Brilliant Sodium *Flex*

Table 1	Package Contents			
Label	Name	Volume	Containers	Storage
Reagent A	ING-2 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 Brilliant Sodium 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

Sodium (Na^+) is one of the most important monovalent metal cations in living organisms. Na^+ channels, Na^+-permeable non-selective monovalent cation channels, and Na^+-coupled transporters play critical roles including modulating neuronal activity, powering transport of nutrients and signaling molecules, and regulating solute balance. Na^+-permeable channel and Na^+ transporter-targeted drugs provide effective treatments for a diversity of indications: epilepsy, pain, bipolar disorder, depression, diuresis, and many others. As a result, interest in Na^+-permeable channels and Na^+ transporters as drug targets remains high.

ION's Brilliant Sodium *Flex* Assay is a total assay solution for multi-well plate-based, high-throughput measurements of changes in intracellular Na^+ mediated through a wide-variety of plasma membrane and intracellular sodium channels and transporters. In multi-well, plate-based formats, the Brilliant Sodium Assay can be used to discover and characterize the effects of many tens-of-thousands of compounds and environmental factors on effectors of intracellular Na^+. ION's Brilliant Sodium *Flex* provides all the reagents necessary for use as a wash or no-wash assay with adherent or non-adherent cells. The optional use of a probenecid solution and an extracellular background masking solution (TRS) offers the ultimate in compatibility for cells types which are difficult to load with fluorescent Na^+ indicators (e.g. Chinese Hamster Ovary, CHO cells) and when performing assays in complete, serum-containing cell culture medium is desired.

ION's Brilliant Sodium *Flex* Assay is compatible with fluorescence microscopes, flow cytometers, and plate readers capable of detecting fluorescein or more optimally, yellow fluorescent protein (YFP).

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 Sodium 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 Sodium

Laboratory Procedures Cont.

Assay package. Notably compounds to be tested are not included, neither are buffers and solvents for the dissolution of those compounds. The Brilliant Sodium Assay package also does not contain reagents necessary for cell culture.

In addition to reagents, a fluorescence plate reader that is capable of providing excitation between 485 - 525 nm and collecting emission at ~545 nm is required. Although kinetic plate readers capable of providing readouts at ~1 Hz, such as WaveFront Panoptic, Hamamatsu FDSS, Molecular Devices FLIPR and Molecular Devices FlexStation, are commonly used - ION's Brilliant Sodium Assay can also be used in an endpoint format (see Figures 2 and 4) on standard plate readers.

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 ING-2 AM and ING-2 AM-containing solutions should be protected from direct light.

1. Add 20 µL DMSO (Reagent B) to the tube containing ING-2 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	Method C
Reagent A	ING-2 AM Solution	20 µL	20 µL	20 µL
Reagent C	DySolv	200 µL	200 µL	200 µL
Reagent D	10X Brilliant Sodium Assay Buffer	1 mL	1 mL	1 mL
Reagent E	TRS*	200 µL	200 µL	-
Reagent F	Probenecid Solution**	200 µL	-	200 µL
	Water	8.4 mL	8.6 mL	8.6 mL
	Total	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.*

***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 ING-2 AM Solution from step 2 to the tube from step 6.
8. Briefly vortex the Dye Loading Solution, 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 30 minutes - 1 hour at 37° C.

Table 3 Wash Solution

Label	Name	Method A	Method B	Method C	Method D
Reagent D	10X Brilliant Sodium 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

12. Steps 12 - 15 are only required if a Dye Loading Solution without TRS (Method C in Table 2) is used. 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 dye-loaded, cell-containing microplate from step 11 or 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 ~ 520 nm***, an emission wavelength of ~ 545 nm and an acquisition frequency of ~1 Hz.**** Begin data acquisition and after 20 seconds add 5 µL of the 5X stimulus solution to the cell-containing plate and continue data acquisition for an additional 90 seconds*****.

****Excitation sources commonly used for fluorescein, Fluo-4, and GFP (480–490 nm) are also compatible with Brilliant Sodium.*

*****For targets where changes in intracellular sodium concentrations are slow or sustained, an endpoint assay format can be used. We recommend acquiring data before the addition of stimulus (F_0) and again 15-30 min after the addition of stimulus.*

******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 ION ING-2 AM (Reagent A)
2. Vortex until Reagent A is fully dissolved.

Table 4 Dye Loading Solution

Label	Name	Method A	Method B
Reagent A	ING-2 AM Solution	20 µL	20 µL
Reagent C	DySolv	400 µL	400 µL
Reagent D	10X Brilliant Sodium 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 assay performance for the target of interest.*

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 ING-2 AM Solution from step 2 to the tube from step 7.
9. Briefly vortex the Dye Loading Solution, 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 30 minutes - 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 ~ 520 nm***, an emission wavelength of ~ 545 nm and an acquisition frequency of ~1 Hz.**** Begin data acquisition and after 20 seconds add 10 µL of the 5X stimulus solution to the cell-containing plate and continue data acquisition for an additional 90 seconds*****.

****Excitation sources commonly used for fluorescein, Fluo-4, and GFP (480—490 nm) are also compatible with Brilliant Sodium.*

*****For targets where changes in intracellular sodium concentrations are slow or sustained, an endpoint assay format can be used. We recommend acquiring data before the addition of stimulus (F_0) and again 15-30 min after the addition of stimulus.*

******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.*

Example Results

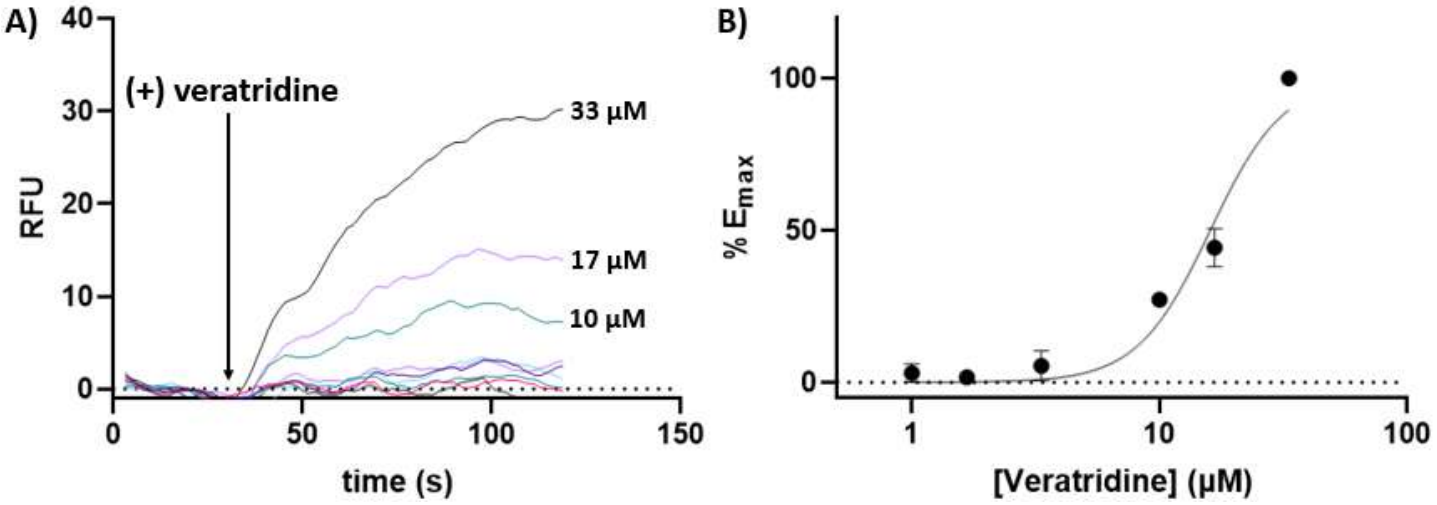
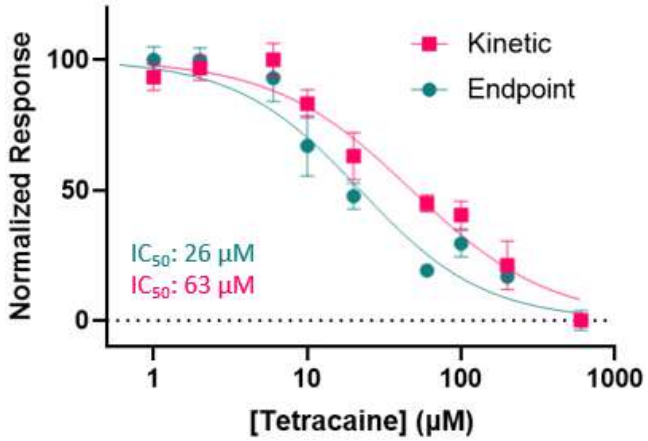


Figure 1. Measuring $Na_v1.3$ activity using ING-2 in engineered HEK $Na_v1.3$ cells. A) Baseline subtracted, kinetic fluorescence data acquired using a Molecular Devices FlexStation® (Ex: 515 nm, Em: 545 nm, Cutoff: 530 nm) for all veratridine concentrations evaluated. Veratridine, an inhibitor of Na_v channel inactivation, was added at 30 sec. B) Veratridine concentration response curve (CRC) in engineered HEK $Na_v1.3$ cells. The estimated EC_{50} is 15 µM, and error bars represent standard deviation (n = 3).

Figure 2. Measuring $Na_v1.3$ inhibition using ING-2 in engineered HEK $Na_v1.3$ cells. Tetracaine concentration response curves (CRC) in HEK Na_v 1.3 cells measured using ING-2. Cells were exposed to tetracaine, a local anesthetic known the block voltage-gated sodium channels, for 10 min. prior to the addition of veratridine (33.3 µM). Fluorescence (Ex: 515 nm, Em: 555 nm, Cutoff: 550 nm) was recorded at ~1 Hz on a Molecular Devices FlexStation® plate reader for 1.5 min. after the addition of veratridine for "Kinetic" data (pink). For "Endpoint" data (blue), a Cytation 5 was used to collect fluorescence (Ex: 525 nm Em: 545 nm) 30 minutes after the addition of veratridine. Error bars represent SEM (n = 3).



Example Results Cont.

Endpoint compatible	For certain targets, such as Na^+/K^+-ATPase and voltage-gated Na^+ channels, any fluorescence plate reader will work. Load ING-2 for 30—60 minutes, acquire initial fluorescence data, add your compounds, wait ~30 minutes, then acquire fluorescence data again. This approach is demonstrated in Fig. 2 and 4.

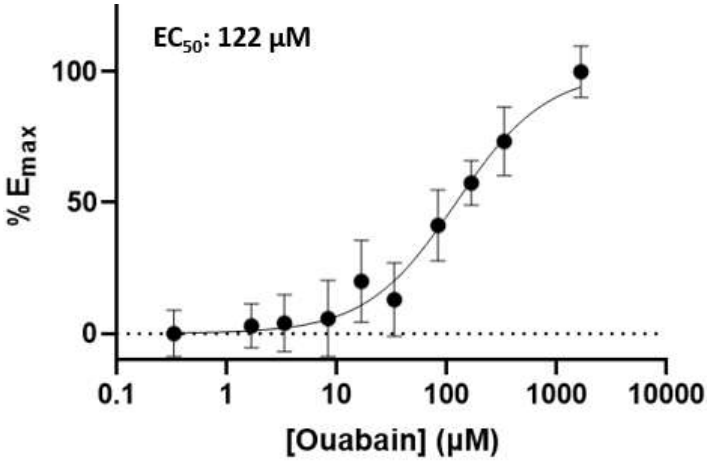


Figure 3. Measuring Na^+/K^+-ATPase inhibition using ING-2. Ouabain concentration response curve (CRC) in CHO K1 (WT) cells measured using ING-2 AM. Fluorescence (Ex: 525 nm, Em: 555 nm, Cutoff: 550 nm) was recorded at ~1 Hz using a Molecular Devices FlexStation® plate reader for 4.5 min. after the addition of ouabain, and (F_{max}-F_0) values were obtained. The estimated EC_{50} is 122 µM. Error bars represent standard deviation (n = 3).

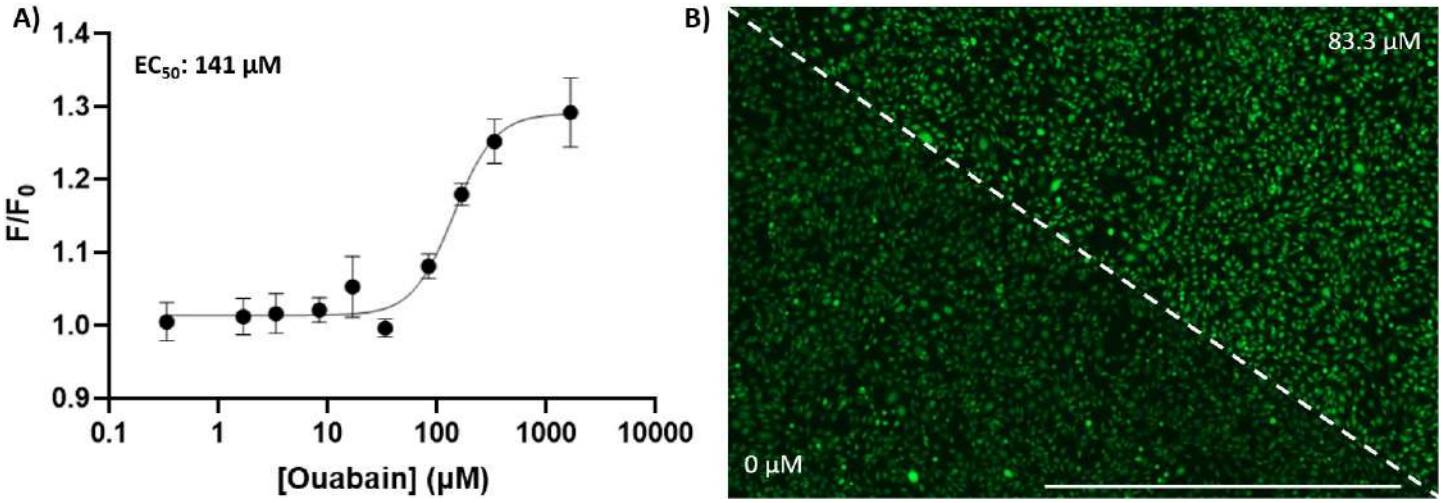


Figure 4. Measuring Na^+/K^+-ATPase inhibition using ING-2 using an endpoint assay. A) Ouabain concentration response curve (CRC) in CHO K1 (WT) cells measured using ING-2. F/F_0 were recorded 30 min. after the addition of ouabain using a Molecular Devices FlexStation® (Ex: 515 nm, Em: 545 nm, Cutoff: 530 nm). The measured EC_{50} is 141 µM, and error bars represent standard deviation (n = 3). B) Representative fluorescence images acquired ~35 min. after the addition of ouabain using a BioTek® Cytation equipped with a GFP filter cube (Ex: 469/35 nm, Em: 525/39 nm) and 4X objective. Corresponding ouabain concentrations are overlayed on each image, and increased fluorescence at higher concentrations of ouabain is observed. Scale bar is 1mm.

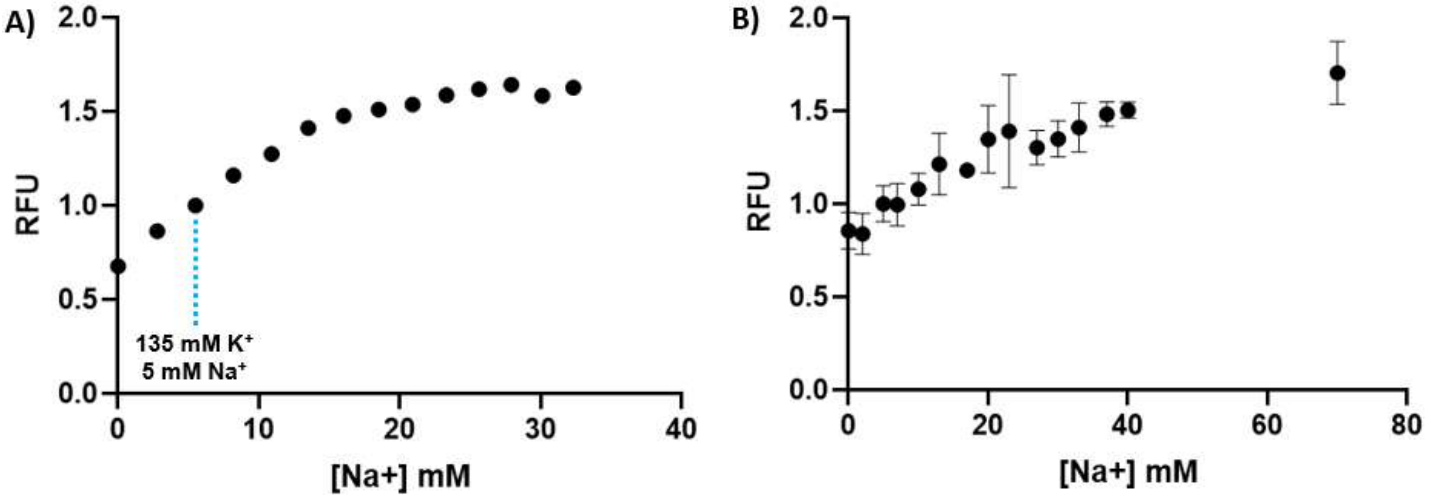


Figure 5. Increases in ING-2 fluorescence in response to [Na^+]. A) Titration of ING-2 in 12.5 mM TRIS-Cl (pH = 7.4) buffer containing BSA (0.25 w/v%) and Mg^{2+} (1.2 mM) over a physiologically relevant range of [Na^+] + [K^+] concentrations. [Na^+] + [K^+] = 140 mM. B) Intracellular calibration of ING-2 loaded in CHO K1 cells. Calibrations were performed using gramicidin (5 µM) and fluorescence was recorded 90 min. after buffer exchange using a Cytation 5 plate reader. All data was normalized to the fluorescence (Ex: 525 nm, Em: 545 nm) at [K^+] = 135 mM and [Na^+] = 5 mM. Error bars represent standard deviation (n = 3).

References

1. Tay B, Stewart TA, Davis FM, Deuis JR, Vetter I. Development of a high-throughput fluorescent no-wash sodium influx assay. *PLoS One*. 2019 Mar 11;14(3):e0213751.
2. Iamshanova, O., Mariot, P., Lehen'kyi, V. *et al.* Comparison of fluorescence probes for intracellular sodium imaging in prostate cancer cell lines. *Eur Biophys J.* 45, 765–777 (2016).
3. Yurinskaya VE, Aksenov ND, Moshkov AV, Goryachaya TS, Vereninov AA. Fluorometric Na^+ Evaluation in Single Cells Using Flow Cytometry: Comparison with Flame Emission Assay. *Cell Physiol Biochem*. 2020 May 29;54(4):556-566.
4. Naumann G, Lippmann K, Eilers J. Photophysical properties of Na^+ -indicator dyes suitable for quantitative two-photon fluorescence-lifetime measurements. *J Microsc*. 2018 Nov; 272(2):136-144.

Related Products

Product Code	Product Name
2011	ING-2 AM
7010s	10X Brilliant Sodium Assay Buffer
7601A	100X Pluronic F-127 Solution
7501A	DySolv
7060A	TRS
7300A	Probenecid Solution