Montana Molecular
Fluorescent Biosensors for Live Cell Discovery

Green Fluorescent Borealis Arrestin Assay Kits

February 8, 2024

Product Info & Protocol

Patent Pending: PCT/US2019/044165 [WO2020028381A1]

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

About these Assays

A kinetic, real time assay for arrestin recruitment through receptor specific arrestin-3 (β-arrestin-2) activation. Kits are available to detect arrestin recruitment at specific GPCRs; your choice of Vasopressin Type 2 (V2R), Angiotensin II-Type 1 (AT1R), Glucagon-like Peptide 1 (GLP-1R), μ-Opioid (MOR), β_2-Adrenergic (β2AR), D_1 Dopamine (D1R), Oxytocin (OTR), Serotonin 5-HT_{2A} (5-HT2AR), κ-Opioid (KOR), Proteinase-Activated 2 (PAR2), δ-Opioid (DOR), Lysophosphatidic Acid (LPA1R), Parathyroid Hormone 1 (PTH1R), Serotonin 5-HT_{2C} (5-HT2CR), and Glucagon (GCGR) receptors. The arrestin sensor is a genetically-encoded biosensor packaged in a BacMam viral vector for easy transduction into a wide variety of cell types. Measure robust signals 24 hours post transduction on standard fluorescence microscopes and automated plate readers.

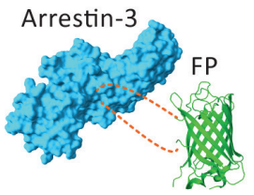


Figure 1. Borealis Arrestin Sensor overview

Fluorescence intensity **decreases** when arrestin is recruited to the GPCR (Fig. 2). The signal is bright, robust, and easy to detect on fluorescence plate readers. For some receptors, co-expression of a GRK enhances the arrestin signal. The recommended GRK has been included in your kit if needed (see Table 3). For sensitive and/or difficult to transduce cell types, such as primary cultures, we offer purified, high-titer BacMam stock. Please contact us to discuss whether purified BacMam is the right product for you.

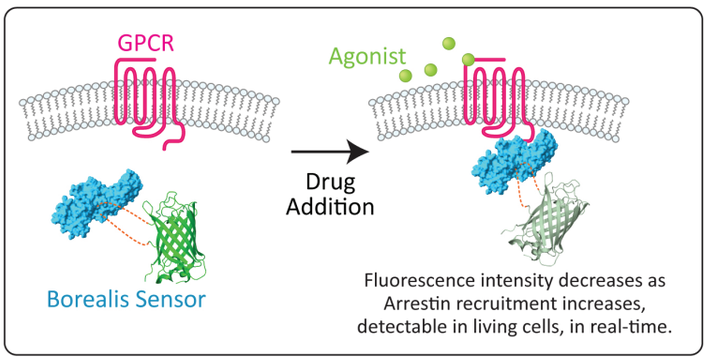


Figure 2. Borealis Arrestin Sensor mechanism

This protocol is optimized for measuring arrestin-3 responses in rapidly dividing, immortalized cell lines on a 96-well plate, and has been validated in live HEK 293T cells [1]. The assay is very robust and can be used for live-cell imaging or for screening on automated fluorescence plate readers. For use in iPSC-derived or adherent cells, see Suggestions for Assays in Adherent Cells section. For use in CHO cells or other cell types, see Alternative Spinoculation Protocol section.

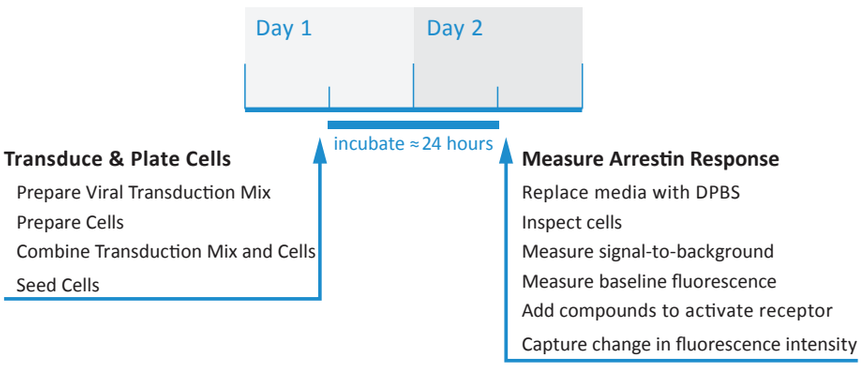


Figure 3. Protocol steps overview

The protocol steps are simple and will be performed over two consecutive days (Fig. 3). Please remember to optimize the assay for your conditions before performing your first experiment.

Table 1: Borealis Arrestin-3 Assay Kits

Product	Description	Promoter	Recommended Use
#D2000G	Borealis — V2R	CMV	Fluorescence imaging and plate reader assay
#D2010G	Borealis — AT1R	CMV	Fluorescence imaging and plate reader assay
#D2020G	Borealis — GLP 1R	CMV	Fluorescence imaging and plate reader assay
#D2030G	Borealis — MOR	CMV	Fluorescence imaging and plate reader assay
#D2040G	Borealis — β2AR	CMV	Fluorescence imaging and plate reader assay
#D2050G	Borealis — D1R	CMV	Fluorescence imaging and plate reader assay
#D2060G	Borealis — OTR	CMV	Fluorescence imaging and plate reader assay
#D2070G	Borealis — 5-HT2AR	CMV	Fluorescence imaging and plate reader assay
#D2080G	Borealis — KOR	CMV	Fluorescence imaging and plate reader assay
#D2090G	Borealis — PAR2	CMV	Fluorescence imaging and plate reader assay
#D2100G	Borealis — DOR	CMV	Fluorescence imaging and plate reader assay
#D2110G	Borealis — LPA1R	CMV	Fluorescence imaging and plate reader assay
#D2120G	Borealis — PTH1R	CMV	Fluorescence imaging and plate reader assay
#D2130G	Borealis — 5-HT2CR	CMV	Fluorescence imaging and plate reader assay
#D2140G	Borealis — GCGR	CMV	Fluorescence imaging and plate reader assay

Assay Kit Materials and Storage

BacMam stocks should be stored at 4°C protected from light in the original package. Aliquot and **store control agonist at -20°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. Store **sodium butyrate at 4°C.**

Table 2. Arrestin-3 Kits Contain:	Details	Storage
Borealis Sensor BacMam (Product #D2222G) ≅ 3 ×10^{10} VG/mL in ESF 921 Insect Culture Medium (Expression Systems, product #96-001-01)	Green fluorescent sensor that changes in fluorescence intensity in response to arrestin recruitment. VG/mL is the number of viral genes per milliliter (see Biosafety Considerations section).	4˚C
GPCR in BacMam, specific to each kit (Table 3) ≅ 2 ×10^{10} VG/mL in ESF 921 Insect Culture Medium (Expression Systems, product #96-001-01)	Kits are available to detect arrestin recruitment at specific GPCRs. If you're interested in a GPCR not listed, please contact us.	4˚C
GRK in BacMam, specific to some kits (Table 3) ≅ 2 ×10^{10} VG/mL in ESF 921 Insect Culture Medium (Expression Systems, product #96-001-01)	For some GPCRs, addition of a G protein-coupled receptor kinase will further optimize the assay.	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
Ra Control Receptor AT1R (Product #Z0800N), in ESF 921 Insect Culture Medium (Expression Systems, product #96-001-01)	The AT1R is provided as a positive control for the purpose of assay optimization.	4˚C
Aa Angiotensin II (Ang II control agonist) 5mM, in H_20 Cayman Chemical #17150	Ang II can be used to stimulate arrrestin recruitment through the positive control, the AT1R receptor.	-20˚C

Table 3. Recommended GPCR-specific agonist and GRK information

Arrestin Kit	GPCR in BacMam	Recommended GPCR Agonist	GRK
#D2000G Borealis — V2R Assay Kit	Vasopressin Type 2 Receptor (V2R) (Product #Z0620N)	Argipressin (arginine vasopressin, AVP) Cayman Chemical, #24154	—
#D2010G Borealis — AT1R Assay Kit	Angiotensin II-Type 1 Receptor (AT1R) (Product #Z0800N)	Angiotensin II (Ang II) Cayman Chemical, #17150	—
#D2020G Borealis — GLP-1R Assay Kit	Glucagon-like Peptide 1 Receptor (GLP-1R) (Product #Z0600N)	Exendin-4 Cayman Chemical, #11096	GRK5 (Product #K0005N)
#D2030G Borealis — MOR Assay Kit	μ-Opioid (MOR) (Product #Z0720N)	DAMGO Cayman Chemical, #21553	GRK2 (Product #K0002N)
#D2040G Borealis — B2AR Assay Kit	β_2 Adrenergic Receptor (B2AR) (Product #Z0500N)	Isoproterenol Sigma Aldrich, #12760	GRK2 (Product #K0002N)
#D2050G Borealis — D1R Assay Kitt	D_1 Dopamine Receptor (D1R) (Product #Z0100N)	Dopamine Hydrochloride Sigma Aldrich, #H8502	GRK2 (Product #K0002N)
#D2060G Borealis — OTR Assay Kit	Oxytocin Receptor (OTR) (Product #Z0900N)	Oxytocin Cayman Chemical, #11799	—
#D2070G Borealis — 5-HT2AR Assay Kit	Serotonin 5-HT_{2A} Receptor (5-HT2AR) (Product #Z1100N)	Serotonin (5-HT) Cayman Chemical, #14332	GRK3 (Product #K0003N)
#D2080G Borealis — KOR Assay Kit	κ- Opioid (Receptor (KOR) (Product #Z0710N)	Dynorphin A Cayman Chemical #18169	GRK5 (Product #K0005N)
#D2090G Borealis — PAR2 Assay Kit	Proteinase-Activated 2 Receptor (PAR2) (Product #Z1200N)	2-furoyl-LIGRLO Cayman #14594	—
#D2100G Borealis — DOR Assay Kit	δ-Opioid Receptor (DOR) (Product #Z0740N)	DADLE Cayman #28928	GRK5 (Product #K0005N)
#D2110G Borealis — LPA1R Assay Kit	Lysophosphatidic Acid Receptor (LPA1R) (Product #Z1300N)	Oleoyl-L-α-lysophosphatidic Acid Sigma #L7260	—
#D2120G Borealis — PTH1R Assay Kit	Parathyroid Hormone 1 Receptor (PTH1R) (Product #Z1400N)	Parathyroid Hormone (1-34) Cayman #24985	—
#D2130G Borealis — 5-HT2CR Assay Kit	Serotonin 5-HT2C Receptor (5-HT2CR) (Product #Z1800N)	Serotonin (5-HT) Cayman Chemical, #14332	—
#D2140G Borealis — GCGR Assay Kit	Glucagon Receptor (GCGR) (Product #Z2000N)	Glucagon Sigma G2044	—

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 1X with Ca^{2+} and Mg^{2+} (10X solution from Gibco #14080055) [2].
3. GPCR-specific agonist, see Table 3.
4. Cells and cell culture media. We recommend viral transduction in EMEM, McCoy's 5A or F12K culture media.

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 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. [10]

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.

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 the assay for your cell type and your particular conditions.** See our suggestions for Assays in Adherent Cell Types, Scaling for 384-well Plates , and Alternative Spinoculation Protocol.

Before Your First Experiment: Optimize Your Assay by Titrating the Sensor

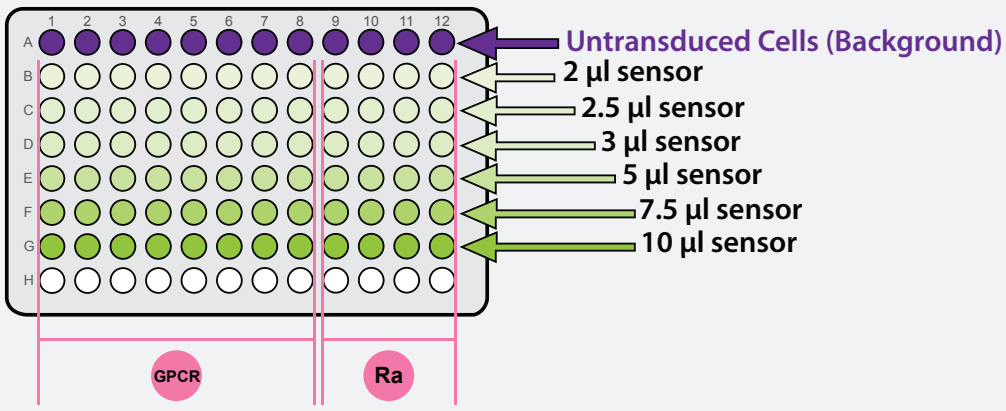
We recommend using a titration series to determine the best combination of signal above background, cell health, and sensor expression. Ideally, the signal in each well before drug addition should be at least 5 times above background.

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).

B. Perform titration to determine optimal sensor volume for your cells, as detailed in plate diagram and tables below.

C. For each sensor volume, include a subset of wells that includes the recommended amount of the GPCR specific to your kit (see Table 4 below). Also include a subset of wells that has 15 µl of **Ra** control receptor for your positive control. If you're working with a receptor that requires co-expression of a GRK (Table 3), the recommended GRK is included in your kit.



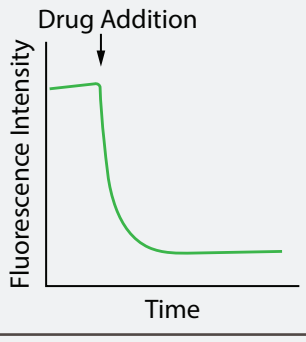
Wells A1-A12 Background (with Untransduced Cells)	
500 mM **SB** (sodium butyrate)	0.6 µL
complete media	49.4 µL
total volume per well	**50 µL**

Wells B1-B8 Transduction Mix	
Arrestin Sensor	2 µL
GPCR (Table 4 lists recommended amount)	15 µL
500 mM **SB** (sodium butyrate)	0.6 µL
complete media	32.4 µL
total volume per well	**50 µL**

Wells B9-B12 Transduction Mix	
Arrestin Sensor	2 µL
Ra control receptor	15 µL
500 mM **SB** (sodium butyrate)	0.6 µL
complete media	32.4 µL
total volume per well	**50 µL**

*If your kit includes a GRK, add 3 µL GRK to each GPCR well and adjust your volume of complete media accordingly to reach 50 µL total volume per well.

Tip: *To optimize your experiment even further, we recommend performing a titration for your receptor volume in addition to your sensor volume titration..*



Measure Parameters to Determine Optimal Conditions

D. Determine optimal sensor volume by analyzing fluorescence above background, cell health, and response to drug. (For details on measuring fluorescence, refer to Day 2 procedure.)

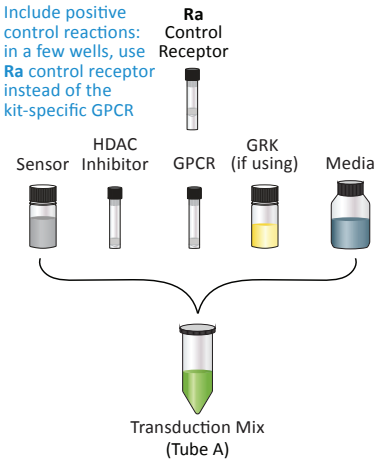
Tip: *Please refer to Table 4 for recommended receptor volume and serum to use for your particular kit.*

Table 4. Recommended Receptor Volume and Serum

GPCR	Recommended Volume (96-well plate)	Recommended Serum
V2R	15 µL	Regular FBS
AT1R	15 µL	Regular FBS
GLP-1R	15 µL	Regular FBS
MOR	15 µL	Regular FBS
B2AR	15 µL	Regular FBS
D1R	15 µL	Regular FBS
OTR	**10 µL**	**Dialyzed FBS**
5-HT2AR	15 µL	**Dialyzed FBS**
KOR	15 µL	Regular FBS
PAR2	15 µL	Regular FBS
DOR	**7.5 µL**	Regular FBS
LPA1R	**7.5 µL**	Regular FBS
PTH1R	15 µL	Regular FBS
5-HT2CR	**10 µL**	**Dialyzed FBS**
GCGR	**20 µL**	Regular FBS

Day 1 – Transduce and Plate Cells for your Experiment

E. **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 tables at right.



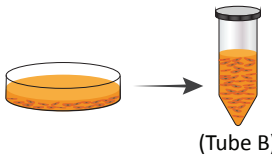
Transduction Mix (Tube A) [no GRK]	
Arrestin Sensor	Variable
SB 500 mM sodium butyrate	0.6 µL
GPCR / Ra (see Table 4)	15 µL
complete media (see Table 4)	(To 50 µL)
total volume per well	**50 µL**

F. If you're working with a receptor that requires **co-expression of a GRK** (see Table 3), add 3 µL of the GRK, and adjust the volume of cell culture media so that the total volume is 50 µL. Mix gently.

Transduction Mix (Tube A) [with GRK]	
Arrestin Sensor	Variable
SB 500 mM sodium butyrate	0.6 µL
GPCR / Ra (see Table 4)	15 µL
GRK	3 µL
complete media (see Table 4)	(To 50 µL)
total volume per well	**50 µL**

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

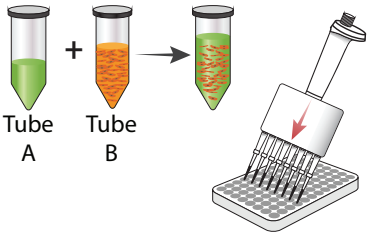
G. **Prepare Cells (Tube B):** Detach cells using standard trypsinization protocol. Resuspend cells in complete culture media (see Table 4 fore recommended serum for each kit) and determine cell count.



Cells (Tube B)	
cells per well	100 µL

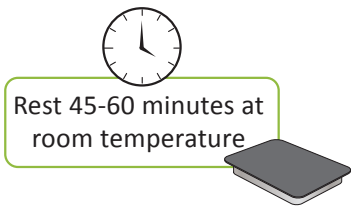
H. **Prepare a dilution of cells at your desired concentration.** (100 µL of this cell resuspension will be required for a single well in a 96-well plate, so prepare enough of the dilution to seed the desired number of wells in the plate).

I. **Combine Transduction Mix and Cells**: Combine Tube A and Tube B (50 µL Tube A + 100 µL Tube B). Mix by pipetting up and down gently, and seed 150 µL per well on the 96-well plate.



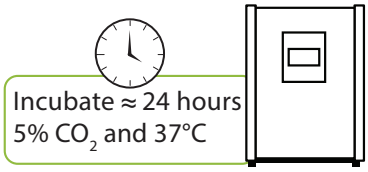
Transduction Mix + Cells (Tube A) + (Tube B)	
transduction mix	50 µL
cell suspension	100 µL
total volume per well	**150 µL**

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



Tip: *90% cell confluence after 24 hours is ideal. For HEK 293T cells 50,000 cells per well works well but plating density is cell type dependent*

K. 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, arrestin assays are compatible with screening primary cultures and iPSC-derived lines, where the cells are plated before transduction. 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 in steps E,F; but add the transduction mix directly to the plated cells (no aspiration of cell media 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), gently aspirate transduction solution (we recommend using a plate washer). Add 100 µL complete growth media with sodium butyrate at a concentration of 2 mM. If cells will not tolerate a full media exchange, partial media exchanges can be done.

Adherent Cells Day 3:

- Measure fluorescence as detailed in Day 2 procedure.

Scaling for 384 Wells (1 plate)

To set up the assay in a 384-well plate, follow all of the protocol steps, adjusting reagent volumes as shown in the tables below. It is important to titrate the sensor (see Before your First Experiment Section) to determine the optimal sensor volume for your cell type. Volumes shown tables are a suggestion.
A good titration range is 0.5-1.75 µl. For receptor and GRK volumes, reference the table.

12.5 µL of the cell resuspension will be required for a single well in a 384-well plate. A plating density of 7,500 cells per well is a good starting point, so prepare the cell suspension at 600,000 cells/mL. Depending on the cell type and plate type, 5,000-15,000 cells per well may be optimal.

Transduction Mix (Tube A) (384-well plate)	
Arrestin Sensor	1 µL
500 mM **SB** sodium butyrate	0.1 µL
GPCR / Ra (see Table 4)	3 µL
complete media	8.4 µL
total volume per well	**12.5 µL**

Cells (Tube B) (384-well plate)	
cells per well	12.5 µL

Transduction Mix + Cells (Tube A) + (Tube B) (384-well plate)	
transduction mix	12.5 µL
cell suspension	12.5 µL
total volume per well	**25 µL**

Tip: *When scaling for 384-well plates, the volume of the cell suspension per well can be increased to 50 µL to improve cell health. Make sure that the well is still receiving 7,500 cells and that you increase the volume of sodium butryate to 0.2 µL per well.*

Alternative Spinoculation Protocol for Other Cell Types

For best results in CHO cells on a fluorescence plate reader, we recommend a modified transduction protocol. This alternative protocol may also be useful for other cell types.

Tip: *For best results when using valproic acid, prepare 50 - 100 µL aliquots and store at -20°C.*

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 (we recommend 22,500 cells/well in a 96-well plate, as a starting point). 50 µL 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 (**50 µL** transduction mix + **50 µL** cells). Mix gently, then seed **100 µL** 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.**
   * We recommend sealing the plate with Breathe-Easy® (Cat. No. 70536-10) during this step to avoid contamination.
7. After spinning the plate, **remove the transduction mix** and replace with fresh media containing **5 mM valproic acid** (100 µL - 150 µL per well).
8. Return plate to normal growth conditions and incubate for 20-24 hours.

Transduction Mix for CHO Spinoculation	
Arrestin Sensor	Variable
GPCR / Ra (see Table 4)	15 µL
GRK (if using; see Table 3)	3 µL
300 mM valproic acid (5 .1 mM in well)	1.7 µL
1M HEPES (pH 7.4)	0.7 µL
cell culture media	(To 50 µL)
total volume per well	**50 µL**

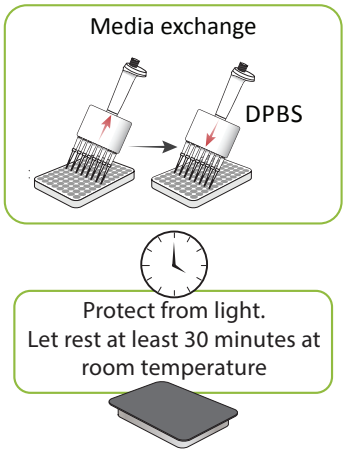
Tip: *Titrate the sensor to determine optimal volume;* see optimization page

---

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 mix. Please make the following adjustments to your transduction mix:

Transduction Mix for CHO Spinoculation	
Arrestin Sensor	Variable
GPCR / Ra (see Table 4)	15 µL
GRK (if using; see Table 3)	3 µL
TransDux	0.34 µL
Max Enhancer	17 µL
300 mM valproic acid (5 .1 mM in well)	1.7 µL
1M HEPES (pH 7.4)	0.7 µL
cell culture media	(To 50 µL)
total volume per well	**50 µL**

Day 2 – Measure Arrestin Response

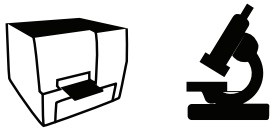


L. Prior to measuring fluorescence, replace culture media with DPBS (1X, containing Ca^{2+} and Mg^{2+}). Wash gently 4 - 5 times, careful not to dislodge cells. We recommend using a plate washer. (We recommend the BioTek 405 TS.)

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

N. Visually inspect cells on microscope to confirm cell health. Transduced cells should be at least 5× brighter compared to untransduced cells. (If you have high background fluorescence, gently wash cell culture media again.)

Measure Fluorescence on Plate Reader or Imaging System



O. Measure fluorescence in transduced and untransduced cells. Transduced cells should be at least 5× brighter compared to untransduced cells.

P. Acquire 10 - 20 baseline fluorescence reads before adding compounds.

Q. Activate the receptor with agonist to induce arrestin recruitment. Resume measurement immediately after adding drug. Measure continuously for a minimum of 30 minutes, (and up to 5 hours at 15 - 60 second intervals on a plate reader). A decrease in fluorescence intensity under standard GFP excitation and emission wavelengths will indicate arrestin recruitment. For wells transduced with the control receptor (**Ra**), add 1-3 µM **Aa** agonist (final concentration in well, amount is dependent on conditions) to activate the receptor. The optimal dose of agonist may need to be determined for a given cell line.

*** Important:** Make sure that the final concentration of any drug solvents in the well (e.g. DMSO) does not exceed 1%. The ideal concentration for DMSO is 0.1%.

Tip: *Add agonists at a concentration of 3 - 4× (in a volume of 50 µL DPBS) to wells containing 100 - 150 µL DPBS. This will result in a 1 : 3 or 1 : 4 dilution, and allow for adequate diffusion.*

Tip: *Always test the addition of the vehicle alone (i.e. DPBS without drug).*

Fluorescence Detection

Our assays are compatible with automated fluorescent plate readers and imaging systems.

We have validated on:

- Agilent (BioTek) Synergy
- Agilent (BioTek) Cytation™
- BMG CLARIOstar®
- Agilent (BioTek) Lionheart
- Agilent (BioTek) Neo
- Hamamatsu FDSS®
- Epifluorescence microscopes

Our customers have reported good results on:

- Molecular Devices FLIPR®
- Molecular Devices Flexstation®
- Perkin Elmer Enspire®
- Perkin Elmer Opera Phenix ®

Tip: *Below are the specific settings recommended for use with our assays for instruments we use at Montana Molecular. To determine the best settings for fluorescence detection on your instrument, please consult the manufacturer.*

Table 5. CLARIOstar® Recommendations

Instrument Settings	
Detection Mode:	FI (Bottom)
Detection Method:	Plate Mode, Kinetic
Scan Mode:	Orbital Averaging
Scan Diameter (mm):	4
Gain/Focal Height:	Adjusted prior to test
Optical Settings	
Excitation:	F 482-16
Dichroic:	LP 504
Emission:	F 530-40

Table 6. Neo & MX™ Recommendations

Instrument Settings	
Detection Method:	Fluorescence Intensity
Read Type:	Endpoint/Kinetic
Optics:	Monochromators
Excitation:	485 nm
Emission:	528 nm
Bandwidth:	20 nm (for both ex and em)
Optics Position:	Bottom
Gain:	100

Table 7. Cytation™ & Lionheart Recommendations

Image Preprocessing	
Image Set:	GFP
Background:	Dark
Rolling Ball Diameter:	Automatic
Image Smoothing:	0 cycles
Cellular Analysis Parameters	
Channel:	Tsf [GFP]
Threshold:	7,000
Background:	Dark
Split Touching Objects:	Checked
Fill Holes in Mask:	Checked
Minimum Object Size:	5 µm
Maximum Object Size:	1,000 µm
Include Primary Edge Objects:	Checked
Analyze Entire Image:	Checked
Advanced Detection Options	
Rolling Ball Diameter:	Automatic
Image Smoothing Strength:	1 cycle of 3×3 average filter
Evaluate Background On:	5%
Primary Mask:	Use threshold mask

Fluorescence Properties

The arrestin sensor is constructed with the very bright, mNeonGreen fluorescent protein [6]. 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.

Tip: *We offer* mNeon BacMam Kits *(products #F0500G and #F0505G). This test kit is a good way to determine BacMam transduction efficiency, evaluate promoter systems, and optimize expression in your cells of choice.*

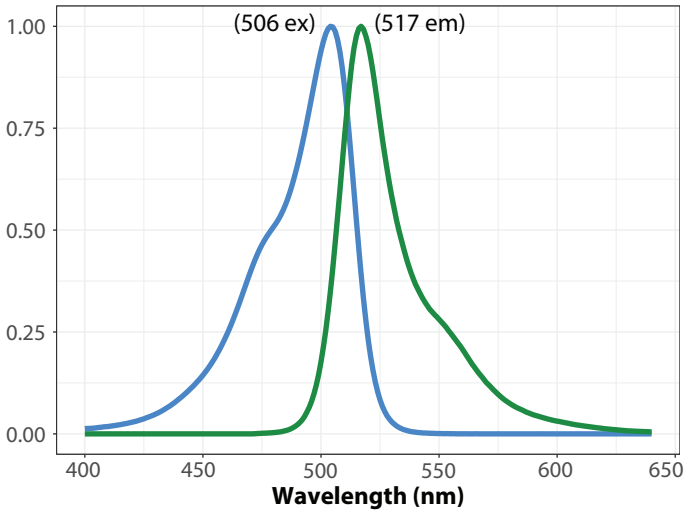


Figure 4. Absorption and emission properties of the mNeonGreen fluorescent protein plotted as a function of wavelength.

Timing

Unlike many arrestin assays, Montana Molecular's assay measures arrestin recruitment in living cells, in real time. For best results, be sure to capture changes in arrestin recruitment during the peak response. In Fig. 5A-5O fluorescence was captured from cells before the addition of the drug and then sampled at regular intervals. Each GPCR-specific figure shows the expected timing to reach the maximal response.

Data Analysis

In the experiment, the baseline fluorescence is recorded in the plate reader before the addition of the GPCR agonist. This provides an internal control for the amount of sensor/cells in the well. To estimate arrestin recruitment, calculate the mean fluorescence at baseline before compound addition (F_0) for each well, and normalize individual fluorescence reads (F) for the same well as follows:

$$\text{Arrestin Recruitment} = 1 - (F/F_0)$$

See Biosensor Data Analysis Overview for more details. See these kinetic data analysis parameters for useful equations to assist with your data analysis [7].

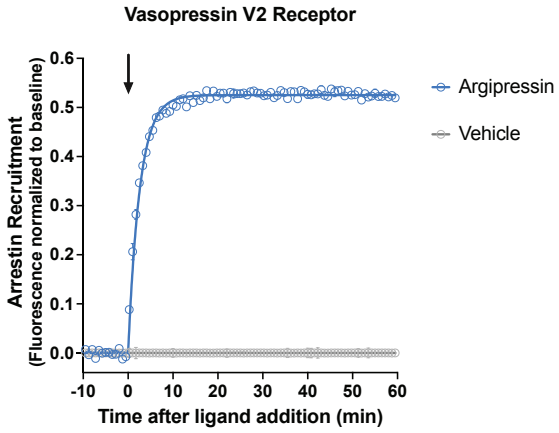


Figure 5A. HEK 293T cells transduced with the Arrestin Sensor and the Vasopressin Type 2 Receptor (V2R), responding to the addition of 2.5 µM Arginine Vasopressin (AVP).

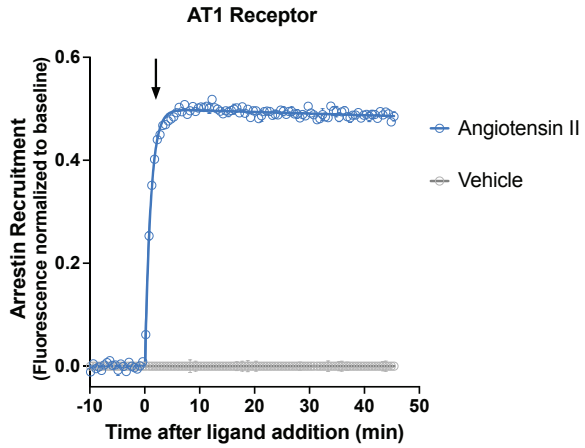


Figure 5B. HEK 293T cells transduced with the Arrestin Sensor and the Angiotensin II Type 1 Receptor (AT1R), responding to the addition of 500 nM Angiotensin II (AngII).

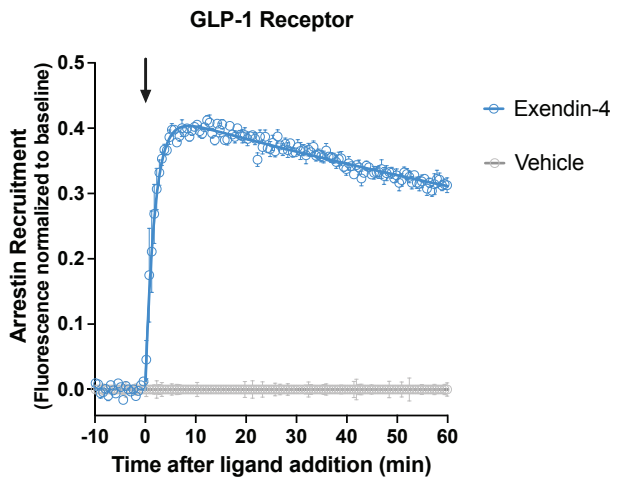


Figure 5C. HEK 293T cells transduced with the Arrestin Sensor, GRK5, and the Glucagon-Like Peptide 1 Receptor (GLP-1R), responding to the addition of 1 µM Exendin-4.

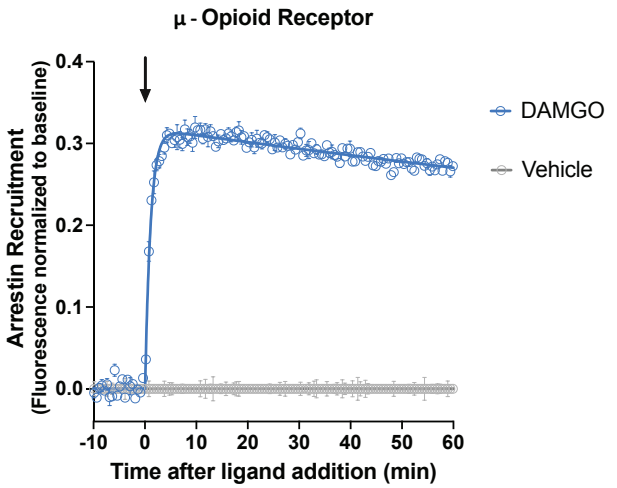


Figure 5D. HEK 293T cells transduced with the Arrestin Sensor, GRK2, and the µ-Opioid Receptor (MOR), responding to the addition of 10 µM DAMGO.

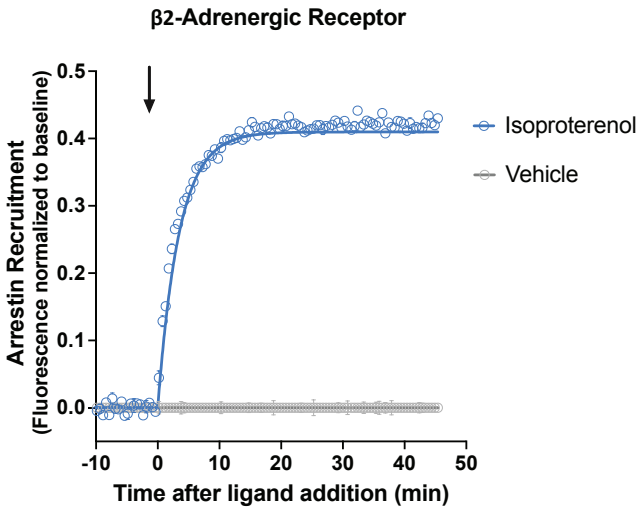


Figure 5E. HEK 293T cells transduced with the Arrestin Sensor, GRK2, and the β2-Adrenergic Receptor (B2AR), responding to the addition of 10 µM Isoproterenol.

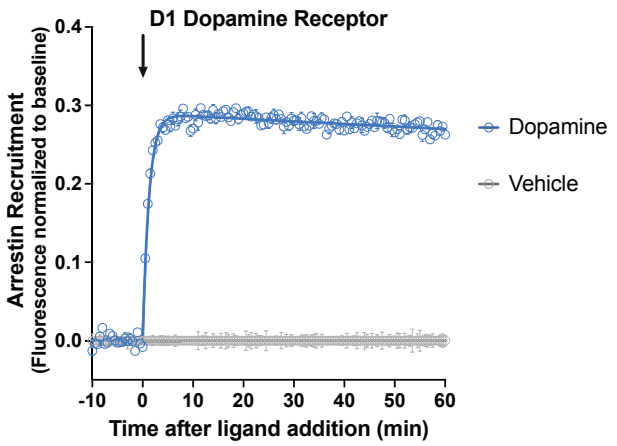


Figure 5F. HEK 293T cells transduced with the Arrestin Sensor, GRK2, and the D1 dopamine receptor (D1R), responding to the addition of 10 µM dopamine.

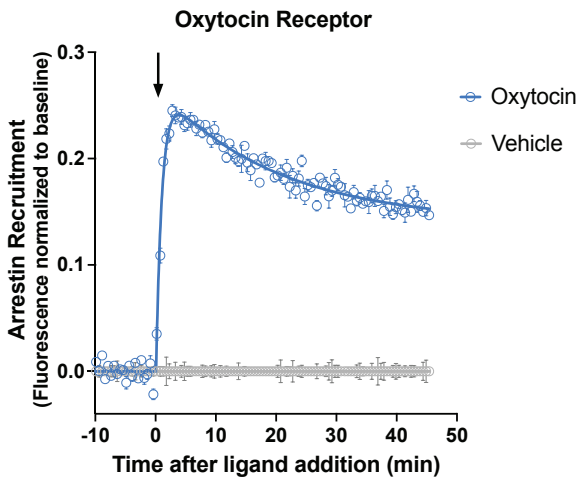


Figure 5G. HEK 293T cells transduced with the Arrestin Sensor, and the Oxytocin Receptor (OTR), responding to the addition of 5µM Oxytocin.

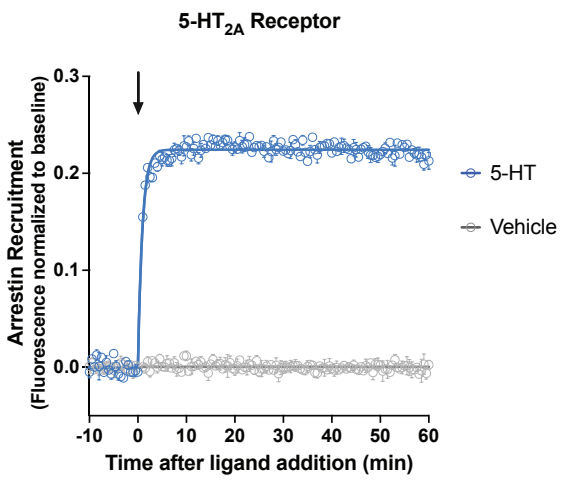


Figure 5H. HEK 293T cells transduced with the Arrestin Sensor, and the 5-HT_{2A} Receptor (5-HT2AR), responding to the addition of 10 µM 5 HT.

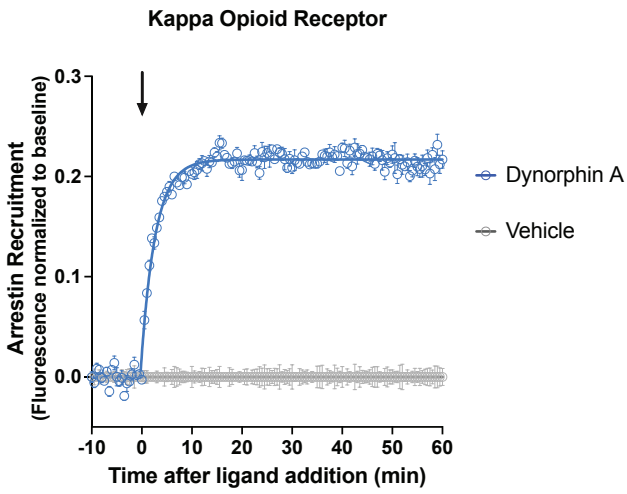


Figure 5I. HEK 293T cells transduced with the Arrestin Sensor, and the κ-Opiod Receptor (KOR), responding to the addition of 5 µM Dynorphin A.

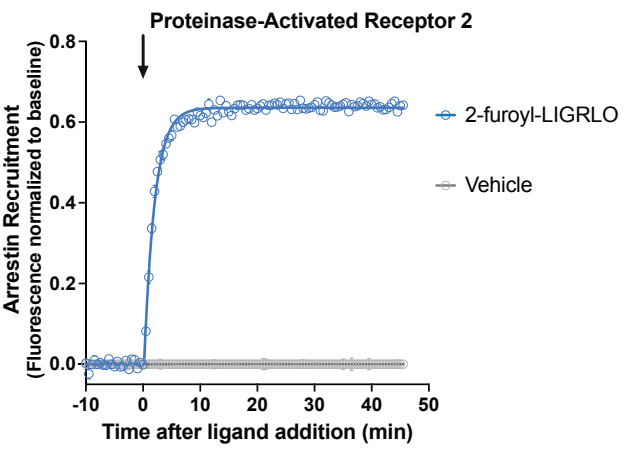


Figure 5J. HEK 293T cells transduced with the Arrestin Sensor, and the Proteinase-Activated Receptor 2 (PAR2), responding to the addition of 1 µM furoyl-LIGRLO.

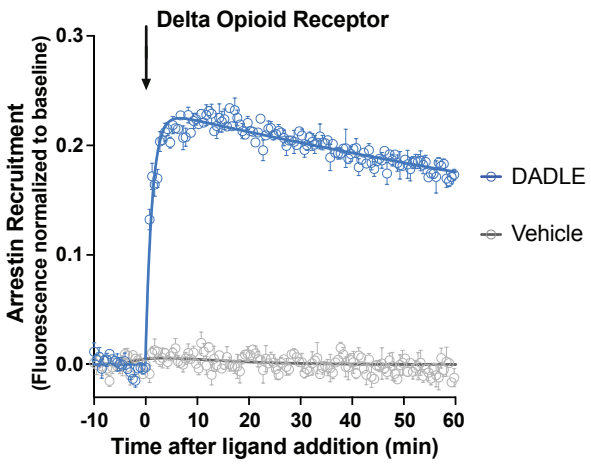


Figure 5K. HEK 293T cells transduced with the Arrestin Sensor, and the δ-Opiod Receptor (DOR), responding to the addition of 1 µM DADLE.

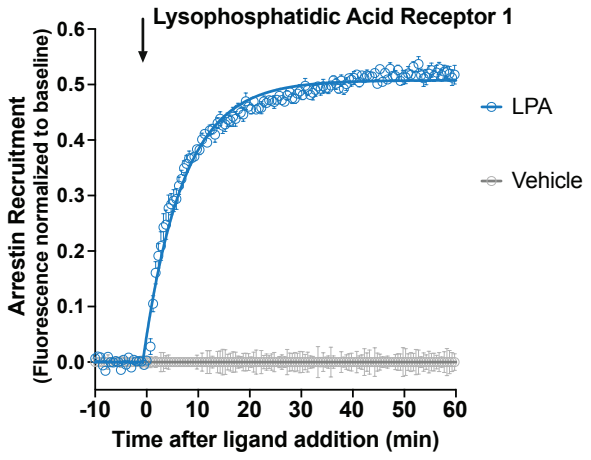


Figure 5L. HEK 293T cells transduced with the Arrestin Sensor, and the LPA1 Receptor (LPA1R), responding to the addition of 10 µM LPA.

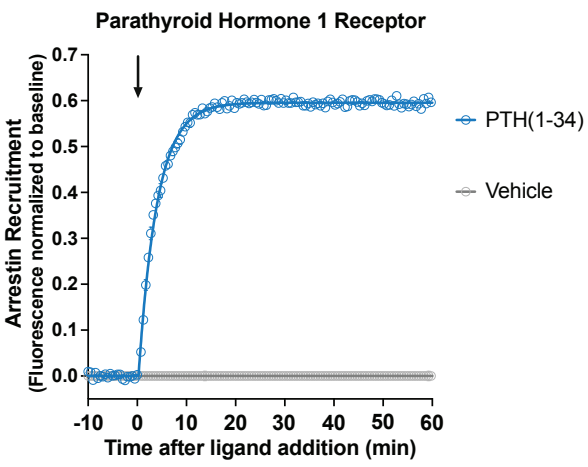


Figure 5M. HEK 293T cells transduced with the Arrestin Sensor, and the PTH1 Receptor (PTH1R), responding to the addition of 1 µM PTH.

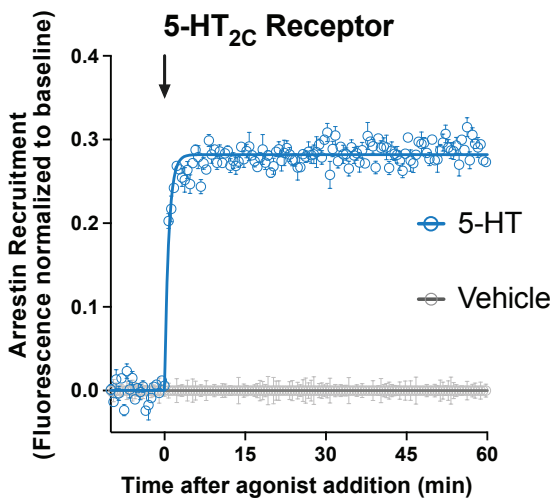


Figure 5N. HEK 293T cells transduced with the Arrestin Sensor, and the 5-HT_{2C} Receptor (5-HT2CR), responding to the addition of 10 µM 5 HT.

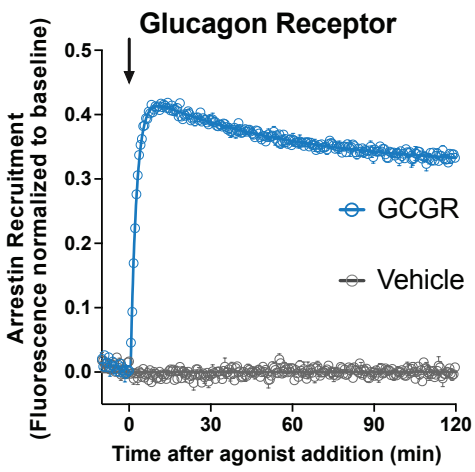


Figure 5O. HEK 293T cells transduced with the Arrestin Sensor, and the Glucagon Receptor (GCGR), responding to the addition of 1 µM glucagon.

Assay Optimization

Optimizing Fluorescence

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

HDAC inhibitors may be used to maintain expression of the sensors. While BacMam transduction alone will result in sensor 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 [4]. 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, McCoy's 5A, and F12K culture media. Cell culture media with high sodium bicarbonate content can interfere with transduction efficiency [8]. If your cell culture media of choice is affecting transduction efficiency, transduction can be conducted in DPBS for 6 hours and then the cells can be returned to complete cell culture media containing 2 mM sodium butyrate.

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 sensor. For example, a range of 2 µL to 10 µL is a good starting range in a 96-well plate (see optimization experiment). A range for of 0.5 µL to 1.75 µL is a good starting range in a 384-well plate. Choose the range that gives you at least 5 - 10 times above background and gives you the highest amount of fluorescence change with your **Ra** control receptor.

Varying the cell density, concentration of sodium butyrate, or trying a new HDAC inhibitor ((valproic acid or trichostatin A)) may boost expression as well.

Please contact us if you would like to use the sensor under the control of a specific promoter system. Sensors under 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 sensor.

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

Use the Positive Control

If the cells are expressing the sensor, and fluorescence is detectable on your instrument, evaluate sensor performance using the positive control receptor included in your kit. Add 15 µL of the **Ra** (control receptor) to a set of control wells and activate with the control agonist included in the kit (Table 3), Fig. 5A-5O.

Optimizing Receptor Expression

If you have titrated the arrestin sensor and determined the optimal volume, but fail to see a receptor mediated signal, the receptor and/or GRK expression level may be the issue. Try titrating the receptor or GRK with a fixed amount of the β-arrestin sensor.

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

Arrestin Assay FAQ

1. Can I use a plate reader with the Arrestin Biosensor assay?

   We've confirmed that the AT1R, β2AR, MOR, GLP-1R, V2R, OTR, 5-HT2AR, KOR, PAR2, DOR, LPA1R, PTH1R, 5-HT2CR, and GCGR receptors produce robust Z' values on standard fluorescence plate readers in HEK293T cells. Other cell types and receptors may require imaging to capture signals from individual cells.

2. Can I detect a response in primary cultures or iPSCs?

   We haven't tried iPSCs or primary cultures for the arrestin biosensors yet, but if you are interested, we can help.

3. What if I have a cell line already expressing my receptor of interest?

   The response will depend on the level of receptor expression in your cell line. If the level is too low in your cell line, we recommend using a BacMam vector to optimize receptor expression. We offer a number of GPCRs in high titer BacMam stock.

4. Can I detect the activity of endogenously expressed receptors with this assay?

   Success will depend on the level of endogenous receptor expression.

5. Can I express the receptor using a plasmid?

   You can, but this will increase variability and reduce Z'. For optimal results, we recommend adding the receptor packaged in BacMam so that you can control the level of receptor expression and reduce cell-to-cell variability.

6. Can I multiplex the Arrestin Biosensor with a red sensor for Ca^{2+}, DAG or cAMP?

   Yes you can. We recommend confirming the positive controls in separate experiments before multiplexing the assay.

7. How do I analyze kinetic data?

   The best way to analyze kinetic data from GPCR signaling is with the Pharmechanics GraphPad Prism module.

8. What Arrestin Biosensor kits are available?

   Receptor-specific kits for AT1R, β2AR, MOR, GLP-1R, V2R, OTR, 5-HT2AR, KOR, PAR2, DOR, LPA1R, PTH1R, 5-HT2CR, and GCGR are shipping now. Don't see what you need? Please let us know info@montanamolecular.com.

9. What if my receptor of interest is not available?

   New receptor-specific kits are coming on line on a regular basis. Please let us know if you do not see the assay you need on our list. We offer custom assay services including:

   • Packaging your receptor in BacMam so you can optimize the arrestin assay.
   The BacMam delivery system allows you to easily control receptor expression and produces low cell-to-cell variability. You receive ~175 mL of high titer BacMam vector, a panel of GRKs, a specific protocol for assay optimization and a 10 mL arrestin biosensor kit.

   • Custom Assay Optimization
   Each receptor is unique when it comes to arrestin biology. To capture the optimal kinetic response it's important to optimize assay conditions for your receptor. We package your receptor in BacMam, and optimize conditions for your receptor. You receive a detailed report including sample data, a specific protocol, and ~175 mL of your receptor in high titer BacMam stock.

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 - 10 µL in 96-well plate format) to identify optimal volume. Too little can result in low expression, too much can result in poor cell health.
	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 Assays in Adherent Cells.
	Suboptimal cell density; too few or too many cells added.	Transduce cells so that the cells will be around 80-90% confluent at the time of the experiment. 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 - 5 mM 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 45 - 60 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 - 6 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. 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.t
	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.

Problem	Possible Cause	Solution
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 DPBS at the time of experiment. If cell culture media is to be used, try using FluoroBrite media.
	Wrong microplate type is being used.	Use black, clear-bottom microplates with low autofluorescence. See our recommended plates
	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 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**.
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 DPBS at the time of experiment.	Exchange media so that cells are in DPBS 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**.
	Cells are detaching from the plate	Coat the plate with poly-D lysine or other appropriate cell attachment factor. See our recommended plates

	Problem	Possible Cause	Solution
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.**	Photobleaching	Reduce exposure time, sampling rate, and/or light intensity.
		Drug is at the wrong concentration	Confirm drug concentration and solubility.
		Drug was not stored properly.	Confirm drug **storage** conditions.
		Drug was added to the cells in a volume that was too low relative to the volume of DPBS/media in the well, resulting in inadequate mixing.	Add drug in a volume that will allow for sufficient diffusion (i.e. 1 : 3 or 1 : 4 drug to total volume)
		Drug was not added in the same solution as the solution in the well/culture dish.	Make sure that the drug preparation and cells are in the same solution.
		Drug addition is producing an artifact.	Make sure to add a vehicle-only control. Make sure drug is added in a solution that is the same as the solution in the well. Do not exceed 1% DMSO final in the well (0.1% or less is ideal)
		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.
		Drug addition was too forceful and dislodged cells.	Add drugs manually or with an on-board dispense function, but do so gently, so as not to dislodge cells.
		Baseline reads were not acquired before adding drug.	Acquire 10 - 20 baseline fluorescence reads before adding drug. Monitor for a change in fluorescence intensity upon addition of drug.
		Gain setting on instrument is too high, and signal is saturating. Gain setting is too low, and signal cannot be detected.	Adjust gain setting.
		Too much sensor has been added to cells and the signal is saturated (i.e. not enough analyte for the amount of sensor in the cell).	Titrate the amount of sensor to determine maximum signal for your cell type. See protocol recommendations for **HEK 293T** and other cell types.
		Target receptor was not added, or expression levels are suboptimal (too little or too much, or receptor has high level of constitutive activity).	Titrate the amount of receptor to optimize the signal for your cell type and receptor.
		Sampling rate is not consistent with sensor kinetics.	Acquire 10 - 20 baseline reads before adding drug. Resume measurement quickly after adding drug (within 5 - 10 seconds for DAG/PIP_2, 60 seconds for cADDis, cGMP, and arrestin and 1 - 2 seconds for GECO Ca^{2+}). Measure long enough to capture max response of sensor.
6.	**Poor cell health, cells detaching from plate.**	Too much BacMam stock was added to cells (e.g. sensor, receptor, Gs mutant).	Titrate lower amounts of BacMam stock to identify the optimal volume for your cells.
		Concentration of HDAC inhibitor is too high, or cells are sensitive to the HDAC inhibitor being used.	Confirm concentration of HDAC inhibitor being used. Make new stock solution. Try a different HDAC inhibitor. Confirm that they are being used at the proper concentration: sodium butyrate - 2 mM valproic acid - 5 mM trichostatin A - 0.25 µM * Perform a titration to determine optimal concentration for the cell type being used.
		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.

Problem	Possible Cause	Solution
6. Poor cell health, cells detaching from plate (continued).	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**.
	DPBS being used does not contain calcium and magnesium.	Use DPBS 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.

Borealis Arrestin Sensor- in the Literature

1. H. Schiff, et al. *ß-arrestin-biased proteinase-activated receptor-2 antagonist C781 limits allergen-induced airway hyperresponsiveness and inflammation.* British Journal of Pharmacology. June 2022.
2. S. Hoare, et al. *Quantifying the Kinetics of Signaling and Arrestin Recruitment by Nervous System G-Protein Coupled Receptors.* Frontiers in Cellular Neuroscience. January 2022.
3. S. Hoare, T. Hughes. *Biosensor Assays for Measuring the Kinetics of G-Protein and Arrestin-Mediated Signaling in Live Cells.* The Assay Guidance Manual. September 2021.
4. S. Hoare, et al. *Analyzing kinetic signaling data for G-protein-coupled receptors.* Nature Scientific Reports. July 2020.
5. S. Hoare, et al. *A kinetic method for measuring agonist efficacy and ligand bias using high resolution biosensors and a kinetic data analysis framework.* Nature Scientific Reports. Feb 2020.

References

1. Graham FL, et al. *Characteristics of a human cell line transformed by DNA from human adenovirus type 5.* J Gen Virol 1977, 36(1):59-74.
2. Dulbecco R and Vogt M. *Plaque formation and isolation of pure lines with poliomyelitis viruses.* The Journal of experimental medicine 1954.
3. Chalfie M, et al., *Green fluorescent protein as a marker for gene expression.* Science 1994.
4. Kost T, et al., *Implementation of BacMam virus gene delivery technology in a drug discovery setting.* Drug Discovery Today 2007, 12(9-10):396-403.
5. Tewson PH, et al., *New DAG and cAMP sensors optimized for live cell assays in automated laboratories.* Journal of Biomolecular Screening 2015.
6. Shaner, N.C., et al., (2013) *A bright monomeric green fluorescent protein derived from Branchiostoma lanceolatum.* Nature Methods, May;10(5):407-9. doi: 10.1038/nmeth.2413.
7. Hoare, S., et al. *Analyzing kinetic signaling data for G-protein-coupled receptors.* Sci Rep. Jul 2020
8. Shen H-C, et al., *Baculovirus-mediated gene transfer is attenuated by sodium bicarbonate.* J Gene Med. Jun. 2007.
9. Hoare, S., et al. *A kinetic method for measuring agonist efficacy and ligand bias using high resolution biosensors and a kinetic data analysis framework*. Sci Rep. 2020.
10. 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

Table 8. Related Products

Product	Description	Promoter	Recommended Use
#D0200G	Green Down cADDis cAMP	CMV	Fluorescence imaging and plate reader assay (Z' > 0.9)
#U0200G	Green Up cADDis cAMP	CMV	Fluorescence imaging and plate reader assay (Z' > 0.85)
#U0200R	Red Up cADDis cAMP	CMV	Fluorescence imaging and plate reader assay (Z' < 0.5)
#D0300G	Green Down DAG	CMV	Fluorescence imaging and plate reader assay (Z' > 0.8)
#U0300G	Green Up DAG	CMV	Fluorescence imaging and plate reader assay (Z' > 0.8)
#D0300R	Red Down DAG	CMV	Fluorescence imaging and plate reader assay (Z' > 0.7)
#U0600G	Green Up G-GECO Ca^{2+}	CMV	Fluorescence imaging and plate reader assay (Z' < 0.5)
#U0600R	Red GECO Ca2+	CMV	Fluorescence imaging and plate reader assay (Z' > 0.5)

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™

Patent Pending: PCT/US2019/044165 [WO2020028381A1]