Montana Molecular
Fluorescent Biosensors for Live Cell Discovery

mNeonGreen BacMam Tools

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Product Info & Protocol

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About mNeonGreen BacMam

The following protocol is relevant to Montana Molecular's BacMam products that express mNeonGreen, including a suite of Cell Painting Tools and test kits to determine BacMam transduction efficiency and optimize expression in your cells of choice.

For sensitive and/or difficult to transduce cell types, such as primary neurons, we offer purified, high-titer BacMam stock. Please contact us to discuss whether purified BacMam is the right product for you.

The following protocol is optimized in rapidly dividing, immortalized cell lines on a 96-well plate, and has been validated in live CHO, NIH 3T3, and HEK 293T cells [1]. For use in iPSC-derived or adherent cells, see Suggestions for Expression in Adherent Cells section. For use in particularly difficult-to-transduce or low expressing cell types, see Alternative Spinoculation Protocol.

Table 1. mNeonGreen BacMam Kits

Product	Description	Promoter	Recommended Use
#F0500G	mNeonGreen BacMam	CMV	Test expression efficiency in your cells
#F0505G	mNeonGreen BacMam	CAG	Test expression efficiency in your cells
#F0520G	Nuclear-Targeted mNeonGreen BacMam	CMV	Expresses Nuclear-targeted mNeon Green FP
#P1000G	Untargeted mNeonGreen	CMV	Expresses untargeted mNeon Green FP
#P1001G	Untargeted mNeonGreen	Synapsin	Expresses untargeted mNeon Green FP
#P1005G	Untargeted mNeonGreen	CAG	Expresses untargeted mNeon Green FP
#P1010G	Green MitoPaint	CMV	Expresses Mitochondria-targeted mNeon Green FP
#Z0200G	Human M1 Muscarinic Receptor - Green	CMV	Constitutively expresses mNeon Green with hM1R
#Z0500G	β2 Adrenergic Receptor - Green	CMV	Constitutively expresses mNeon Green with β2AR

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*

BacMam Kit Materials and Storage

BacMam stocks should be stored at 4°C protected from light in the original package. **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
mNeon in BacMam ≅ 2 ×10^{10} VG/mL in ESF 921 Insect Culture Medium (Expression Systems, product #96-001-01)	mNeon fluorescent protein in BacMam.	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

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+} (DPBS) available from VWR [2].
3. Cells and cell media of your choice.

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. 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 for your cell type and your particular conditions.

See our suggestions for Assays in Adherent Cell Types, Alternative Spinoculation Protocol.

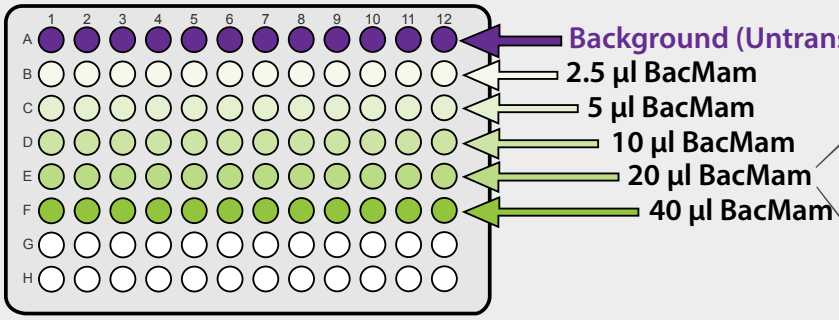
Optimize Fluorescent Protein Expression

We recommend using a titration series to determine optimal BacMam volume based on signal- above-background compared with cell health.

Day 1

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 volume for your cells.



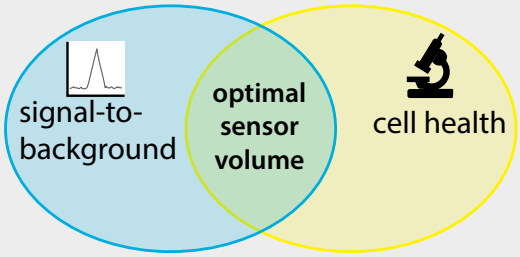
Transduction Reaction Per Well	
mNeon BacMam	20µL
500mM SB (sodium butyrate)	0.6µL
complete media	29.4µL
total volume per well	**50µL**

Day 2

iii. Evaluate fluorescent protein expression

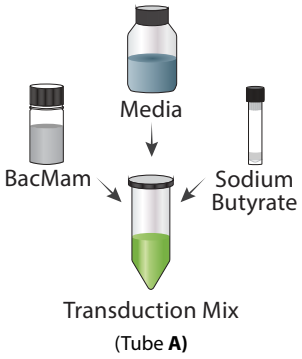
iv. **Refer to detailed protocol** Day 2 procedure **on page 8**

Check your cells for these 2 parameters to determine **optimal BacMam volume.**



Day 1 – Transduce and Plate Cells for your Experiment

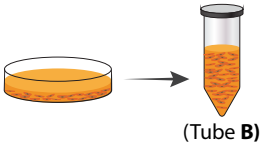
A. **Prepare Viral Transduction Mix (Tube A)**: For each transduction reaction (i.e. one well in a 96-well plate), prepare the transduction mix as detailed in table at right (using the optimal volume of sensor that was determined in your optimization experiment). Mix gently.



Transduction Mix (Tube A)	
mNeon BacMam	Variable
SB 500mM (sodium butyrate)	0.6µL
complete media	(To 50µL)
total volume per well	**50µL**

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

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

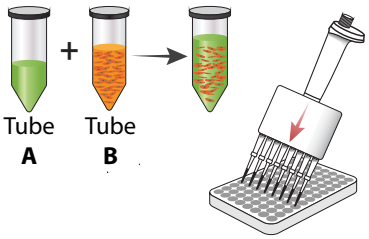


Cells (Tube B)	
cells per well	100µL

Tip: *500,000 cells/mL works well for HEK293T cells. This will result in 50,000 cells/well in a 96 well plate. But remember, your optimal cell density is cell type dependent*

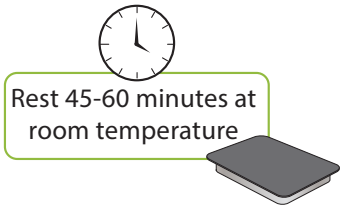
C. **Prepare a dilution of cells at your desired concentration.** (100µL of this cell resuspension will be required for a single well in a 96-well plate, so prepare enough of the dilution to seed the desired number of wells in the plate).

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.

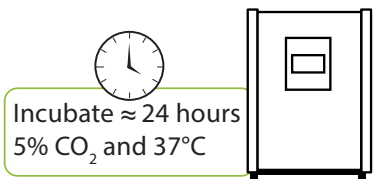


Transduction Mix + Cells (Tube A) + (Tube B)	
transduction mix	50µL
cell suspension	100µL
total volume per well	**150µL**

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



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



Suggestions for Assays in Adherent Cells

This protocol is optimized for rapidly dividing immortalized cells.

However, mNeon BacMam 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.

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 **100µL** 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.

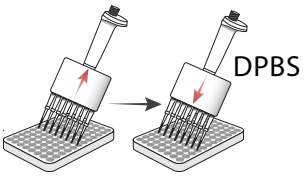
Alternative Transduction Mix	
mNeon BacMam	Variable
500mM **SB** (sodium butyrate)	0.6µL
1M HEPES (pH 7.4)	0.7µL
cell culture media	(To 50µL)
total volume per well	**50µL**

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:

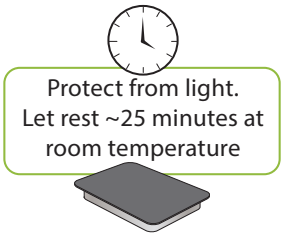
Transduction Mix + Enhancer Reagents	
mNeon BacMam	Variable
TransDux	0.34µL
Max Enhancer	17µL
500mM **SB** (sodium butyrate)	0.6µL
1M HEPES (pH 7.4)	0.7µL
cell culture media	(To 50µL)
total volume per well	**50µL**

Day 2 – Measure Fluorescence



G. Prior to measuring fluorescence, replace culture media with DPBS (1×, containing Ca^{2+} and Mg^{2+}). Wash gently 4-5 times, careful not to dislodge cells. We recommend using a plate washer. (We recommend the BioTek 405 TS.)

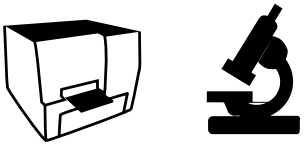
H. Cover the cells. Allow to equilibrate at room temperature at least 30 mintues before measuring fluorescence Experiments can be performed at room temperature



I. Visually inspect cells on microscope to confirm cell health, mNeon BacMam expression, and transduction efficiency.

* **Important:** Make sure that the final concentration of any drug solvents in the well (e.g. DMSO or Ethanol) does not exceed 1.0%.

Fluorescence Detection



Our mNeonGreen BacMam constructs are easily detectable with standard green fluorescence instrumentation. It performs well on a variety of epifluorescence microscopes with lenses ranging from 10×, 20×, 0.9 N.A. to 63×, 1,4 N.A. Depending on the cell type and targeting motif, fluorescence from the mNeonGreen fluorescent tools may be compatible on plate readers as well.

Fluorescence Properties

mNeonGreen BacMam expresses 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.

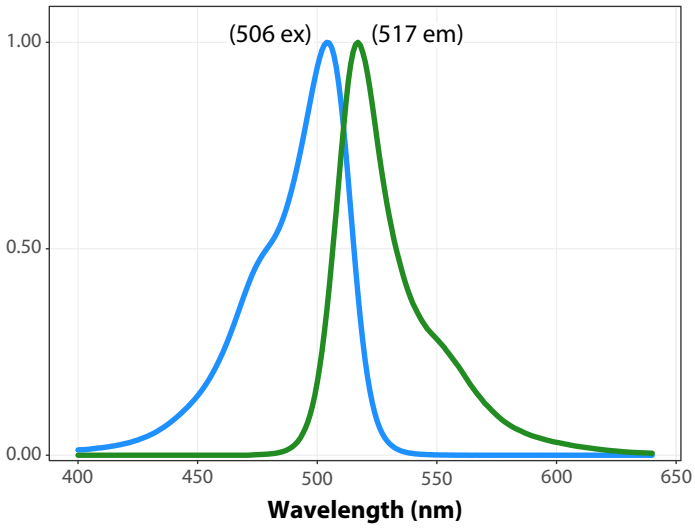


Figure 1. Absorption and emission properties of the mNeonGreen fluorescent protein plotted as a function of wavelength.

Optimization

Optimizing Fluorescence

Twenty-four hours after transduction, check your cells for fluorescence. Wells that were transduced with the mNeonGreen BacMam 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 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 BacMam under the control of a specific promoter system. 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 construct.

Purified viral preparations, which can increase expression in particularly sensitive or difficult to transduce cell types, are available upon request.

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. 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 Expression 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 - 0.25µM 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 DPBS and adding to cells. Replace transduction mix with media after 2-4 hours.

Problem	Possible Cause	Solution
(continued) **Low 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 expression, low transduction efficiency.	See solutions for Problem 1.
	Excitation/emission settings are not optimal for mNeon.	Refer to protocol for the fluorescence spectra of the mNeon. 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 mNeon 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.**

References

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2. Dulbecco R and Vogt M: Plaque formation and isolation of pure lines with poliomyelitis viruses. The Journal of experimental medicine 1954.
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4. 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.
5. 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.

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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