AssayGenie

Technical Manual

GenieHTS Sodium Flux Assay Kit

- **Catalogue Code: ASIB005**
- **Size: 10 plates**
- **Research Use Only**

Introduction

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.

Assay Genies' GenieHTS Sodium Flux 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 GenieHTS Sodium Flux 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^+. Assay Genies' GenieHTS Sodium Flux Assay 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.

Assay Genies' GenieHTS Sodium Flux Assay is compatible with fluorescence microscopes, flow cytometers, and plate readers capable of detecting fluorescein or more optimally, yellow fluorescent protein (YFP).

Kit Features:

- **Excitation:** 5250nm
- **Emission:** 545nm
- **MW:** 1084

Kit Components

Table 1

Component Name	Size	Storage
GenieHTS Sodium Flux Indicator	Lyophilized (10)	-20°C
DMSO	225µL	4°C
Dye Solvent	4mL	4°C
10X Sodium Assay Buffer	20mL	4°C
TRS	4mL	4°C
Probenecid Solution	4mL	4°C

Materials needed but not provided

- Compounds to be tested.
- Buffers and solvents for dissolution.
- Reagents necessary for cell culture.
- A fluorescence plate reader ~ 490 nm /~ 520 nM.
- Plate reader capable of collect kinetic data (1 Hz) e.g. WaveFront Panoptic, Hamamatsu FDSS, Molecular Devices FLIPR and Molecular Devices FlexStation.

Assay Procedure

Adherent Cells: Wash Method

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 Assay Genie Potassium Ion indicator and ION Brilliant Thallium indicator-containing solutions should be protected from direct light.

1. Add 20 µL DMSO to the tube containing GenieHTS Sodium Flux Indicator.
2. Vortex until GenieHTS Sodium Flux Indicator 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 to tube from step 3.
5. Add 200 µL of Dye Solvent to the tube from step 4.
6. If desired add 200 µL of Probenecid Solution to the tube from step 5.

Table 2: Dye Loading Solution

Component	Method 1	Method 2	Method 3
GenieHTS Sodium Flux Assay Kit	20µL	20µL	20µL
Dye Solvent	200µL	200µL	200µL
10X Sodium Assay Buffer	1mL	1mL	1mL
TRS*	200µL	200µL	-
Probenecid Solution**	-	200µL	200µL
Water	8.4mL	8.6mL	8.6mL
Total	**10mL**	**10mL**	**10mL**

*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 GenieHTS Sodium Flux 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

Component	Method 1	Method 2	Method 3	Method 4
10X Sodium Assay Buffer	1mL	1mL	1mL	1mL
TRS*	-	200µL		200µL
Probenecid Solution	-	-	200µL	200µL
Water	9mL	8.8mL	8.8mL	8.6mL
Total	**10mL**	**10mL**	**10mL**	**10mL**

*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. Steps 12 - 15 are only required if a Dye Loading Solution without TRS (Method 3 in Table 2) is used. Prepare Wash Solution in a 15 mL centrifuge tube by adding the appropriate amounts of water, 10X Assay Buffer 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 Sodium Flux Assay.
****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 (F0) 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.

Adherent Cells: No-wash Method

1. Add 20 µL DMSO to the tube containing GenieHTS Sodium indicator.
2. Vortex until the Sodium Indicator Solution is fully dissolved.

Table 4: Dye Loading Solution

Component	Method 1	Method 2
GenieHTS Sodium Indicator Solution	20µL	20µL
Dye Solvent	400µL	400µL
10X Sodium Assay Buffer	1mL	1mL
TRS*	400µL	400µL
Probenecid Solution**	-	400µL
Water	8.2mL	7.8mL
Total	**10mL**	**10mL**

*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 Sodium Assay Buffer to tube from step 3.
5. Add 400 µL of Dye Solvent to the tube from step 4.
6. Add 400 µL of TRS to the tube from step 5.
7. If desired add 400 µL of Probenecid Solution to the tube from step 6.
8. Add 20 µL of GenieHTS Sodium Indicator 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 ~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 (F0) 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.

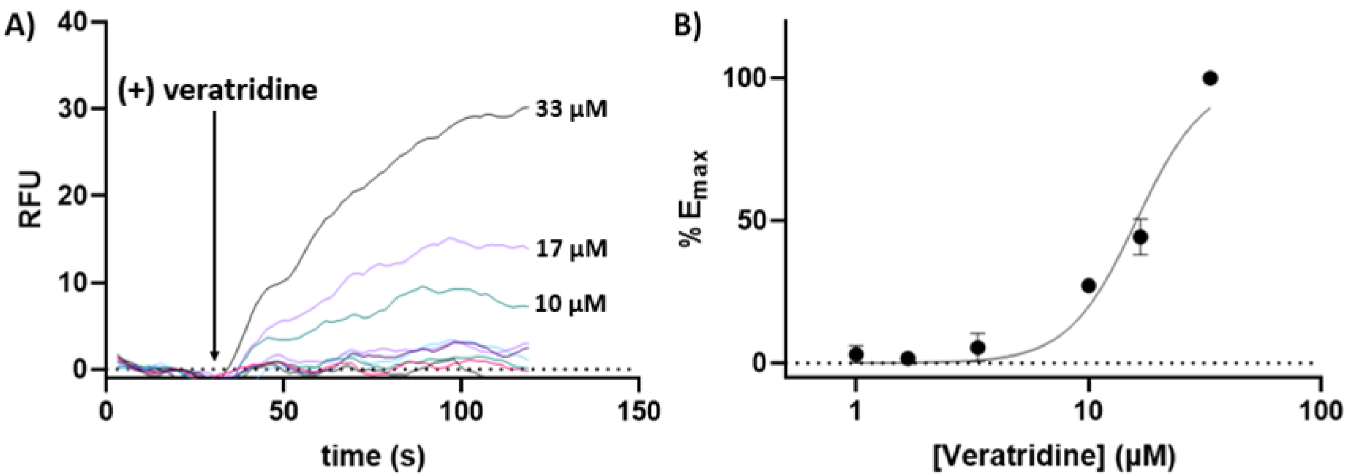
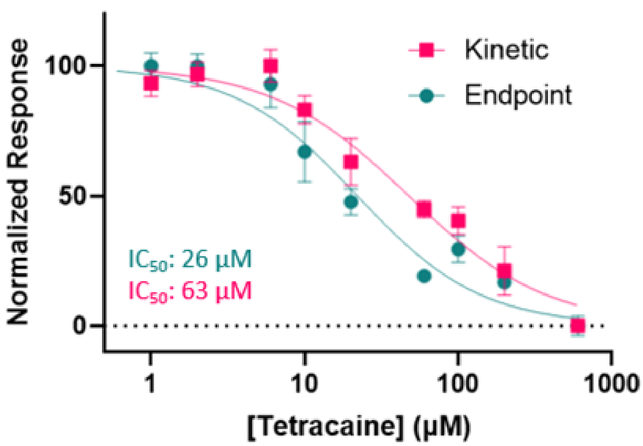


Figure 1: Measuring Nav1.3 activity using ING-2 in engineered HEK Nav1.3 cells. A) Baseline subtracted, kinetic fluorescence data acquired using a Molecular Devices FlexStation® (Ex: 515 nm, Em: 545 nm, Cutoff: 530 nm) for all veratridine concentrations evaluated. Veratridine, an inhibitor of Nav channel inactivation, was added at 30 sec. B) Veratridine concentration response curve (CRC) in engineered HEK Nav1.3 cells. The estimated EC50 is 15 µM, and error bars represent standard deviation (n = 3).



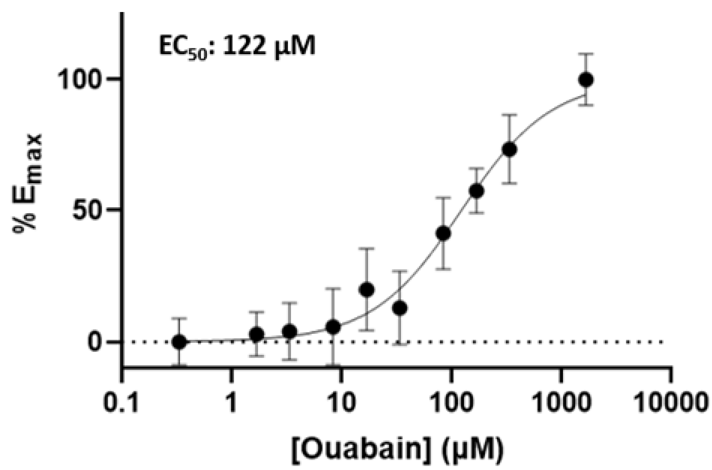


Figure 2: Measuring Nav1.3 inhibition using ING-2 in engineered HEK Nav1.3 cells. Tetracaine concentration response curves (CRC) in HEK Nav 1.3 cells measured using ING-2. Cells were exposed to tetracaine, a local anesthetic known the block voltage-gated sodium channels, for 10 min. prior to the addition 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).

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 (Fmax-F0) values were obtained. The estimated EC50 is 122 µM. Error bars represent standard deviation (n = 3).

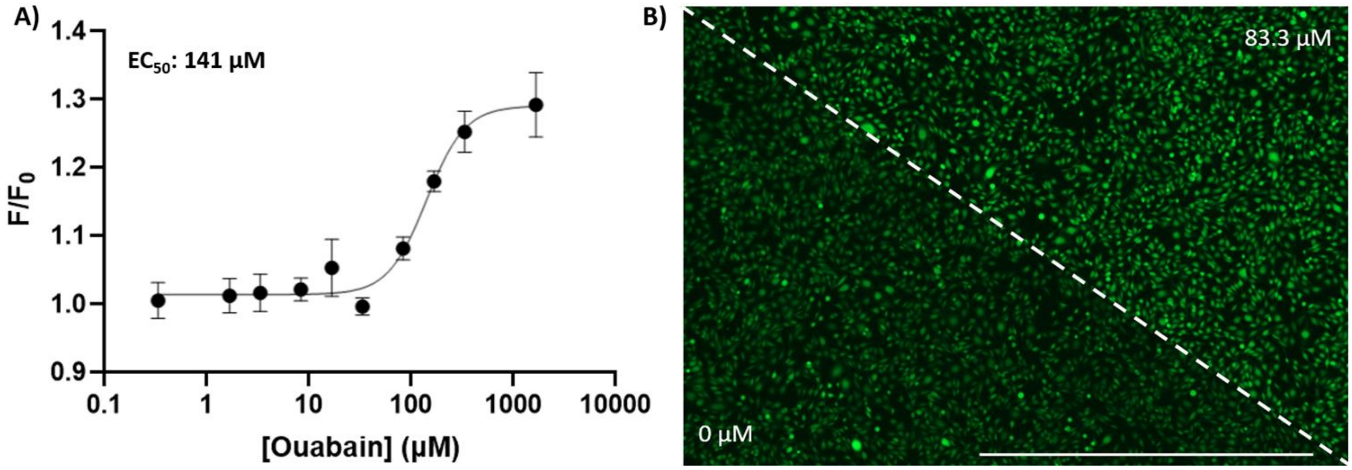


Figure 4: Measuring Na^+ /K^+ -ATPase inhibition using ING-2 using an endpoint assay. A) Ouabain concentration response curve (CRC) in CHO K1 (WT) cells measured using ING-2. F/F0 were recorded 30 min. after the addition of ouabain using a Molecular Devices FlexStation® (Ex: 515 nm, Em: 545 nm, Cutoff: 530 nm). The measured EC50 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.

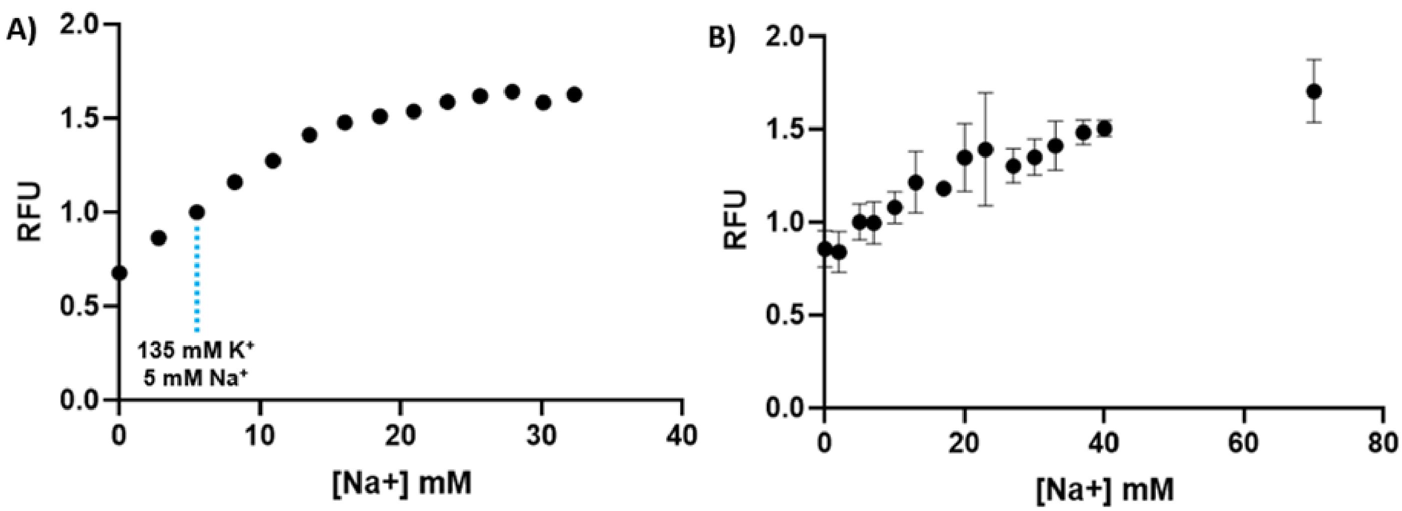


Figure 5: Increases in ING-2 fluorescence in response to [Na^+]. A) Titration of ING-2 in 12.5 mM TRIS-Cl (pH = 7.4) buffer containing BSA (0.25 w/v%) and Mg^{2+} (1.2 mM) over a physiologically relevant range of [Na^+] + [K^+] concentrations. [Na^+] + [K^+] = 140 mM. B)Intracellular calibration of ING-2 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

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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).

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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.

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