Brilliant Calcium *Flex*

Table 1	Package Contents			
Label	Name	Volume	Containers	Storage
Reagent A	Fluo-4 AM (50 µg)	Dry	10	-20° C
Reagent B	DMSO	225 µL	1	-20° C *
Reagent C	DySolv	4 mL	1	4° C
Reagent D	10X Assay Buffer	20 mL	1	4° C
Reagent E	TRS	4 mL	1	4° C
Reagent F	Probenecid Solution	4 mL	1	4° C

**Hygroscopic DMSO should be stored at -20° C with desiccant pack to prevent the solvent from becoming wet. Wet solvent causes difficulties with the solubility of the dye. Use the DMSO within 6 months of receipt.*

Description

ION Biosciences Brilliant Calcium Assay is a total assay solution for multi-well plate-based, high-throughput measurements of changes intracellular Ca^{2+} mediated through a wide-variety of plasma membrane and intracellular calcium channels and transporters. The ION Biosciences Brilliant Calcium Assay is also useful for investigating numerous effectors of ion channels and transporters including G protein-coupled receptors, lipid kinases and protein kinases. In multi-well, plate-based formats, the Brilliant Calcium Assay can be used to discover and characterize the effects of many tens-of-thousands of compounds and environmental factors on effectors of intracellular Ca^{2+}. For the last 25 years, fluorescence-based measures of Ca^{2+} 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 Brilliant Calcium *Flex* provides all the reagents necessary for use as a washed or no-wash assay with adherent or non-adherent cells. The optional use of a probenecid solution and an extracellular background masking solution offers the ultimate in compatibility for cells types which are difficult to load with fluorescent Ca^{2+} indicators (e.g. Chinese Hamster Ovary, CHO cells) and when performing assays in complete, serum-containing cell culture medium is desired.

Laboratory Procedures

Getting Started

Before you begin, make sure that you have all the additional reagents and materials you will need for the successful completion of your experiment. While the ION Brilliant Calcium Assay package contains all the reagents you will need to prepare your cells for testing, your experiments will likely include other reagents which are not included in your ION Brilliant Calcium Assay package. Notably compounds to be tested are not included, neither are buffers and solvents for the dissolution of those compounds. The Brilliant Calcium Assay package also does not contain reagents necessary for cell

Laboratory Procedures

Getting Started

In addition to reagents, a fluorescence plate reader that is capable of providing excitation at ~ 490 nm and collecting emission at ~ 520 nM is required. Ideally this plate reader will be able to collect kinetic data at an interval of once per second (1 Hz). Examples of plate readers of this type are the WaveFront Panoptic, Hamamatsu FDSS, Molecular Devices FLIPR and Molecular Devices FlexStation.

Wash Method – Adherent Cells

The instructions given below are for one, 384-well microplate. Certain aspects of the instructions may need to be altered, as appropriate, for multiple microplates or other assay formats (e.g. 96-well microplates or non-adherent cells). The ION Brilliant Calcium indicator and ION Brilliant Calcium indicator-containing solutions should be protected from direct light.

1. Add 20 µL DMSO (Reagent B) to the tube containing Fluo-4 AM (Reagent A)
2. Vortex until Reagent A is fully dissolved.
3. Add appropriate volume of water (Table 2) to a 15 mL centrifuge tube.
4. Add 1 mL of 10X Assay Buffer (Reagent D) to tube from step 3.
5. Add 200 µL of DySolv (Reagent C) to the tube from step 4.
6. If desired add 200 µL of Probenecid Solution (Reagent F) to the tube from step 5.

Table 2 Dye Loading Solution

Label	Name	Method A	Method B
Reagent A	Fluo-4 AM Solution	20 µL	20 µL
Reagent C	DySolv	200 µL	200 µL
Reagent D	10X Assay Buffer	1 mL	1 mL
Reagent F	Probenecid Solution*	-	200 µL
	Water	8.8 mL	8.6 mL
	Total	10 mL	10 mL

**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.*

7. Add 20 µL of Fluo-4 AM Solution from step 2 to the tube from step 6.
8. Briefly vortex the tube from step 7 to mix.
9. Remove the cell-culture medium from the 384-well microplate containing the cells of interest.
10. Add 20 µL per well of the Dye Loading Solution from step 8 to the microplate from step 9.
11. Incubate the microplate containing the cells and Dye Loading Solution for 1 hour at 37° C.

Table 3	Wash Solution				
Label	Name	Method A	Method B	Method C	Method D
Reagent D	10X Assay Buffer	1 mL	1 mL	1 mL	1 mL
Reagent E	TRS*	-	200 µL	-	200 µL
Reagent F	Probenecid Solution	-	-	200 µL	200 µL
Reagent H	Water	9 mL	8.8 mL	8.8 mL	8.6 mL
	Total	10 mL	10 mL	10 mL	10 mL

**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.*

12. Prepare Wash Solution in a 15 mL centrifuge tube by adding the appropriate amounts of water, 10X Assay Buffer (Reagent D) and other components if desired as shown in Table 3.
13. Briefly vortex the tube from step 12 to mix.
14. Remove Dye Loading Solution from microplate in step 11.
15. Add 20 µL per well of the Wash Solution prepared in step 13 to the microplate from step 14.
16. Transfer the washed, dye-loaded, cell-containing microplate from step 15, along with an additional microplate containing a stimulus solution of interest, to a kinetic-imaging plate reader (e.g. WaveFront Panoptic, Hamamatsu FDSS, Molecular Devices FLIPR or Molecular Devices FlexStation).
17. Acquire data using an excitation wavelength of ~ 490 nm, an emission wavelength of ~ 520 nm and an acquisition frequency of 1 Hz. Begin data acquisition and after 10 seconds add 5 µL of the stimulus solution to the cell-containing plate and continue data acquisition for an additional 90 seconds**.

***The timing of and volume of stimulus solution addition may vary. Some 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.*

No-wash Method – Adherent Cells

1. Add 20 µL DMSO (Reagent B) to the tube containing Fluo-4 AM (Reagent A)
2. Vortex until Reagent A is fully dissolved.

Table 4 Dye Loading Solution

Label	Name	Method A	Method B
Reagent A	Fluo-4 AM Solution	20 µL	20 µL
Reagent C	DySolv	400 µL	400 µL
Reagent D	10X Assay Buffer	1 mL	1 mL
Reagent E	TRS*	400 µL	400 µL
Reagent F	Probenecid Solution**	-	400 µL
	Water	8.2 mL	7.8 mL
	Total	10 mL	10 mL

**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*

3. Add appropriate volume of water (Table 4) to a 15 mL centrifuge tube.
4. Add 1 mL of 10X Assay Buffer (Reagent D) to tube from step 3.
5. Add 400 µL of DySolv (Reagent C) to the tube from step 4.
6. Add 400 µL of TRS (Reagent E) to the tube from step 5.
7. If desired add 400 µL of Probenecid Solution (Reagent F) to the tube from step 6.
8. Add 20 µL of Fluo-4 AM Solution from step 2 to the tube from step 7.
9. Briefly vortex the tube from step 8 to mix.
10. Add 20 µL per well of the Dye Loading Solution from step 9 to the cell-containing microplate. Do not remove the cell culture medium.
11. Incubate the microplate containing the cells and Dye Loading Solution for 1 hour at 37° C in a cell culture incubator.

12. Transfer the dye-loaded, cell-containing microplate from step 11, along with an additional microplate containing a stimulus solution of interest, to a kinetic-imaging plate reader (e.g. WaveFront Panoptic, Hamamatsu FDSS, Molecular Devices FLIPR or Molecular Devices FlexStation).
13. Acquire data using an excitation wavelength of ~ 490 nm, an emission wavelength of ~ 520 nm and an acquisition frequency of 1 Hz. Begin data acquisition and after 10 seconds add 10 µL of the stimulus solution to the cell-containing plate and continue data acquisition for an additional 90 seconds**.

***The timing of and volume of stimulus solution addition may vary. Some 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.*