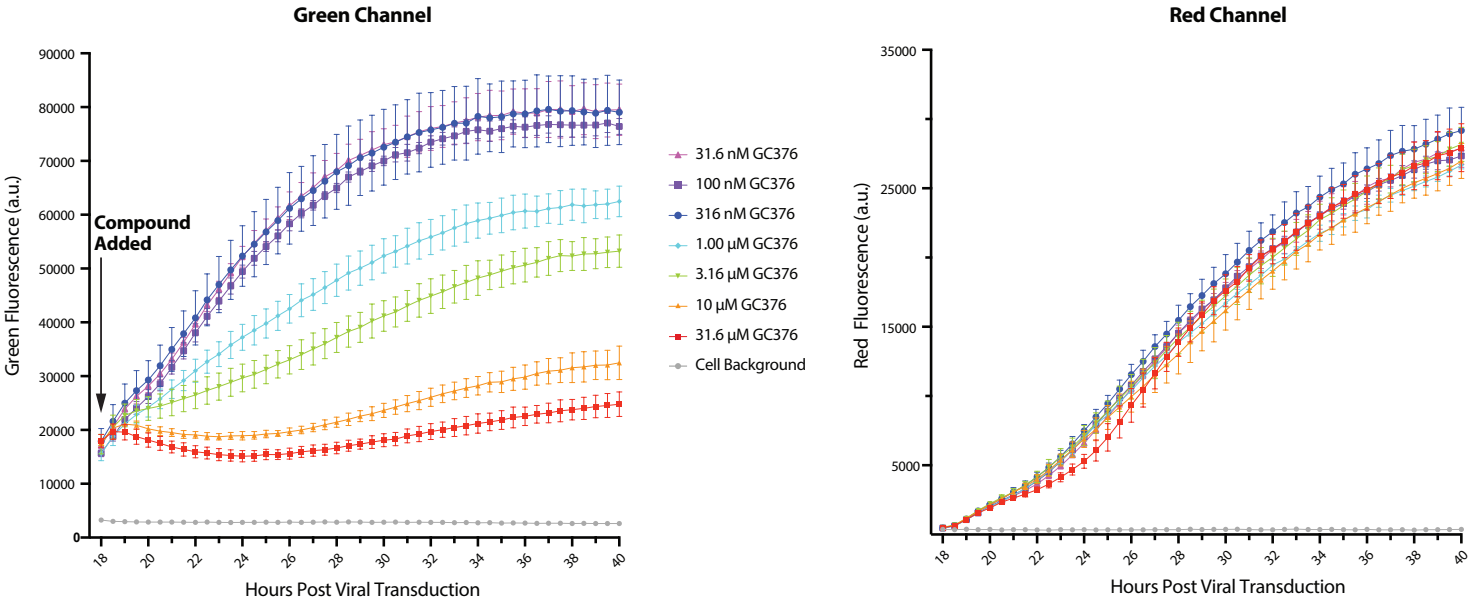


Figure 5. HEK 293T were transduced with 3CLglow Sensor and 3CLpro Enzyme in BacMam **[on Day 2]**. Eighteen hours after the transduction, media was changed to low-background media and a dilution series of inhibitor GC376 was added to cells (n=3) **[on Day3]**. Immediately after drug addition, fluorescent measurements were taken in the green channel (ex: 485 nm, em: 528 nm) and red channel (ex: 558 nm, em: 603 nm) every 30 minutes for 22 hours with a BioTek Synergy™ plate reader. The kinetic data above shows the increases in green fluorescence at low inhibitor concentrations and inhibited green fluorescence at higher concentrations. The red channel steadily increases over time, showing that GC376 was not toxic to the cells.

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.

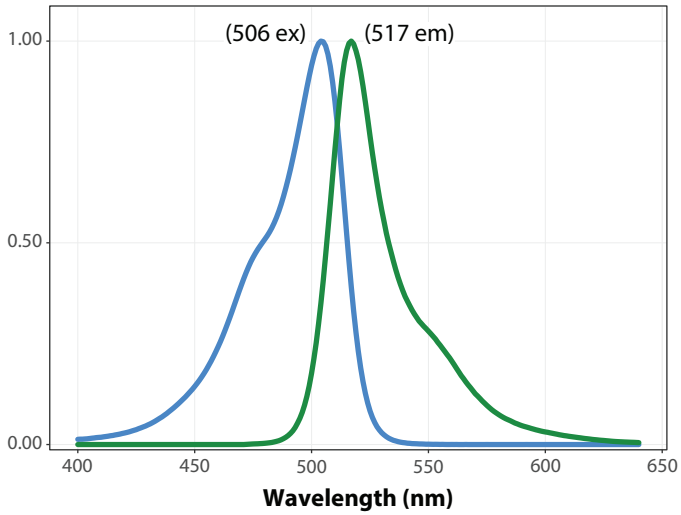


Figure 4A. Absorption and emission properties of the mNeonGreen fluorescent protein plotted as a function of wavelength.

For the red FP, 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.

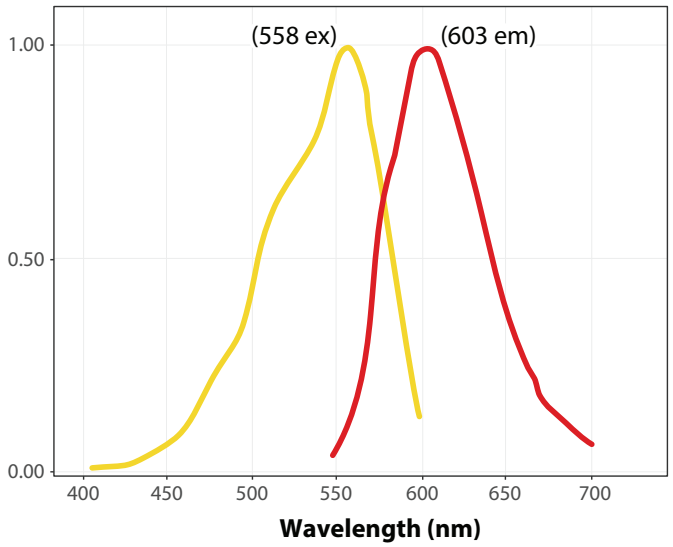


Figure 4B. Absorption and emission properties of the red fluorescent protein plotted as a function of wavelength.

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.	Increase 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.

`

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.

Patent Pending, US 63/077,096

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*

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
#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
#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™