$G_{i/o}$ GPCR-GIRK Thallium Flux Assay

Catalog No. 11100-a/b

Introduction

G protein-gated inwardly rectifying potassium channels (GIRK) are a large family of inwardly rectifying potassium channels that play important roles in neurophysiology and cardiac physiology. Mammals express four GIRK subunits ($K_{ir}3.1$-$K_{ir}3.4$); the predominant form of GIRK in the brain is a $K_{ir}3.1/K_{ir}3.2$ heterotetramer. GIRK is opened by the direct binding of the G protein βγ subunit (Gβγ), which is released from the heterotrimeric G protein ($G\alpha_i\beta\gamma$) upon the activation of G protein coupled receptors (GPCRs). GIRK is exclusively associated with $G_{i/o}$ GPCRs such as dopamine receptors, serotonin receptors, muscarinic receptors and opioid receptors. Due to its critical role of regulating cell excitability, GIRK presents a potential therapeutic target for epilepsy, Parkinson's disease, Down's syndrome, pain, drug addiction, and many more pathologies.

Commonly used methods to study GPCR-GIRK signaling include patch clamp, fluorescence resonance energy transfer (FRET), cAMP, and bioluminescence resonance energy transfer (BRET). These methods face the challenges of complicated procedures of protein tag engineering and assay miniaturization to a high-throughput format for drug screening. ION's $G_{i/o}$ GPCR-GIRK Thallium Flux Assay Kit is a total assay solution for multi-well plate-based, high-throughput measurements of GIRK function elicited by GPCR agonism (Figure 1). This kit provides all reagents necessary, including a stable HEK293 cell line that expresses human $K_{ir}3.1$ and $K_{ir}3.2$, to conduct $G_{i/o}$ GPCR high-throughput screening. A simple transduction step allows the flexibility to study a variety of GPCRs that regulate GIRK using Tl^+ as surrogate cation for potassium channel activation. Over the past 15 years, fluorescence-based measures of Tl^+ flux have brought about the discovery of small-molecule modulators of a host of ion channels, transporters, GPCRs and other targets of interest for both drug discovery and basic research. ION's GPCR-GIRK Thallium Flux Assay Kit generates high quality screening assays and EC_{50} values that are consistent with other methods.

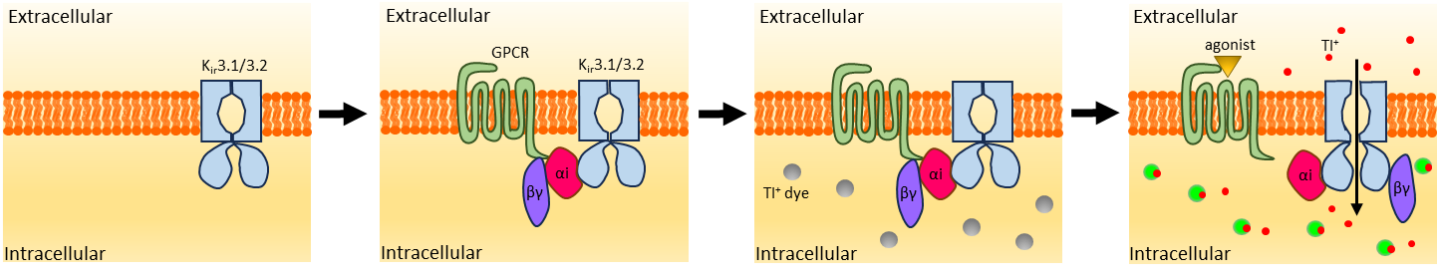

Figure 1. $G_{i/o}$ GPCR-GIRK Thallium Flux Assay Principle. 1) A stable HEK293 cell line (HEK293-hu$K_{ir}3.1/3.2$) that expresses human $K_{ir}3.1$ and $K_{ir}3.2$ is provided in $G_{i/o}$ GPCR-GIRK Thallium Flux Assay. 2) A GPCR of interest is transiently expressed into HEK293-hu$K_{ir}3.1/3.2$ cells; GPCR-$G\alpha_i$-$K_{ir}3.1/3.2$ complex is pre-formed through the interaction of $G\alpha_i$ αA helix. 3) Cells are preincubated with dye loading solution; Tl^+ dye reagents diffuse cross the cell membrane. 4) GIRK is opened by the direct binding of the G protein βγ subunit (Gβγ), which is released from the heterotrimeric G protein (Gαβγ) upon the activation of GPCR by an agonist. Then extracellular Tl^+ ions rapidly enter the cells though open GIRK channels. Intracellular Tl^+ ions bind to Tl^+ dye reagent generating a fluorescence increase.

$G_{i/o}$ GPCR-GIRK Thallium Flux Assay Flow Chart

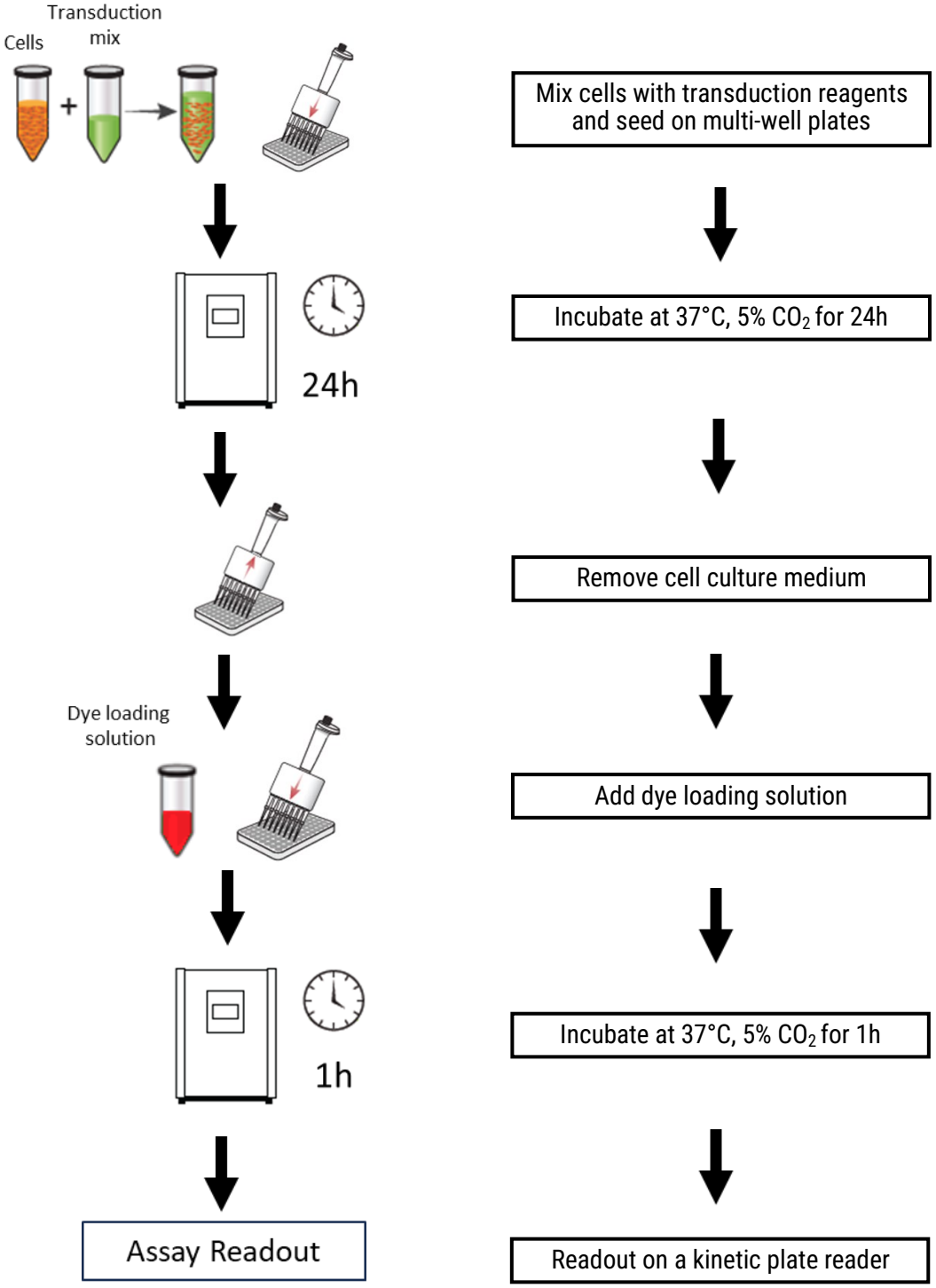

Figure 2. $G_{i/o}$ GPCR-GIRK Thallium Flux Assay Flow Chart. 1) Formulate transduction mix by adding media, GPCR transduction reagent, and sodium butyrate. 2) Combine transduction mix with cells and seed on multi-well plates. 3) Incubate plate at 37°C, 5% CO_2 for 24 hours. 4) Completely remove culture media and add dye loading solution. Incubate plate at 37°C, 5% CO_2 for 1 hour. 5) Prepare the compound plate and transfer both the cell plate and the compound plate to a kinetic plate reader for data collection.

Storage and Stability

Upon receipt, store components at the temperatures indicated on each label. Kit reagent components are stable for up to 12 months from date of shipment when stored as directed. Frozen cell lines can be stored at −80°C for up to 6 months, but for longer term storage should be held at −196°C.

Table 1	Materials Provided (Kit Components)	Amounts Supplied in Kit			
Label	Component Name	Size	Cat# 11100-a	Cat# 11100-b	Storage
N/A	HEK293-huK$_{ir}$3.1/3.2	2X10^6 cells	1 vial	Not Included	< -80°C
Reagent A	Thallos AM[6]	25 µg	10 vials	10 vials	-20°C
Reagent B	DMSO[1]	225 µL	1 vial	1 vial	-20°C
Reagent C	50X DySolv	4 mL	1 bottle	1 bottle	4°C
Reagent D	10X Brilliant Assay buffer	20 mL	1 bottle	1 bottle	4°C
Reagent E	50X TRS[2]	4 mL	1 bottle	1 bottle	4°C
Reagent F	50X Probenecid Solution[2]	4 mL	1 bottle	1 bottle	4°C
Reagent G	10X Chloride-Free Stimulus Buffer	10 mL	1 bottle	1 bottle	4°C
Reagent H	50 mM Thallium Sulfate Solution	20 mL	1 bottle	1 bottle	20—25°C

Table 2	Materials Required But Not Supplied	
Category	Name	Recommendations
Reagents	Cell culture media	Advanced MEM (ThermoFisher Cat#: 12492013)
	GlutaMax™	GlutaMAX™ Supplement (ThermoFisher Cat#: 35050061)
	Fetal Bovine Serum (FBS)	Fetal bovine serum (Corning Cat#: 35-016-CV)
	Blasticidin	Blasticidin S HCl (ThermoFisher Cat#: A1113903)
	Puromycin	Puromycin Dihydrochloride (ThermoFisher Cat#: A1113803)
	TrypLE™	TrypLE™ Express Enzyme (ThermoFisher Cat#: 12605028)
	GPCR transduction reagent	GPCR BacMam transduction reagents (Montana Molecular)
	Compounds to be tested	N/A
Equipment	Multi-well plates	96-well Poly-D-Lysine Black/Clear microplate (Greiner Cat# 655948) or 384-well Poly-D-Lysine Black/Clear microplate (Corning Cat# 356697)
	Kinetic plate reader	WaveFront Panoptic, Molecular Devices FlexStation 3, or similar instrument

Laboratory Procedures

Cell culture

1. Construct complete cell culture medium by mixing 25 mL of FBS, 5 mL of 100X GlutaMax, 250 µL of 10 mg/mL Blasticidin, and 150 µL of 10 mg/mL Puromycin with 470 mL 1X Advance MEM media for the final concentration of 5% FBS, 1X GlutaMax, 5 µg/mL Blasticidin and 3 µg/mL Puromycin.
2. Aliquot 9 mL complete cell culture medium in a 15 mL conical tube.
3. Thaw the frozen cell vial in a 37°C water bath for ~3 min.
4. Add the cells into the 15 mL tube dropwise.
5. Gently mix the cells and centrifuge at 500xg, 25°C for 5 min.
6. Aspirate the supernatant carefully, and resuspend the cell pellet with 1 mL of complete culture medium.
7. Culture cells in a T75 flask until they reach confluency (we recommend changing media every 2~3 days. Cell doubling time is ~36h).

Cell Preparation

1. Harvest cells from T75 flask and resuspend the cells in complete culture medium without selection antibiotics.
2. Perform cell count (A confluent T75 flask normally contains ~$10x10^6$ cells).
3. Dilute cell suspension to desired concentration of 300,000 cells/mL with complete culture medium without selection antibiotics.

Transduction and Seeding[4]

1. Equilibrate all buffers and reagents to room temperature.
2. Create transduction mix by combining 'complete culture medium without selection antibiotics: GPCR transduction reagent : SB' in the ratio of '29.4 µL : 20 µL : 0.6 µL.' [5]
3. Combine transduction mix and diluted cells (300,000 cells/mL) in the ratio of 1 : 2 (Vol : Vol) and gently mix.
4. Seed the cell-transduction mixture in Poly-D-Lysine coated multi-well plates. For 96-well plate seed 100 µL/well, or 20,000 cells/well; for 384-well plate seed 50 µL/well, or 10,000 cells/well.
5. Cover plate to protect from light and let rest at room temperature for 30 min.
6. Incubate plates under normal cell growth conditions (5% CO_2 and 37°C) for 24 hours, protected from light.

Dye Loading

1. Add 20 µL DMSO (Reagent B) to the tube containing Thallos AM[6](Reagent A).
2. Vortex until Reagent A is fully dissolved.
3. Add appropriate volume of water (Table 3, next page) to a 15 mL centrifuge tube.
4. Add 1 mL of 10X Assay Buffer (Reagent D) to tube from step 3.

Procedure Continues on Next Page

Dye-Loading Continued

5. Add 200 µL of 50X DySolv (Reagent C) to the tube from step 4.
6. Add 200 µL of 50X TRS[2] (Reagent E) to the tube from step 5.
7. Add 200 µL of Probenecid Solution[2] (Reagent F) to the tube from step 6.
8. Add 20 µL of Thallos AM Solution[6] from step 2 to the tube from step 7.
9. Briefly vortex the Dye Loading Solution, tube from step 8, to mix.

Table 3 Dye Loading Solution

Label	Name	Method A	Method B	Method C	Method C
Reagent A+B	Thallos AM Solution	20 µL	20 µL	20 µL	20 µL
Reagent C	50X DySolv	400 µL	400 µL	400 µL	400 µL
Reagent D	10X Brilliant Assay Buffer	1 mL	1 mL	1 mL	1 mL
Reagent E	50X TRS[2]	-	400 µL	-	400 µL
Reagent F	50X Probenecid[2]	-	-	400 µL	400 µL
	Water	8.6 mL	8.2 mL	8.2 mL	7.8 mL
	Total	10 mL	10 mL	10 mL	10 mL

10. Completely remove the cell culture medium from the multi-well plates containing cells.
11. Add 100 µL/well of Dye Loading Solution to 96-well plates; or add 20 µL/well of Dye Loading Solution to 384-well plates.
12. Incubate the multi-well plates containing the cells and Dye Loading Solution for 1 hour at 37° C.

Compound Plate Preparation

1. Prepare a Stimulus Solution in a 15 mL centrifuge tube by adding the appropriate amounts of water, 1 mL of 10X Brilliant Chloride-Free Stimulus Buffer (Reagent G), 0.5 mL of 50 mM Thallium Sulfate Solution (Reagent H), and 3X concentrated test compound (supplied by the customer), as shown in Table 4 below.
2. Load 70 µL/well of Stimulus Solution to V-shaped 96-well reagent plates; or load 20 µL/well of Stimulus solution to V-shaped 384-well reagent plates.

Table 4 Stimulus Solution

Label	Name	Volume
Reagent G	10X Brilliant Chloride-Free Simulus Buffer	1 mL
Reagent H	50 mM Thallium Sulfate	0.5 mL
(Customer Supplied Material)	Compound to be Tested	Enough for 3X concentration at total volume
	Water	Q.S. to total volume
	Total	10 mL

Assay Readout

1. Transfer the dye-loaded, cell-containing multi-well plates and compound plate(s) to a kinetic-imaging plate reader (e.g. WaveFront Panoptic, Hamamatsu FDSS, Molecular Devices FLIPR or Molecular Devices FlexStation).
2. Acquire data using an excitation wavelength of ~490 nm, and emission wavelength of ~520 nm and an acquisition frequency of ~1 Hz. Begin data acquisition to collect baseline for 20 seconds.
3. Add 50 µL of Stimulus solution into the cell-containing 96-well plates; or add 10 µL of Stimulus solution into the cell-containing 384-well plates.
4. Continue data acquisition for an additional 90 seconds.

Data Analysis

1. Collect relative fluorescence units (RFU) over time as the raw kinetic data, and define the initial 10-20 data points as a baseline. (Figure 3)
2. Average baseline RFU as F_0 and calculate the ratio of F/F_0 as normalized kinetic data. (Figure 4)
3. On the normalized kinetic data, run simple linear regression for the first 10 time points right after drug addition and calculate the slope as V_{max}*. (Figure 5)
4. Plot dose response curve of V_{max} against drug concentration to calculate EC_{50}. (Figure 6)

*Note: *Some kinetic plate readers can calculate V_{max} (e.g. Molecular Devices FlexStation 3). In this case, data analysis instructions can be skipped.*

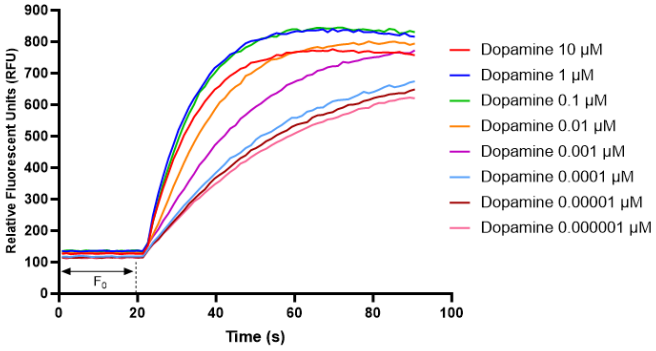

Figure 3. Raw Kinetic Data. Dopamine D2 receptor is transduced in HEK293-huK$_{ir}$3.1/3.2 cells. Assay was performed using FlexStation 3 with excitation wavelength 490 nm, emission wavelength 520 nm, and an cutoff filter of 515 nm. Baseline RFUs were collected for 20 s, then various concentrations of dopamine were added to the cell plate and data collection were continued for additional 70s. RFU rapidly increased at 22s where the stimulus solution was addition completed.

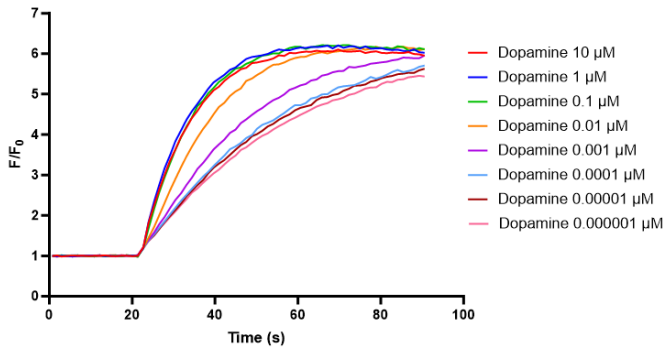

Figure 4. Normalized Kinetic Data. Based on the raw kinetic data from Figure 3, first calculate the mean RFU of the initial 20 s baseline and defined it as F_0. Then calculate the ratio of RFU over F_0 at each timepoint, defined as F/F_0. Plot F/F_0 against time to generate normalized kinetic data.

Data Analysis Continued

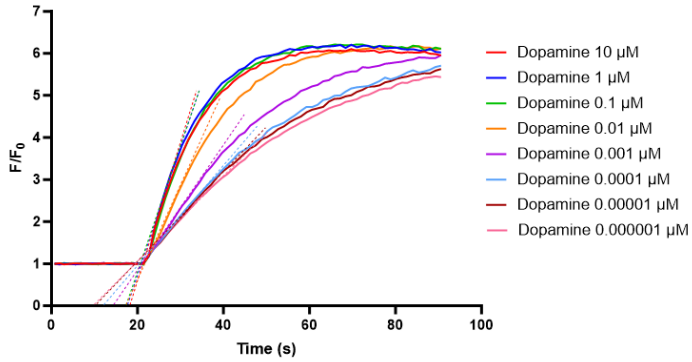

Figure 5. V_{max} Calculation. Based on the normalized kinetic data from Figure 4, run a simple linear regression in Prism for the first 10 time point right after the stimulus addition. Then calculate the slope and define it as V_{max}. Higher dopamine concentration showed higher V_{max}, indicating faster Tl^+ flux with GIRK activation.

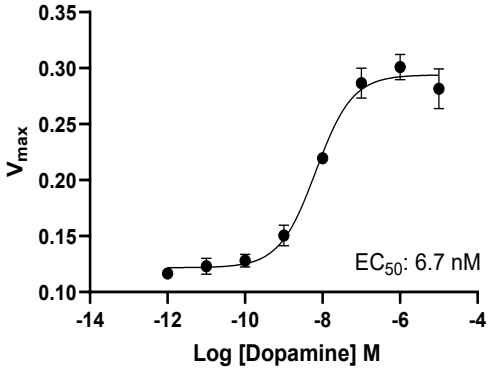

Figure 6. Dose Response Curve. Based on V_{max} values generated from Figure 5, plot V_{max} against log[dopamine]. Run nonlinear regression log(agonist) vs. response in Prism to generate a dose response curve, and calculate an EC_{50} (6.7 nM).

Example Results

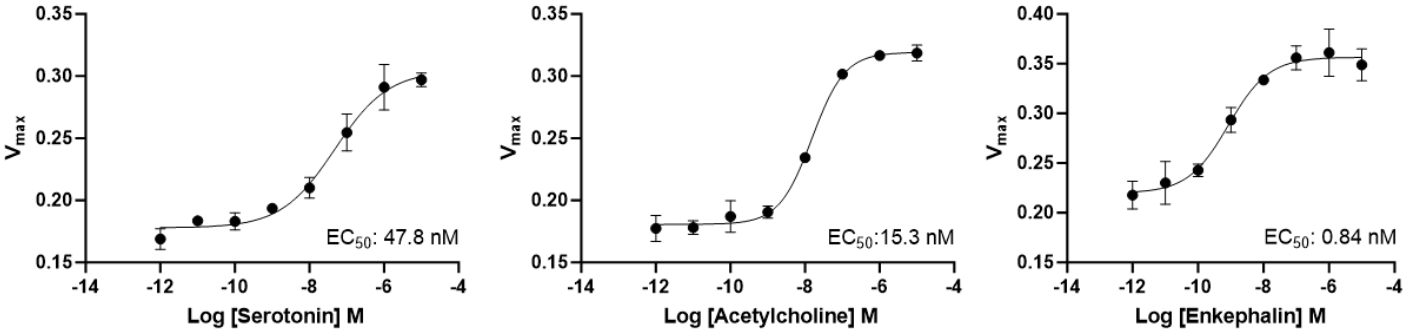

Figure 7. 96-well $G_{i/o}$ GPCR-GIRK Thallium Flux Assay Dose Response Curves. Serotonin 1A receptor, Muscarinic M2 receptor, and delta opioid receptor were transduced in HEK293-huK_{ir}3.1/3.2 cells using the optimized amount of transduction reagent, 20 µL for Serotonin 1A receptor and Muscarinic M2 receptor; 8 µL for delta opioid receptor (see transduction optimization on page 9). 20,000 cells/well were seeded in the plate and incubated for 24 hours for transduction. Assay was performed using FlexStation 3 kinetic plate reader with excitation wavelength 490 nm and emission wavelength 520 nm. Serotonin, Acetylcholine, and Enkephalin were tested with the highest concentration of 10 µM. Dose response curves generated serotonin EC_{50}: 47.8 nM, acetylcholine EC_{50}: 15.3 nM, and Enkephalin EC_{50}: 0.84 nM.

Example Results Continued

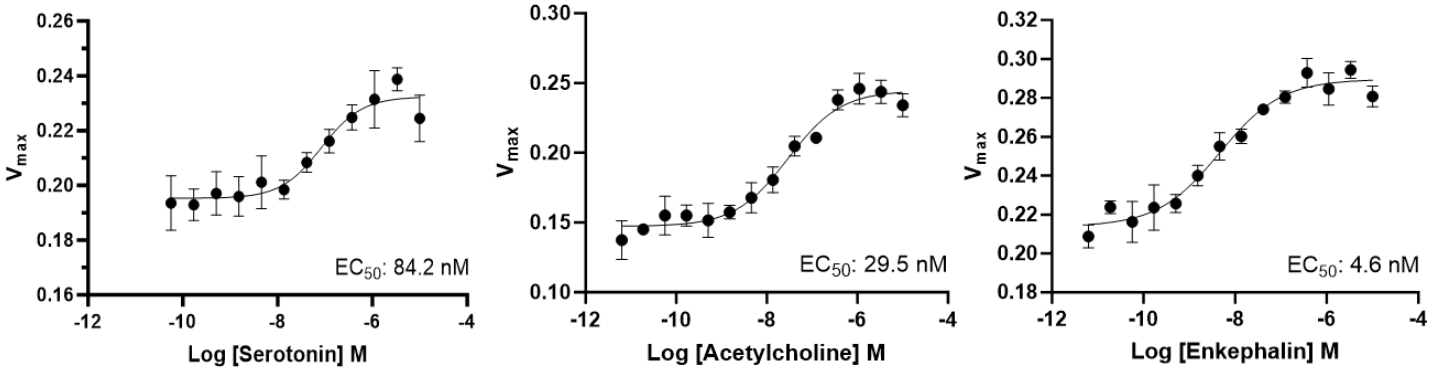

Figure 8. 384-well $G_{i/o}$ GPCR-GIRK Thallium Flux Assay Dose Response Curves. Serotonin 1A receptor, Muscarinic M2 receptor, and delta opioid receptor were transduced in HEK293-huK$_{ir}$3.1/3.2 cells using the optimized amount of transduction reagent, 20 µL for Serotonin 1A receptor and Muscarinic M2 receptor; 8 µL for delta opioid receptor (see transduction optimization on page 9). 10,000 cells/well were seeded in the plate and incubated for 24 hours for transduction. Assay was performed using Panoptic kinetic plate reader with excitation wavelength 477 nm and emission wavelength 515 nm, acquisition 1Hz. Serotonin, Acetylcholine, and Enkephalin were tested with the highest concentration of 10 µM. Dose response curves generated serotonin EC_{50}: 84.2 nM, acetylcholine EC_{50}: 29.5 nM, and Enkephalin EC_{50}: 4.6 nM.

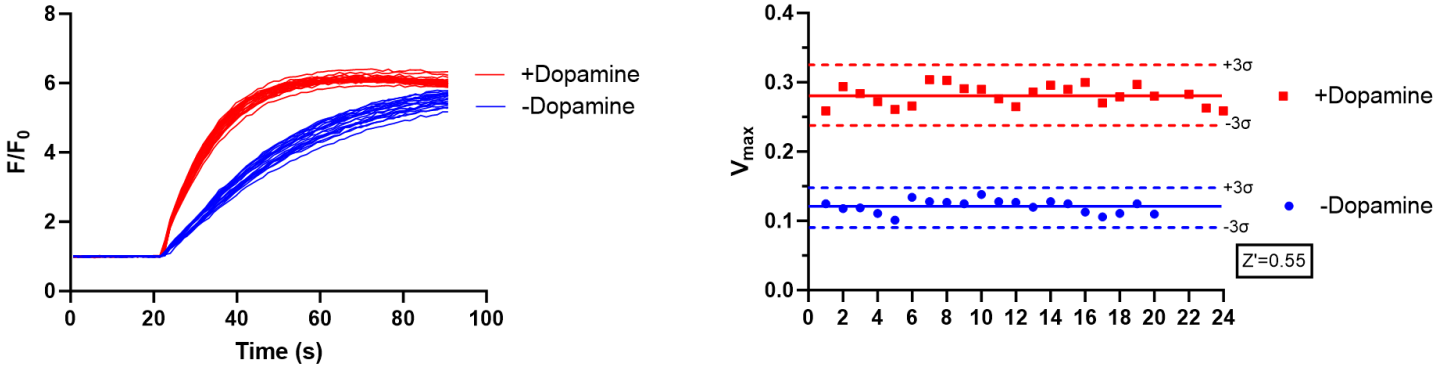

Figure 9. 96-well $G_{i/o}$ GPCR-GIRK Thallium Flux Assay Z' Factor Analysis. Dopamine D2 receptor was transduced in HEK293-huK$_{ir}$3.1/3.2 cells using the optimized amount (20 µL) of transduction reagent. 20,000 cells/well were seeded in the plate and incubated for 24 hours for transduction. Assay was performed using FlexStation 3 kinetic plate reader with excitation wavelength 490 nm and emission wavelength 520 nm. For positive control group 10 µM of dopamine was used to activate dopamine D2 receptor; for negative control gorup buffer alone was added to the plate. Normalized kinetic data showed a clear distinguish between positive and negative control groups. The calcualted Z' factor is 0.55 indicating a high quality screening assay.

$G_{i/o}$ GPCR-GIRK Thallium Flux Assay Optimization

GPCR Transduction Amount

Overexpressing of certain GPCRs might cause damage to cell health. We recommend performing a titration experiment to optimize the transduction conditions with the maximum 20 µL for each new GPCR studied. For example, the delta opioid receptor showed overall decreasing V_{max} while increasing the transduction amount from 0 µL to 20 µL; and at 8 µL the V_{max} fold change with and without enkephalin reached the maximum of 2 (Figure 11). Consistently, reducing the transduction amount of delta opioid receptor to 8 µL significantly improved the enkephalin dose response and EC_{50} (Figure 12).

Cell Density

We recommend to seed 20,000 cells/well for 96-well plates and 10,000 cells/well for 384-well plates. Cell density optimization could be combined with transduction titration. For example, 384-well Dopamine D2 receptor assay at 4,000 cells/well achieved a V_{max} fold change of 1.6 at 4 µL; while at 10,000 cells/well the V_{max} fold change increased to 1.9 at 20 µL (Figure 13). Dopamine dose response curves showed the best EC_{50} at 10,000 cells/well (Figure 14).

Assay Temperature

We recommend to conduct the assay at 37°C. In our studies, an improvement of acetylcholine EC_{50} was observed at 37°C compared to 25°C (Figure 15).

Fluorescence Data Collection Settings

We recommend an excitation wavelength of ~490 nm[7], emission wavelength of ~520 nm[7] or FTIC settings, and an acquisition frequency of 1 Hz. Some instruments (e.g. WaveFront Panoptic) allow users to manually change the exposure time. In this case we recommend optimizing exposure time (Figure 16). Using a Read Mode of "Bottom" read mode only can also significantly improve the quality of the collected fluorescence data.

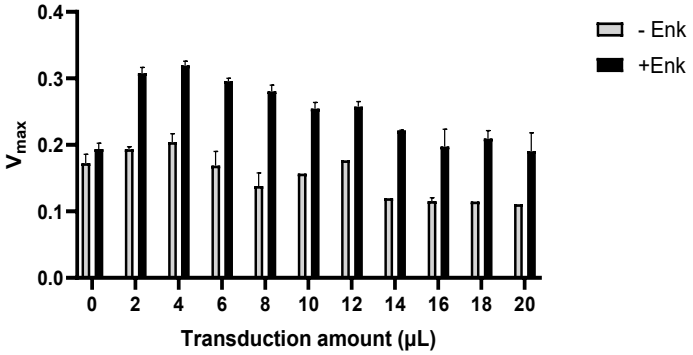

Figure 11. 96-well Delta Opioid Receptor (DOR) Transduction titration. Transduction mix is made by combining culture medium : transduction reagent : SB at the ration of 49.4-29.4 µL : 0-20 µL : 0.6 µL (total 50 µL). Assay was performed using FlexStation 3 kinetic plate reader with excitation wavelength 490 nm and emission wavelength 520 nm. For positive control group, 10 µM of enkepahlin was used to activate DOR receptor; for negative control group buffer alone was added to the plate.

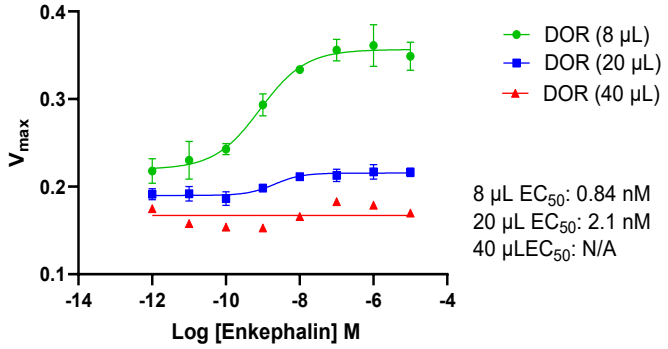

Figure 12. 96-well Dose Response with Different Transduction Amount of Delta Opioid Receptor (DOR). Transduction mix is made by combining culture medium : transduction reagent : SB at the ration of 41.4/29.4/9.1 µL : 8/20/40 µL : 0.6 µL (total 50 µL). Assay was performed using FlexStation 3 kinetic plate reader with excitation wavelength 490 nm and emission wavelength 520 nm. Dose response curves generated the best EC_{50} value of 0.84 nM at 8 µL of transduction reagent.

$G_{i/o}$ GPCR-GIRK Thallium Flux Assay Optimization Continued

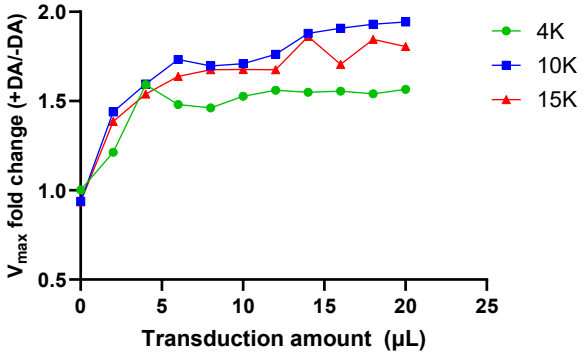

Figure 13. 384-well Dopamine D2 Receptor Transduction Titration with Different Cell Density. Transduction mix is made by combining culture medium : transduction reagent : SB at the ration of 49.4-29.4 µL : 0-20 µL : 0.6 µL (total 50 µL). After mixing with cells, seed 20 µL/50 µL/75 µL per well in a 384-well plate resulting different cell density of 4,000 cells/well, 10,000 cells/well , and 15,000 cells/well. Assay was performed using Panoptic kinetic plate reader with excitation wavelength 477 nm and emission wavelength 515 nm. 10 µM of dopamine was used to activate dopamine D2 receptor.

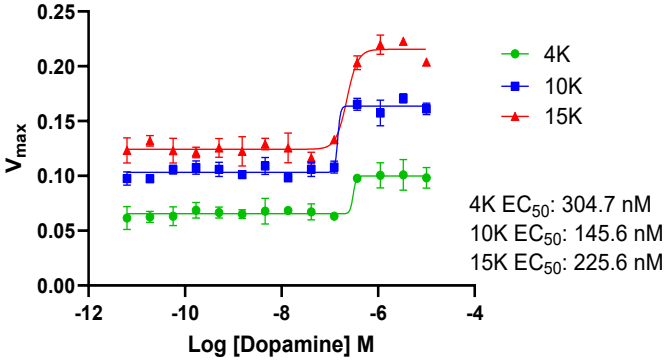

Figure 14. 384-well Dopamine Dose Response with Different Cell Density. Transduction mix is made by combining culture medium : transduction reagent : SB at the ration of 29.4 µL : 20 µL : 0.6 µL. After mixing with cells, seed 20 µL/50 µL/75 µL per well in a 384-well plate resulting different cell density of 4,000 cells/well, 10,000 cells/well , and 15,000 cells/well. Assay was performed using Panoptic kinetic plate reader with excitation wavelength 477 nm and emission wavelength 515 nm. Dose response curves generated the best EC_{50} value of 145.6 nM at 10,000 cells/well.

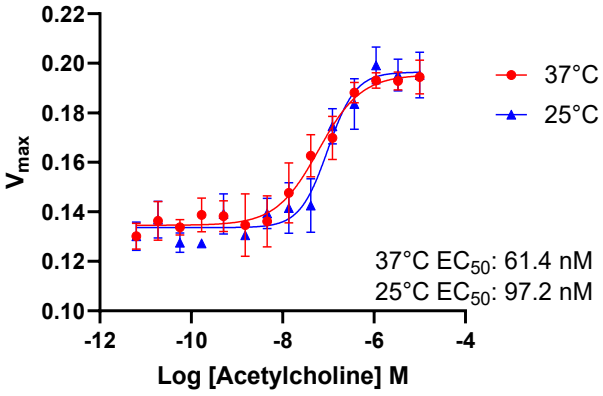

Figure 15. 384-well Acetylcholine Dose Response Curve at Different Temperatures. Muscarinic M2 receptor was transduced in HEK293-huK$_{ir}$3.1/3.2 cells using the optimized amount 20 µL of transduction reagent. Assay was performed using Panoptic kinetic plate reader with excitation wavelength 477 nm and emission wavelength 515 nm at either 37°C or 25°C. Dose response EC_{50} value at 37°C is slightly improved compared to 25°C.

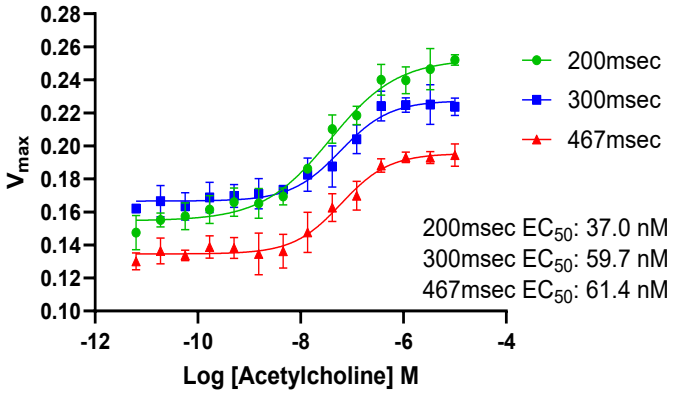

Figure 16. 384-well Acetylcholine Dose Response Curve with Different Exposure Times. Muscarinic M2 receptor was transduced in HEK293-huK$_{ir}$3.1/3.2 cells using the optimized amount 20 µL of transduction reagent. Assay was performed using Panoptic kinetic plate reader with excitation wavelength 477 nm and emission wavelength 515 nm with exposure time setting of 200 msec/300 msec/467 msec. Dose response curves generated the lowest EC_{50} value of 37.0 nM with 200 msec exposure time.

Table 5	Recommended Instrument Settings
Setting	Recommendation
Read Mode	'Bottom' read mode only
Ex/Em wavelengths[7]	~490 nm/520 nm
Cutoff wavelength	515 nm
Filter selection	GFP or FITC
Contact support@ionbiosciences.com for additional recommendations and guidance on optimizing your application.	

Laboratory Procedures - Footnotes

1 DMSO is hygroscopic and should be stored tightly closed at -20° C with desiccant pack to prevent the solvent from becoming wet. Wet solvent causes difficulties with dissolution of the dye. Use the DMSO within 6 months of receipt.

2 Caution is advised when using Probenecid and/or TRS as they may have undesirable effects on assay performance for the target of interest. Probenecid may be included in the Dye Loading Solution to aid in dye retention. This may be particularly important in certain cell lines (e.g. CHO cells). TRS contains a membrane-impermeant dye useful for masking extracellular fluorescence.

3 The timing of and volume of the Stimulus Solution addition may vary. In some cases, experiments may include the addition of other solutions to the cell-containing microplate prior to the addition of the Stimulus Solution. In these cases, the volume of the Stimulus Solution addition should be altered to account for the additional volume of solution in the cell-containing microplate.

4 In this protocol we use Montana Molecular GPCR BacMan transduction reagents. Other transduction/transfection methods are suitable as well.

5 The amount of transduction reagent (Reagent A) needs to be optimized according to each individual GPCR. Overexpression of some GPCRs might cause damage to cell health. Please see $G_{i/o}$ GPCR-GIRK Thallium Flux Assay Optimization on Page 9 for more details.

6 Thallos AM and Thallos AM Solution should be protected from light.

7 To prevent bleed-through or spectral overlap, the Ex/Em wavelengths may need to be optimized by broadening the interval between the wavelengths. Results generated on a Molecular Devices Flexstation 3.

Additional Information

Additional dye indicator and buffer reagents can be purchased either directly from our website or by contacting our Sales Department. Custom and bulk sizes are also available. Contact Sales for more information.

Table 8	Additional Reagents	Available Sizes	
Kit Label	Name	Size	Catalog #
Reagent A	Thallos AM	500 µg x 1 Vial	1381C
		50 µg x 10 Vials	1381F
		50 µg x 3 Vials	1381G
Reagent C	50X DySolv	20 mL Bottle	7501A
Reagent D	10X Brilliant Assay Buffer	10 mL Bottle	7010X
Reagent E	50X TRS	20 mL Bottle	7060A
Reagent F	50X Probenecid	20 mL Bottle	7300P-50
Reagent G	10X Brilliant Chloride-Free Stimulus Buffer	10 mL Bottle	7020B
Reagent H	50 mM Thallium Sulfate	5 mL Bottle	7040S

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