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

Catalog No. 11100-10

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.

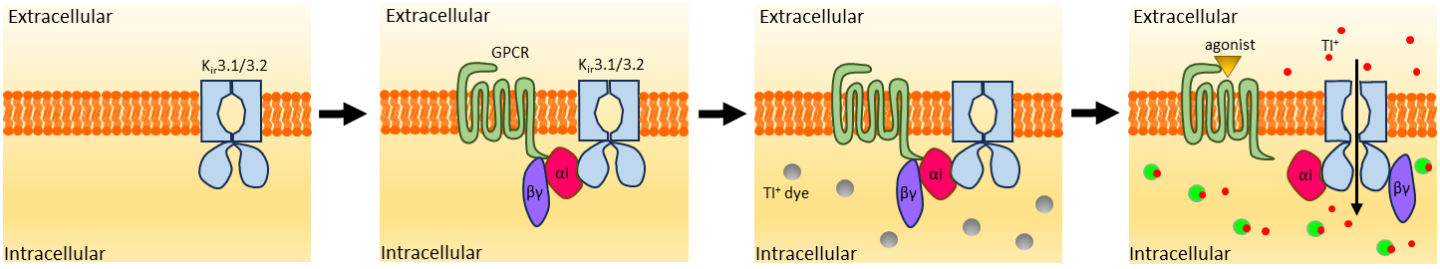

Figure 1. $G_{i/o}$ GPCR-GIRK Thallium Flux Assay Principle. 1) A stable HEK293 cell line (HEK293-huK$_{ir}$3.1/3.2) that expresses human $K_{ir}3.1$ and $K_{ir}3.2$ is provided in $G_{i/o}$ GPCR-GIRK Thallium Flux Assay. 2) A GPCR of interest is transiently expressed into HEK293-huK$_{ir}$3.1/3.2 cells; GPCR-Gα_i-K$_{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

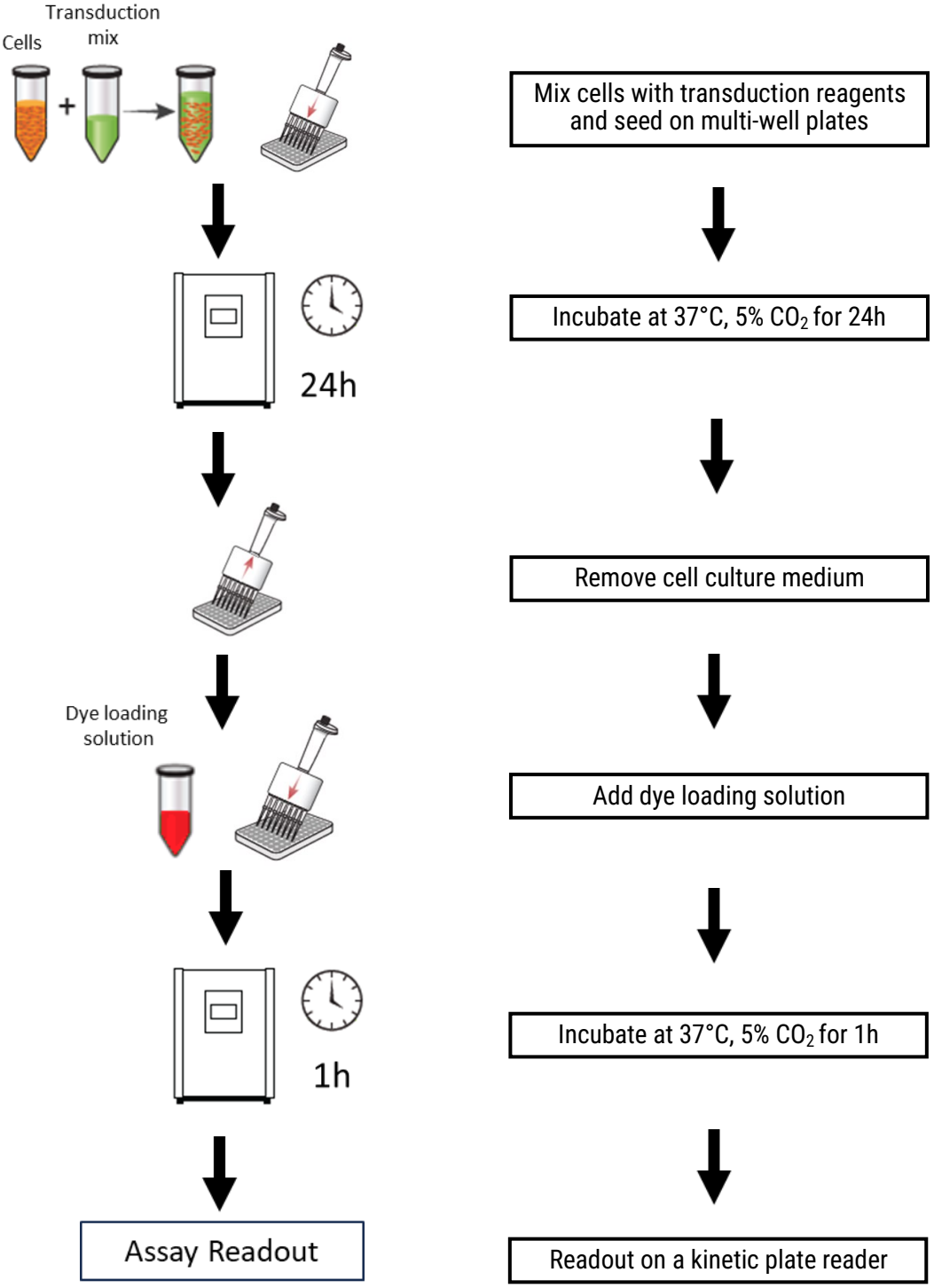

Figure 2. $G_{i/o}$ GPCR-GIRK Thallium Flux Assay Flow Chart. 1) Formulate transduction mix by adding media, GPCR transduction reagent, and sodium butyrate. 2) Combine transduction mix with cells and seed on multi-well plates. 3) Incubate plate at 37°C, 5% CO_2 for 24 hours. 4) Completely remove culture media and add dye loading solution. Incubate plate at 37°C, 5% CO_2 for 24 hours. 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 labels. Kit components are stable for up to 12 months from date of shipment when stored as directed.

Materials Provided (Kit Components)

Label	Name	Volume	Containers	Storage	Cat#
N/A	HEK293-huK$_{ir}$3.1/3.2	2X10^6 cells	1	< -80°C	C1101
Reagent A	Thallos AM (25 µg)	Dry	10	-20°C	1381
Reagent B	DMSO	225 µL	1	4–25°C	N/A
Reagent C	10X Assay buffer	20 mL	1	4°C	7010C
Reagent D	50X DySolv	4 mL	1	4°C	7501A
Reagent E	50X TRS	4 mL	1	4°C	7060A
Reagent F	50X Probenecid Solution	4 mL	1	4°C	7300P-50
Reagent G	10X Chloride-Free Stimulus Buffer	10 mL	1	4°C	7020B
Reagent H	50 mM Thallium Sulfate Solution	20 mL	1	20–25°C	7030S

Materials Required But Not Supplied

Category	Name	Recommendations
Reagents	Cell culture media	Advanced MEM (ThermoFisher Cat#: 12491013)
	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

Laboratory Procedures

A. 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).

B. 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.

C. Transduction* and seeding

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
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.

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

***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.*

Laboratory Procedures Cont.

D. Dye loading

1. Equilibrate all reagents to room temperature.
2. Add 20 µL DMSO (**Reagent B**) to the tube containing Thallos AM* (**Reagent A**).
3. Vortex until Reagent A is fully dissolved.
4. Add 8.38 mL of water to a 15 mL centrifuge tube.
5. Add 1 mL of 10X Assay Buffer (**Reagent C**) to tube from **step 4.**
6. Add 200 µL of 50X DySolv (**Reagent D**) to the tube from **step 5.**
7. Add 200 µL of 50X TRS** (**Reagent E)** to the tube from **step 6.**
8. Add 200 µL of Probenecid Solution*** (**Reagent F)** to the tube from **step 7.**
9. Add 20 µL of Thallos AM Solution from **step 3** to the tube from **step 8.**
10. Briefly vortex the **Dye Loading Solution**, tube from **step 9**, to mix.
11. Completely remove the cell culture medium from the multi-well plates containing cells.
12. 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.
13. Incubate the multi-well plates containing the cells and Dye Loading Solution for 1 hour at 37° C

*Note: *Thallos AM and Thallos AM solution should be protected from light.*

***TRS contains a membrane-impermeant dye useful for masking extracellular fluorescence. Caution is advised when using TRS or other extracellular masking solutions 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 dye retention. This may be particularly important in certain cell lines (e.g. CHO cells). However, caution is advised when using Probenecid as it may have undesirable effects on assay performance for the target of interest.*

E. Compound plate preparation

1. Prepare Thallium Stimulus Solution in a 15 mL centrifuge tube by adding 8.5 mL of water, 1 mL of 10X Chloride-Free Stimulus Buffer (**Reagent G**) and 0.5 mL of 50 mM Thallium Sulfate Solution (**Reagent H**)
2. Prepare a **Stimulus solution** with 3X concentrated compound in Thallium Stimulus Solution.
3. 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.

Laboratory Procedures Cont.

F. 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.

G. 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.*

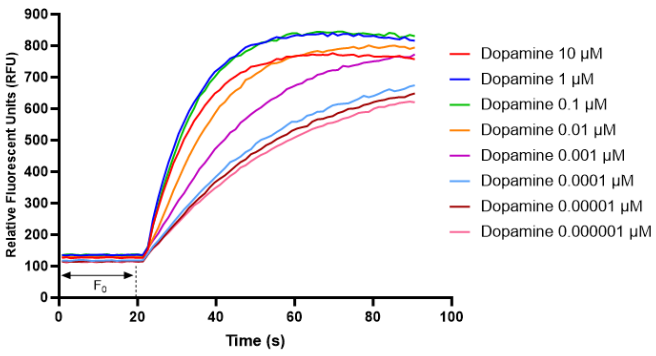

Figure 3. Raw Kinetic Data. Dopamine D2 receptor is transduced in HEK293-huK$_{ir}$3.1/3.2 cells. Assay was performed using FlexStation 3 with excitation wavelength 490 nm, emission wavelength 520 nm, and an cutoff filter of 515 nm. Baseline RFUs were collected for 20 s, then various concentrations of 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.

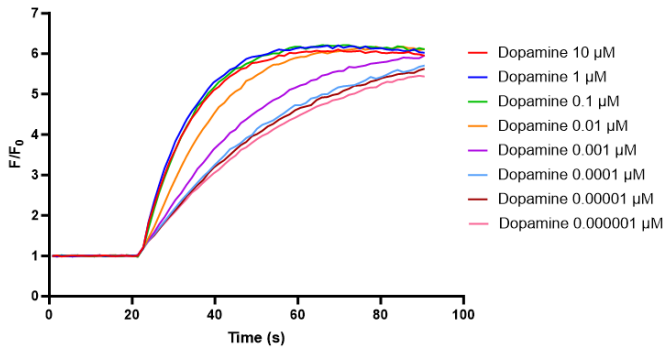

Figure 4. Normalized Kinetic Data. Based on the raw kinetic data from Figure 3, first calculate the mean RFU of the initial 20 s baseline and defined it as F_0. Then calculate the ratio of RFU over F_0 at each timepoint, defined as F/F_0. Plot F/F_0 against time to generate normalized kinetic data.

Laboratory Procedures Cont.

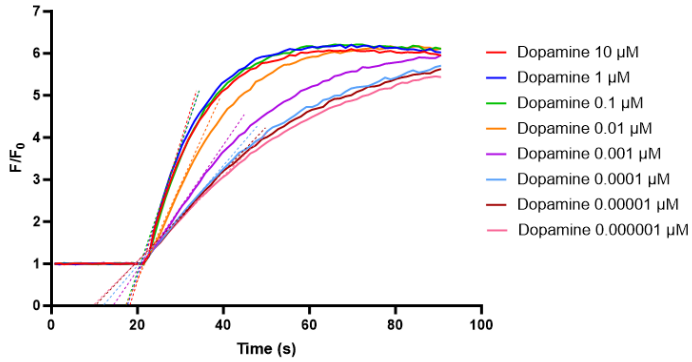

Figure 5. V_{max} Calculation. Based on the normalized kinetic data from Figure 4, run a simple linear regression in Prism for the first 10 time point right after the stimulus addition. Then calculate the slope and define it as V_{max}. Higher dopamine concentration showed higher V_{max}, indicating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

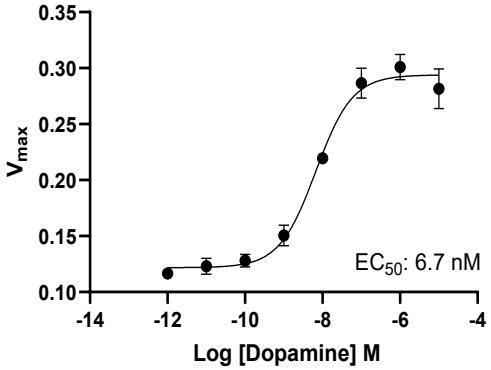

Figure 6. Dose Response Curve. Based on V_{max} values generated from Figure 5, plot V_{max} against log[dopamine]. Run nonlinear regression log(agonist) vs. response in Prism to generate a dose response curve, and calculate an EC_{50} (6.7 nM).

Example Results

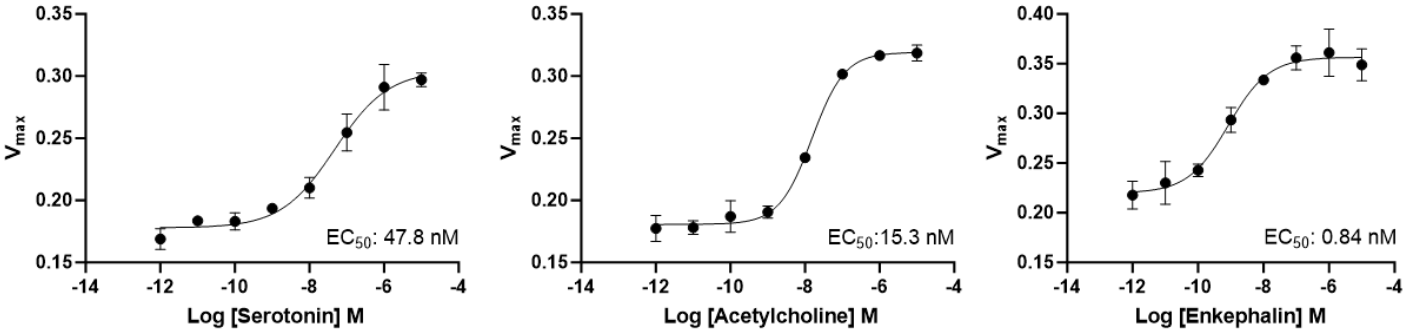

Figure 7. 96-well $G_{i/o}$ GPCR-GIRK Thallium Flux Assay Dose Response Curves. Serotonin 1A receptor, Muscarinic M2 receptor, and delta opioid receptor were transduced in HEK293-huK$_{ir}$3.1/3.2 cells using the optimized amount of transduction reagent, 20 µL for Serotonin 1A receptor and Muscarinic 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 Count.

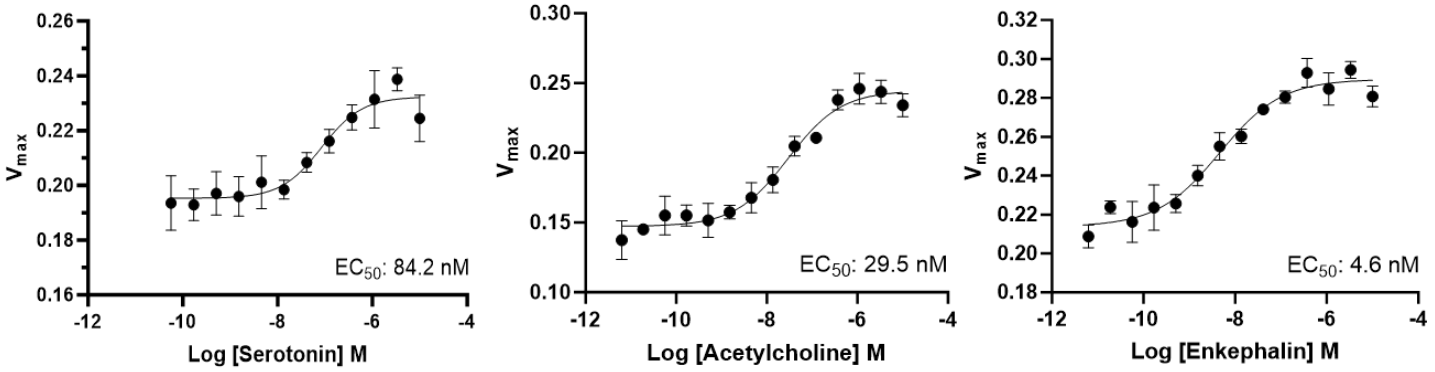

Figure 8. 384-well $G_{i/o}$ GPCR-GIRK Thallium Flux Assay Dose Response Curves. Serotonin 1A receptor, Muscarinic M2 receptor, and delta opioid receptor were transduced in HEK293-huK$_{ir}$3.1/3.2 cells using the optimized amount of transduction reagent, 20 µL for Serotonin 1A receptor and Muscarinic 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.

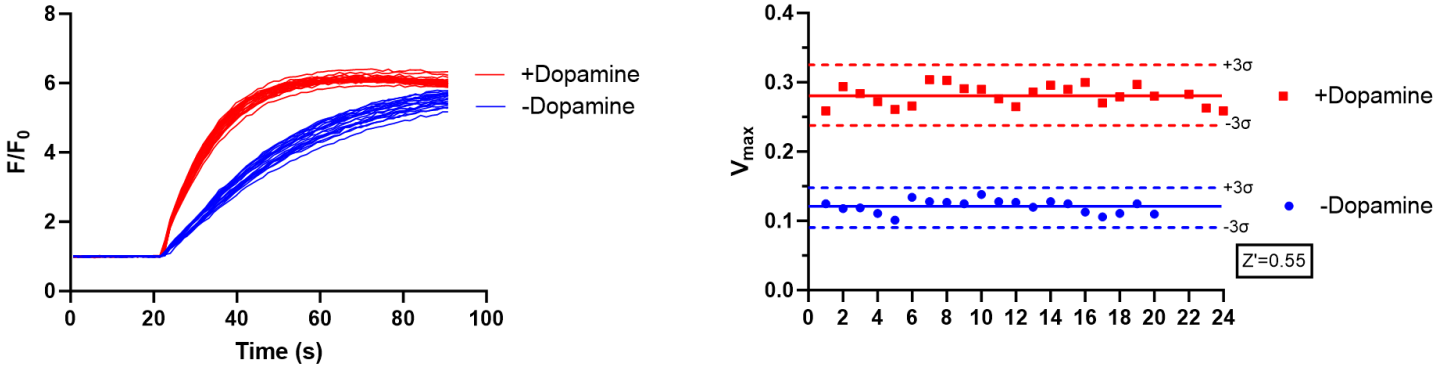

Figure 9. 96-well $G_{i/o}$ GPCR-GIRK Thallium Flux Assay Z' Factor Analysis. Dopamine D2 receptor was transduced in HEK293-huK$_{ir}$3.1/3.2 cells using the optimized amount (20 µL) of transduction reagent. 20,000 cells/well were seeded in the plate and incubated for 24 hours for transduction. Assay 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

A. 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**).

B. 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**).

C. 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**).

D. Fluorescence data collection settings

We recommend an excitation wavelength of ~490 nm, emission wavelength of ~520 nm 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**).

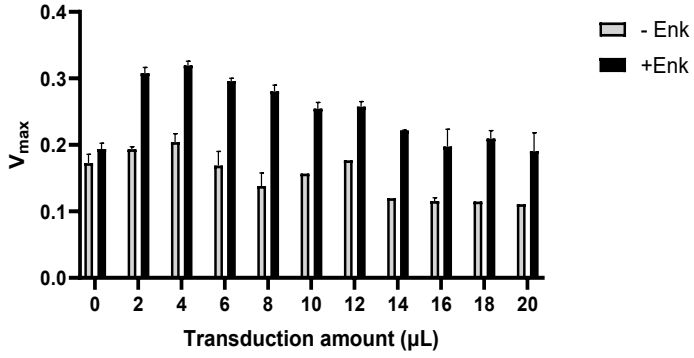

Figure 11. 96-well Delta Opioid Receptor (DOR) Transduction titration. Transduction mix is made by combining culture medium : transduction reagent : SB at the ration of 49.4-29.4 µL : 0-20 µL : 0.6 µL (total 50 µL). Assay was performed using FlexStation 3 kinetic plate reader with excitation wavelength 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.

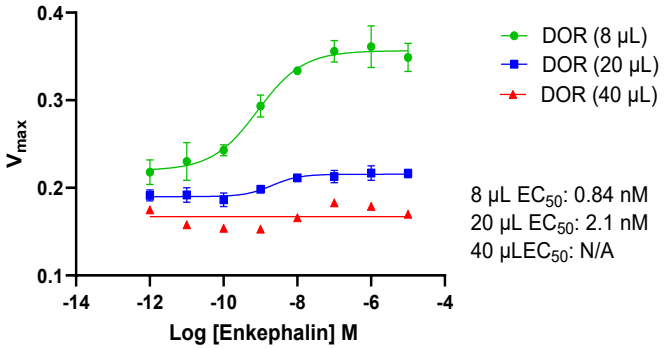

Figure 12. 96-well Dose Response with Different Transduction Amount of Delta Opioid Receptor (DOR). Transduction mix is made by combining culture medium : transduction reagent : SB at the ration of 41.4/29.4/9.1 µL : 8/20/40 µL : 0.6 µL (total 50 µL). Assay was performed using FlexStation 3 kinetic plate 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 Count.

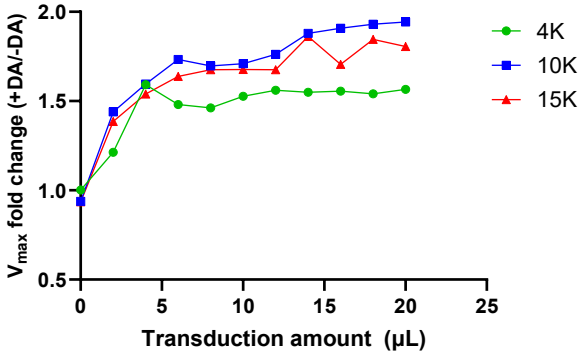

Figure 13. 384-well Dopamine D2 Receptor Transduction Titration with Different Cell Density. Transduction mix is made by combining culture medium : transduction reagent : SB at the ration of 49.4-29.4 µL : 0-20 µL : 0.6 µL (total 50 µL). After mixing with cells, seed 20 µL/50 µL/75 µL per well in a 384-well 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.

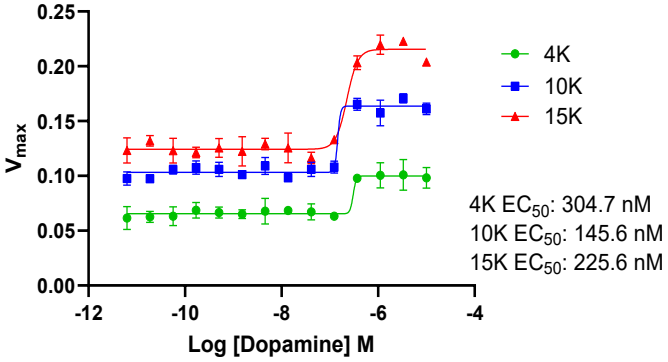

Figure 14. 384-well Dopamine Dose Response with Different Cell Density. Transduction mix is made by combining culture medium : transduction reagent : SB at the ration of 29.4 µL : 20 µL : 0.6 µL. After mixing with cells, seed 20 µL/50 µL/75 µL per well in a 384-well plate resulting different cell density 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.

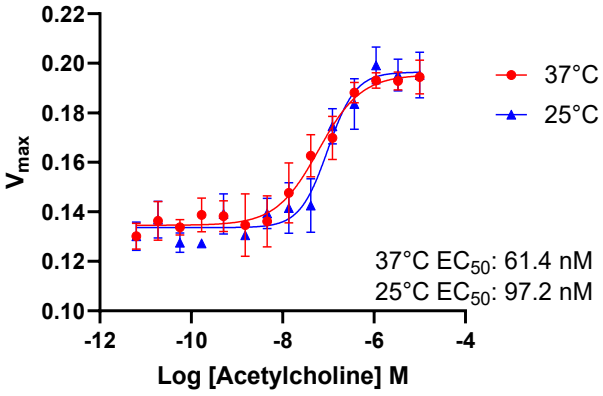

Figure 15. 384-well Acetylcholine Dose Response Curve at Different Temperatures. Muscarinic M2 receptor was transduced in HEK293-huK$_{ir}$3.1/3.2 cells using the optimized amount 20 µL of transduction reagent. Assay was performed using Panoptic kinetic plate reader with excitation wavelength 477 nm 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.

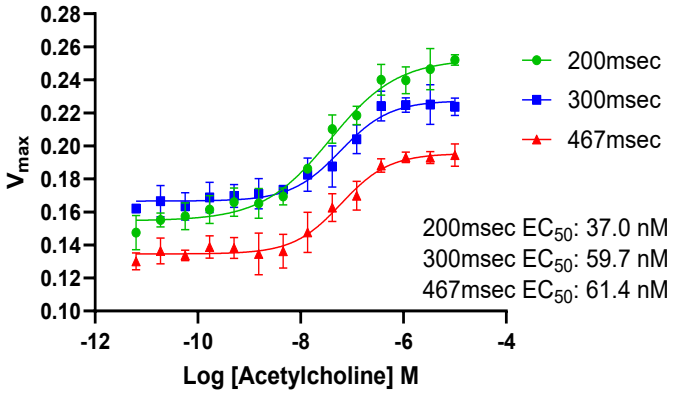

Figure 16. 384-well Acetylcholine Dose Response Curve with Different Exposure Times. Muscarinic M2 receptor was transduced in HEK293-huK$_{ir}$3.1/3.2 cells using the optimized amount 20 µL of transduction reagent. Assay was performed using Panoptic kinetic plate reader with excitation wavelength 477 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.

References

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