FluxOR™ II Green Potassium Ion Channel Assay

Catalog Number F20015, F20016, F20017

Pub. No. MAN0016084 **Rev.** B.0

Table 1 Contents and storage

Material	Amount			Concentration	Storage*
	F20015	F20016	F20017		
FluxOR™ II Green Reagent (Component A)	2 vials** (for 2 plates)	10 vials** (for 10 plates)	10 vials† (for 100 plates)	1000X after reconstitution in DMSO	• ≤8°C • Desiccate • Protect from light
Dimethyl sulfoxide (DMSO) (Component B)	100 µL	200 µL	2 × 1 mL	N/A	• ≤25°C • Desiccate
FluxOR™ II Assay Buffer (Component C)	4 mL	20 mL	200 mL	10X Concentrate	≤8°C
PowerLoad™ Concentrate (Component D)	400 µL	2 × 1 mL	20 mL	100X Concentrate	
Probenecid (Component E)	1 × 77 mg	2 × 77 mg	20 × 77 mg	100X after solubilization in water	• ≤25°C • Desiccate
Chloride-free Stimulus Buffer (Component F)	4 mL	20 mL	200 mL	5X Concentrate	≤8°C
Potassium Sulfate (K_2SO_4) Concentrate (Component G)	4 mL	20 mL	200 mL	125 mM in water	
Thallium Sulfate (Tl_2SO_4) Concentrate (Component H)	4 mL	20 mL	200 mL	50 mM in water	
FluxOR™ II Background Suppressor (Component I)	4 mL	20 mL	200 mL	10X Concentrate	

* All products are shipped on wet ice. When stored as directed, the product is stable for at least 6 months upon receipt.
** Each vial contains sufficient reagents for assaying 1 microplate (96-or 384-well).
† Each vial contains sufficient reagents for assaying 10 microplates (96-or 384-well).
N/A: Not applicable.

Number of assays: Sufficient reagents are supplied for 2 (Cat. No. F20015), 10 (Cat. No. F20016), or 100 (Cat. No. F20017) microplates (96-or 384-well), based on the protocol below.

Approximate fluorescence excitation/emission maxima: 490/525 nm.

Product information

The FluxOR™ II Green Potassium Ion Channel Assay is a solution for high-throughput screening (HTS) of potassium ion channel and transporter activities. The FluxOR™ II Green assay takes advantage of the permeability of potassium channels to thallium (Tl^+) ions.[1,2] When thallium is added to the extracellular solution with a stimulus to open channels, thallium flows down its concentration gradient into the cells, and channel or transporter activity is detected with the FluxOR™ II Green indicator dye that increases in cytosolic fluorescence. In this way, the fluorescence reported in the FluxOR™ II Green system becomes an indicator of any ion channel activity or transport process that allows thallium into cells.

The FluxOR™ II Green Potassium Ion Channel Assay provides all necessary buffers and solutions needed for performing the assay in 2 (Cat. No. F20015), 10 (Cat. No. F20016), or 100 (Cat. No. F20017) 96- or 384-well microplates. The kits allow maximum flexibility and ease of operation in a homogenous format against a variety of potassium ion channel and transporter targets.

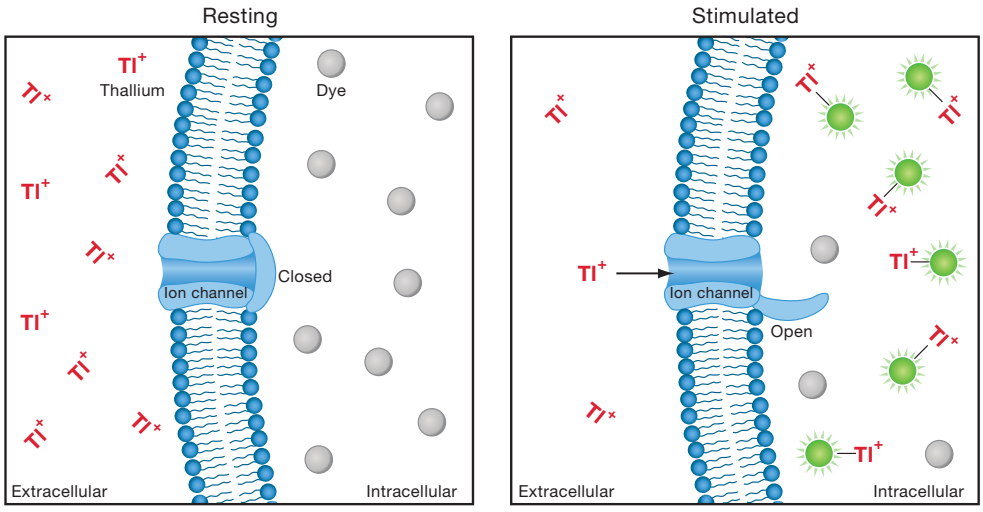

Figure 1. Thallium redistribution in the FluxOR™ II Green assay. Basal fluorescence from cells loaded with FluxOR™ II Green dye is low prior to stimulation of the potassium channels (left panel). When thallium is added to the assay with the stimulus, the thallium flows down its concentration gradient into the cells, activating the FluxOR™ II Green dye (right panel).

Materials required but not provided

- Cells expressing potassium channels of interest and corresponding culture medium for the growth and maintenance of the cells
- Vessels for preparing the assay, loading, and stimulus buffers
- 96- or 384-well microplates

Caution

Thallium sulfate (Component H) is toxic. Use caution when handling thallium sulfate and all solutions prepared containing thallium sulfate. Properly dispose of any waste containing thallium in compliance with all pertaining local regulations.

DMSO (Component B) is known to facilitate the entry of organic molecules into tissues. Handle reagents containing DMSO using equipment and practices appropriate for the hazards posed by such materials. Dispose of the reagents in compliance with all pertaining local regulations.

Procedural guidelines

You can perform the FluxOR™ II Green Potassium Ion Channel Assay with or without the wash step, which removes the loading buffer and growth medium from the culture and replaces them with assay buffer.

Wash method For most high-throughput screening (HTS) and target profiling applications, we recommend the Wash method (page 4). Removing the medium for the loading procedure leaves the cells in a physiologically defined buffer that is free of potentially interfering components from the medium. You can use the 10X FluxOR™ II Background Suppressor (Component I) to remove stray fluorescence from the well and the surrounding solution or compound library, and confine the signal to the inside of the cells.

No-wash method For rapid assay development and for improving the signal-to-noise ratio and the Z factor, we recommend the No-wash method (page 6). Serum-shift assays or experiments using poorly-adhered cell types that cannot tolerate solution exchanges also benefit from the No-wash method. Note that the presence of serum in the growth medium can shift the apparent activity of known pharmacological agents.

Before you begin

Thaw and prepare reagents

1.1 To prepare 100X Probenecid stock solution, add 1 mL of deionized water to each vial containing 77 mg of Probenecid (Component E) and vortex until the powder is completely dissolved. Use the solution on the day of preparation or aliquot and store any unused portions at –20°C for up to 6 months. Avoid repeated freeze-thaw cycles.

Note: 200 µL of 100X Probenecid is needed per microplate.

1.2 To prepare 1000X FluxOR™ II Green dye stock solution, thaw the DMSO (Component B) and briefly centrifuge the vial.

- For the 2 or 10 microplate kits (Cat. Nos. F20015, F20016), add 20 µL of DMSO to a single vial of FluxOR™ II Green Reagent (Component A) to yield a 1000X dye stock solution.
- For the 100 microplate kit (Cat. No. F20017), add 200 µL of DMSO to a single vial of FluxOR™ II Green Reagent (Component A) to yield a 1000X dye stock solution.

Vortex briefly to dissolve the dye completely. The dye stock solution should be colorless. Aliquot and store any unused portions at –20ºC, protected from light and moisture. Avoid repeated freeze-thaw cycles.

Note: 10 µL of 1000X dye stock solution is needed per microplate for the Wash method. 20 µL of 1000X dye stock solution is needed per microplate for the No-wash method.

1.3 Thaw Components C, F, G, and H at room temperature, and store at 4°C until use.

1.4 Thaw the PowerLoad™ Concentrate (Component D) and the FluxOR™ II Background Suppressor (Component I) at 37°C to ensure complete dissolution of the concentrated stock and to remove any precipitate that might have formed during storage. Store at 4°C until use.

1.5 Mix all previously frozen solutions well before use.

Prepare cells

Resuspend the cells expressing the potassium ion channels of interest to 2×10^5 cells/mL in complete growth medium, and plate onto Poly-D-Lysine-coated microplates (80 µL/well for 96-well plates or 20 µL/well for 384-well plates). Allow the cells to recover and adhere for at least 4 hours.

Note: The use of Poly-D-Lysine-coated plates helps cells to attach and stay in place during the kinetic read; however, these plates are not a requirement for assay performance.

Note: Cell culture conditions and plating density should be optimized for each target and cell type.

FluxOR™ II Green Potassium Ion Channel Assay—Wash method

In the Wash method, the medium is removed and the cells are loaded for 60 minutes with 1X Loading Buffer. The Loading Buffer is then removed and replaced with Assay Buffer and the optional FluxOR™ II Background Suppressor. Cells are then treated with drug or control, and the intracellular fluorescence is measured in a kinetic assay.

Prepare 1X Loading Buffer

2.1 For each microplate prepare 10 mL of 1X Loading Buffer. Add the components in the order indicated:

PowerLoad™ Concentrate, 100X (Component D)	100 µL
FluxOR™ II Reagent, reconstituted in DMSO (Step 1.2)	10 µL
Deionized water	8.8 mL
FluxOR™ II Assay Buffer, 10X (Component C)	1 mL
Probenecid, reconstituted in deionized water (Step 1.1)	100 µL
Total volume	10 mL

Load cells

2.2 Remove growth medium from the cells and add 20 µL (for a 384-well plate) or 80 µL (for a 96-well plate) of 1X Loading Buffer (Step 2.1) to each well.

2.3 Incubate the cells at 18–24°C for 60 minutes. During this time, prepare the Assay Buffer and the Stimulus Buffer.

Note: Optimal loading times and incubation temperatures vary for different cell types. Incubate poorly loading cells at 37°C to facilitate dye entry.

Prepare Assay Buffer

2.4 Prepare 10 mL of Assay Buffer:

Deionized water	7.9 mL
FluxOR™ II Assay Buffer, 10X (Component C)	1 mL
FluxOR™ II Background Suppressor (Component I)	1 mL
Probenecid, reconstituted in deionized water (Step 1.1)	100 µL
Total volume	10 mL

Note: The amount of FluxOR™ II Background Suppressor should be optimized for each target and cell type. To prepare the 1X Assay Buffer without the optional FluxOR™ II Background Suppressor, replace the background suppressor solution with deionized water.

Prepare Stimulus Buffer

2.5 Prepare 10 mL of Stimulus Buffer:

- For voltage-gated channels, prepare the **High Potassium Stimulus Buffer**.
- For non-voltage-gated channels, prepare the **Basal Potassium Stimulus Buffer.**

IMPORTANT! The concentrations of potassium and thallium recommended in the following buffers are starting points for further optimization. We recommend that you prepare and test a matrix of varying potassium and thallium concentrations in Stimulus Buffer and choose the concentrations that result in optimal performance and pharmacology for each target and cell type.

High Potassium Stimulus Buffer (for voltage-gated channels)

This 5X formulation injects an elevated level of potassium to depolarize the cells and open voltage gated (Kv type) potassium ion channels. Prepared as below, this stimulus buffer elevates extracellular potassium by 10 mM and adds 2 mM thallium (final) to the solution. Note that the improved sensitivity of the FluxOR™ II dye allows you to use final thallium concentrations in the range of 0.5–1 mM.

Chloride-free Stimulus Buffer (Component F)	2 mL
Potassium Sulfate (K_2SO_4) (Component G)	2 mL
Thallium Sulfate (Tl_2SO_4) (Component H)	1 mL
Deionized water	5 mL
Total volume	10 mL

Basal Potassium Stimulus Buffer (for non-voltage-gated channels)

This 5X formulation injects the thallium stimulus in an isotonic solution to a final concentration of 2 mM to measure the activity of tonically open "resting " or Kir type potassium ion channels. Prepared as below, this stimulus buffer adds 2 mM thallium (final) to the solution. Note that the improved sensitivity of the FluxOR™ II dye allows you to use final thallium concentrations in the range of 0.5–1 mM.

Chloride-free Stimulus Buffer (Component F)	2 mL
Thallium Sulfate (Tl_2SO_4) (Component H)	1 mL
Deionized water	7 mL
Total volume	10 mL

Perform assay

2.6 Remove the Loading Buffer and replace it with 20 µL/well (for a 384-well plate) or 80 µL/well (for a 96-well plate) of Assay Buffer (from Step 2.4) in each well.

2.7 *Optional:* Add the test compounds and incubate for 10–30 minutes at 18–24°C.

2.8 Perform the FluxOR™ II assay using a kinetic dispense microplate reader:

a. Set up the instrument with standard FITC green filters or set the excitation wavelength to 460–490 nm and the emission wavelength to 520–540 nm.

b. After 10 seconds of recording, add Potassium Channel Stimulus buffer to the cells at a 1:5 final dilution (5 µL/well for a 384-well plate or 20 µL/well for a 96-well plate).

c. Read the plate every 1–2 seconds for 1–3 minutes.

FluxOR™ II Green Potassium Ion Channel Assay—No-wash method

In the No-wash method, cells are loaded in complete culture medium for 60 minutes with 2X Loading Buffer. Cells are then treated with drug or control without removing the mixture of Loading Buffer and culture medium, and the intracellular fluorescence is measured in a kinetic assay.

The following protocol describes an initial starting point for using the FluxOR™ II Potassium Ion Channel Assay No-wash method to interrogate potassium ion channel activity. Further signal optimization may be necessary, which you can achieve by adjusting the levels of FluxOR™ II Background Suppressor in the Loading Buffer and the levels of potassium and thallium in the Stimulus Buffer.

Prepare 2X Loading Buffer

3.1 For each microplate prepare 10 mL of 2X Loading Buffer. Add the components in the order indicated:

PowerLoad™ Concentrate, 100X (Component D)	200 µL
FluxOR™ II Reagent, reconstituted in DMSO (Step 1.2)	20 µL
Deionized water	6.5 mL
FluxOR™ II Assay Buffer, 10X (Component C)	1 mL
FluxOR™ II Background Suppressor (Component I)	2 mL
Probenecid, reconstituted in deionized water (Step 1.1)	200 µL
Total volume	10 mL

Load cells

3.2 Add 2X Loading Buffer (from Step 3.1) to each well at a volume equal to the growth medium (20 µL/well for a 384-well plate or 80 µL/well for a 96-well plate).

3.3 Incubate the cells at 18–24°C for 60 minutes, **protected from light**. During this time, prepare the Stimulus Buffer.

Note: Optimal loading times and incubation temperatures vary for different cell types. Incubate poorly loading cells at 37°C to facilitate dye entry.

3.4 Prepare 10 mL of High Potassium or Basal Potassium Stimulus Buffer (see Step 2.5, page 5).

Perform assay

3.5 *Optional:* Add the test compounds and incubate for 10–30 minutes at 18–24°C.

3.6 Perform the FluxOR™ II assay using a kinetic dispense microplate reader:

- **a.** Set up the instrument with standard FITC green filters or set the excitation wavelength to 460–490 nm and the emission wavelength to 520–540 nm.
- **b.** After 10 seconds of recording, add Potassium Channel Stimulus buffer to the cells at a 1:5 final dilution (10 µL/well for a 384-well plate or 40 µL/well for a 96-well plate).
- **c.** Read the plate every 1–2 seconds for 1–3 minutes.

Using high thallium concentrations to detect difficult targets

When using high thallium concentrations to detect difficult targets, replace the FluxOR™ II Assay Buffer (Component C) in the working assay buffer solution (Step 2.4) with Chloride-free Stimulus Buffer (Component F) after the cell loading procedure.

To prepare the working assay buffer, mix 2 mL of Chloride-free Stimulus Buffer (Component F) with 8 mL of deionized water and 100 µL of Probenecid (Component E). These conditions may help in obtaining signals from poorly expressing targets or targets with limited thallium permeability.

Sufficient Chloride-free Stimulus Buffer (Component F) and Thallium Sulfate (Tl_2SO_4) Concentrate (Component H) are included in the kit to run the assay under high-thallium conditions, allowing up to 8 mM free thallium after dilution into the microtiter plate.

Appendix: Assay principle

The FluxOR™ II Green Reagent (Component A) is a fluorogenic indicator dye, which is loaded into cells as a membrane-permeable AM ester (Figure 1, page 2). Before loading into the cells, the FluxOR™ II Green Reagent is dissolved in DMSO (Component B) and further diluted with FluxOR™ II assay buffer (Component C). Loading is assisted by the proprietary PowerLoad™ concentrate (Component D), a formulation of Pluronic™ surfactants, which acts to disperse and stabilize AM ester dyes for optimal loading in aqueous solution. Once inside the cell, the non-fluorescent AM ester form of the FluxOR™ II Green dye is cleaved by endogenous esterases into a fluorogenic thallium-sensitive indicator. The thallium sensitive form is retained in the cytosol and its extrusion is inhibited by water-soluble Probenecid (Component E), which blocks organic anion pumps.

For most applications, cells are loaded with the dye at room temperature. For best results, the dye-loading buffer is then replaced with fresh, dye-free FluxOR™ II assay buffer (Component C) containing Probenecid, before the HTS assay. During the HTS assay, a small amount of thallium is added to the cells with a stimulus solution that opens potassium-permeant ion channels with a mild depolarization or agonist addition. Thallium then passes into cells through open potassium channels according to a strong inward driving force. Upon binding cytosolic thallium, the de-esterified FluxOR™ II Green dye exhibits a strong increase in fluorescence intensity at its peak emission of 525 nm. Baseline and stimulated fluorescence is monitored in real time to give a dynamic, functional readout of thallium redistribution across the membrane with no interference from quencher dyes.

The FluxOR™ II Green assay may also be used to study enzymatic potassium transport processes that accommodate the transport of thallium into cells.[2] Voltage gated potassium channels are opened by the co-administration of potassium and thallium in the stimulus buffer. Resting and inward rectifier potassium channels such as Kir1.1 and Kir2.1 are assayed by adding stimulus buffer with thallium alone, as are potassium ion transporters and cells interrogated for GPCR activity by way of coupling to calcium (KCa) or G protein activated (GIRK) potassium channels.

The unique FluxOR™ II Green Reagent formulation allows the use of the dye in physiological saline, without the need to load or assay cells in chloride-free conditions. This is a major advantage over traditional approaches to thallium flux assays that utilize completely chloride-free conditions to load cells with the dye.[3] Thallium chloride is an insoluble precipitate that forms when concentrations of free thallium and chloride in the solution are greater than about 4 mM. Because the FluxOR™ II Green Reagent is extremely sensitive to thallium, the reagent is added to cells for most applications at a final concentration of 2 mM after dilution into the assay plate. The kits include concentrates of Tl_2SO_4 and K_2SO_4 that allow you to optimize the dose of surrogate (thallium) and stimulus (potassium) used together in the assay to depolarize voltage gated potassium channels.

For ligand gated channels, resting potassium channels, and potassium transporters, the extra potassium is left out of the stimulus buffer, and the stimulus buffer is composed of the channel opener and thallium concentrate, which are necessarily prepared in the chloride-free buffer provided in the kit. However, for low expressing or poorly thallium-permeant targets requiring high extracellular thallium concentrations, enough chloride-free buffer is supplied for the assay and stimulation, allowing for maximum ease and flexibility of use against a wide range of targets. In these cases, cells are still loaded with the FluxOR™ II Green Reagent in FluxOR™ II assay buffer which is removed and replaced with 1X chloride-free buffer containing Probenecid prior to running the assay.

References

1. Assay Drug Dev Technol 6, 765 (2008); **2.** J Biol Chem 284, 14020 (2009); **3.** J of Biomol Screen 9, 671 (2004); **4.** J Gen Physiol 61, 669 (1973); **5.** Nature Biotech 23, 567 (2005); **6.** Trends in Biotech 20, 173 (2002); **7.** Drug Discovery Today 12, 396 (2007); **8.** Assay Drug Dev Technol 5, 417 (2007); **9.** Expert Opin Drug Discov 2, 1669 (2007); **10.** Adv Virus Res 68, 255 (2006); **11.** Expression and Analysis of Recombinant Ion Channels: From Structural Studies to Pharmacological Screening, Clare, J.J. & Trezise, D.J. Eds. Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany, pp.79-109.

Frequently asked questions

Q: Why is there no change in signal from my cells?
A: Make sure there is thallium present in the stimulus buffer only.

Q: How can I increase the signal window?
A: Prepare fresh stimulus buffer, and run a matrix of thallium and potassium doses to determine the optimal ratio for your target of interest.

Q: What causes a small signal window?
A: There are several possible reasons for a small signal window: 1) low level expression of target, 2) low thallium permeability of target, 3) cells were loaded too long (>2 hours), 4) cells were loaded too short (<30 minutes), or 5) Probenecid was not included.

Q: Why is there no detectable signal from the cells?
A: Cells may not have been loaded with dye. FluxOR™ II dye should be readily visible with an FITC filter on a fluorescence microscope. Check for even, cytoplasmic labeling of the cells after loading. If cells are not labeled, check that the dye was properly resuspended and stored, and that the reagents in the loading buffer were mixed in the proper order.

Q: Why I am getting high variability from well to well?
A: Incomplete removal of solutions from the wells is the likely cause. Make sure to aspirate or remove all culture medium before loading the cells with the loading buffer. Also, carefully remove all of the dye before replacing it with fresh assay buffer. Ensure that cells are not removed during the process. An additional wash step following the removal of the dye may also increase fidelity in the assay.

Ordering information

Cat. No.	Product name	Unit size
F20015	FluxOR™ II Green Potassium Ion Channel Assay *for 2 microplates*	1 kit
F20016	FluxOR™ II Green Potassium Ion Channel Assay *for 10 microplates*	1 kit
F20017	FluxOR™ II Green Potassium Ion Channel Assay *for 100 microplates*	1 kit
Related products		
P10020	PowerLoad™ concentrate, 100X	5 mL
P36400	Probenecid, water soluble	10 × 77 mg

Documentation and support

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  - User guides, manuals, and protocols
  - Certificates of Analysis
  - Safety Data Sheets (SDSs; also known as MSDSs)

Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

Revision history: Pub. No. MAN0016084

Revision	Date	Description
B.0	06 November 2017	Add instructions to prepare dye stock solution for 100 microplate kits.
A.0	12 August 2016	New document

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