APPLICATION NOTE

High-throughput Sodium Flux Assay for $Na_V1.5$ using Assay Ready Cells

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Abstract

Voltage-gated sodium channels (Na_Vs) present tremendous therapeutic potential for treating pain, epilepsy, neuromuscular disorders, cardiovascular diseases, respiratory disorders, and cancer. $Na_V1.5$ is the primary Na_V channel in the heart, and the well-established molecular target for antiarrhythmic drugs and local anesthetics. A fluorescence-based high-throughput sodium flux assay was developed to screen small molecule modulators targeting $Na_V1.5$. This assay utilizes ION Biosciences' Brilliant Sodium Assay and acCELLerate's assay ready CHO-NaV1.5-DUO™ instaCELLs to provide an effective, simple, and fast method to identify $Na_V1.5$ activators/inhibitors.

Introduction

Voltage-gated sodium channels ($Na_V1.X$) are expressed in the cell membranes of excitable and non-excitable cells. In humans there are nine different Na_V α subunits, $Na_V1.1$ – $Na_V1.9$. Membrane depolarization facilitates a conformational change of the α subunit, resulting in the opening of the Na^+-selective channel and inward current of Na^+. Na_V channels work in concert to initiate action potentials; therefore, it is no surprise that their dysfunction plays a role in numerous neuronal and cardiac disorders. Sodium channel blockers have been developed as anticonvulsant, antiarrhythmic, and local anesthetic drugs in the past. Currently, enormous efforts are underway to find and study the effects of subtype-selective Na_V modulators in pre-clinical disease models [1].

$Na_V1.5$ channels, encoded by gene SCN5A, are mostly expressed in human heart tissue. $Na_V1.5$ channel mutations are associated with many cardiac dysfunctions including Brugada syndrome, long QT syndrome, and atrial fibrillation [2]. $Na_V1.5$ is also a therapeutic target for antiarrhythmic drugs and local anesthetics such as lidocaine and tetracaine [3], and is part of the Comprehensive in vitro Proarrhythmia Assay (CiPA) panel for evaluation of cardiac risk. Thus, there is high interest in screening for $Na_V1.5$ modulators.

Here we present a fluorescence-based high-throughput sodium flux assay that is suitable for screening for modulators targeting $Na_V1.5$. This assay utilizes ION Biosciences' Brilliant Sodium Assay and acCELLerate's assay ready CHO-NaV1.5-DUO™ instaCELLs to provide a rapid and simple method to identify $Na_V1.5$ activators/inhibitors in less than 24 hrs. ION Biosciences Brilliant Sodium Assay contains ION Natrium Green-2 (ING-2), a sodium selective-indicator, and all other reagents necessary for running

a HTS assay. CHO-NaV1.5-DUO™ instaCELLs are qualified frozen aliquots of cells from a recombinant cell line which stably express $Na_V1.5$. The cell line had been developed and validated by B'SYS GmbH, Switzerland and is distributed by acCELLerate in the assay ready instaCELL format. Instantly after thawing and without prior cultivation, CHO-NaV1.5-DUO™ instaCELLs display a high viability (>90%) and exhibit a strong functional expression of $Na_V1.5$.

Experimental overview

Thaw assay ready CHO-NaV1.5-DUO™ instaCELLs and dilute into the desired concentration with complete cell culture media. Seed cells into black wall/clear bottom multi-well plates (30,000 cells/well for 96-well plate and 15,000 cells/well for 384-well plate) and incubate overnight to allow the cells to attach. The next day, make a 1X dye loading solution according to the Brilliant Sodium Assay protocol, remove cell culture media and add dye loading solution into each cell-containing well (100µL/well for 96-well plate and 20µL/well for 384-well plate). Incubate the plates for 1 hr. During the incubation, prepare a 3X concentrated compound plate. Finally, perform assay readout on a kinetic plate reader using the parameters listed below.

Assay format	Instrument	Ex*, Em	Frequency	Baseline read	Drug addition	Post-addition read
96-well	Flexstation 3	515 nm, 545 nm	1 Hz	20 s	50 µL	130 s
384-well	Panoptic	518 nm, 562 nm	1 Hz	20 s	10 µL	300 s

*ING-2 Na^+ indicator also works with standard 488nm light sources.

When testing for $Na_V1.5$ inhibitors, add inhibitors 15 min prior to your read, then introduce a high concentration of veratridine, a blocker of Na_V inactivation, during the kinetic read. If a kinetic plate reader isn't available, the assay can be conducted in endpoint mode using a standard plate reader or fluorescence microscope using either GFP or YFP filters.

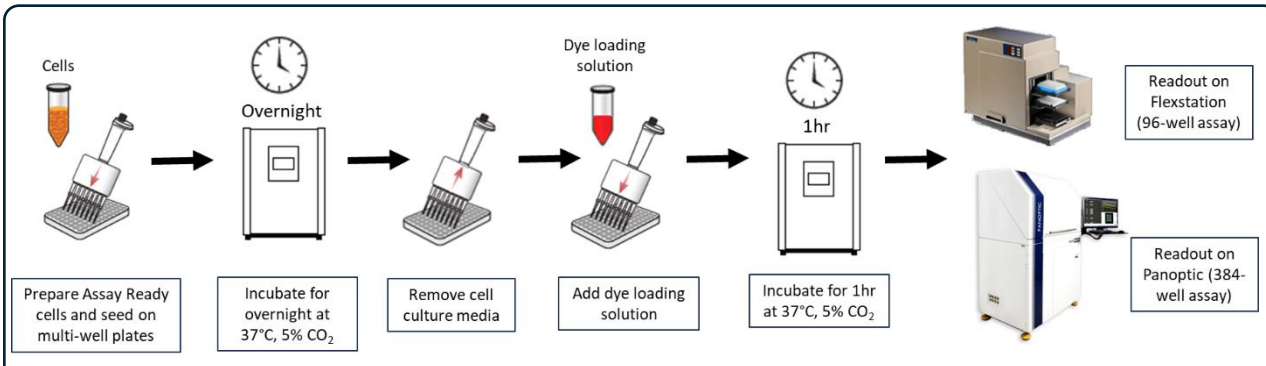

Figure 1. $Na_V1.5$ Sodium Flux Assay Flow Chart. 1) Assay ready CHO-NaV1.5-DUO™ cells are thawed and diluted to the desired concentration. 2) Seed cells on multi-well plate. 3) Incubate plate at 37°C, 5% CO_2 for overnight. 4) Completely remove culture media and add dye loading solution. 5) Incubate plate at 37°C, 5% CO_2 for 1 hour. 6) Prepare the compound plate, and transfer both the cell plate and the compound plate to a kinetic reader for data acquisition.

Materials and reagents

Category	Name	Discription	Cat. #	Supplier
Assay reagnets	Brilliant Sodium Assay Flex Kit	Assay kit includes Na^+ indicator ING-2 and all other reagnets needed (e.g., assay buffer, DySolv, Probenecid, and TRS)	9000-10	ION Biosciences
Cell culture	CHO-NaV1.5-DUO™	Assay ready CHO-K1 cells that overexpress $Na_V1.5$	RE305	acCELLerate/B'SYS GmbH
	Advanced MEM	Base media (Complete media contain 5% FBS and 1X GlutaMAX)	12491013	ThermoFisher
	Fetal Bovine Serum (FBS)	Fetal Bovine Serum	35-016-CV	Corning
	GlutaMAX™ Supplement	200 mM L-alanyl-L-glutamine dipeptide in 0.85% NaCl	35050061	ThermoFisher
Tool compounds	Veratridine	Voltage-gated Na^+ channel activator		
	Tetracaine	Na^+ channbel blocker		

Results

Veratridine (VTD) is a steroidal alkaloid neurotoxin isolated from the seeds of the Central America and Mexican plant sabadilla lily. Veratridine causes sodium channels to stay open during a sustained membrane depolarization by abolishing inactivation [4]. Because of its ability to open Na_V channels, veratridine is used in cell-based drug screening assays in which controlling the membrane voltage is

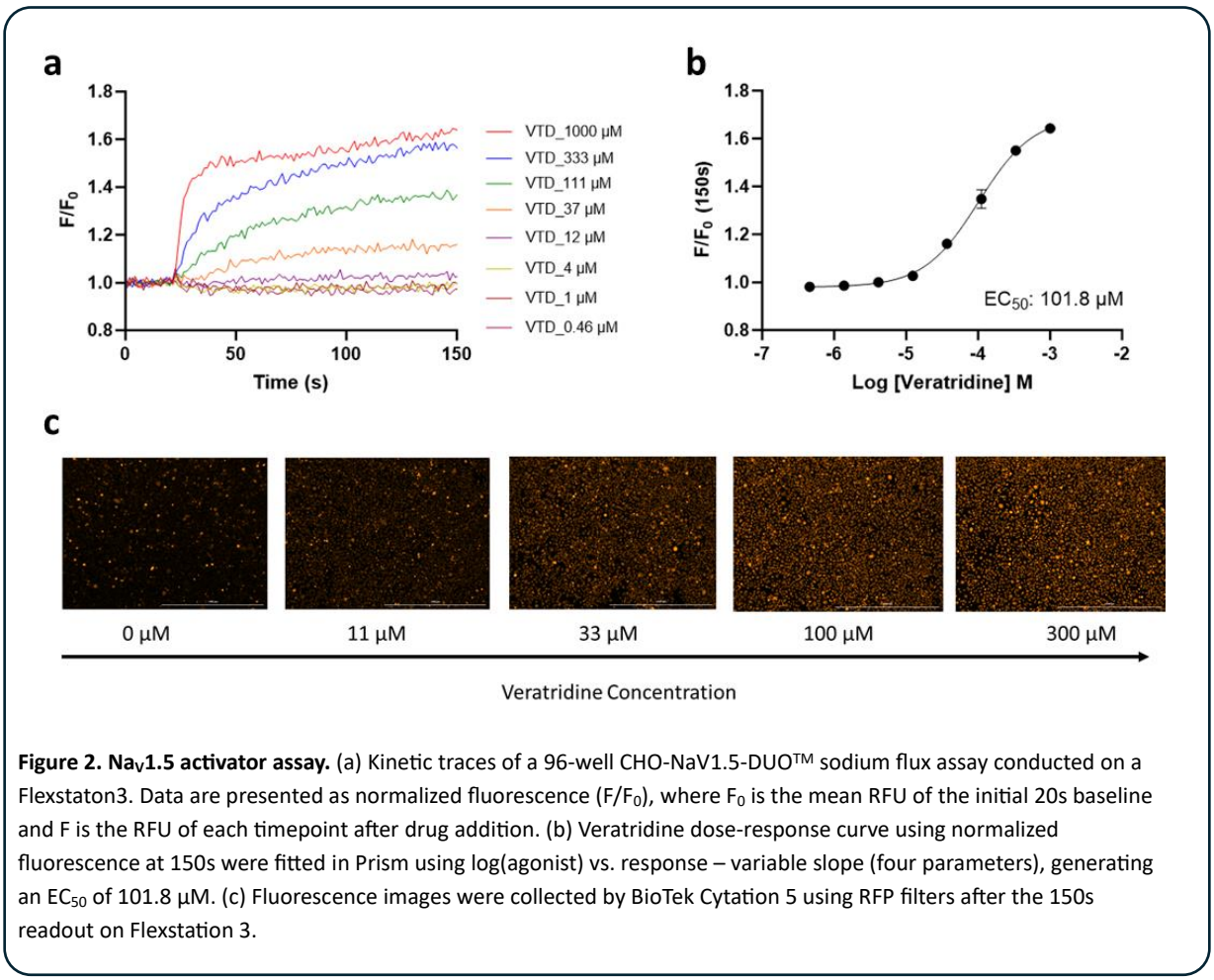

Figure 2. $Na_V1.5$ activator assay. (a) Kinetic traces of a 96-well CHO-NaV1.5-DUO™ sodium flux assay conducted on a Flexstaton3. Data are presented as normalized fluorescence (F/F_0), where F_0 is the mean RFU of the initial 20s baseline and F is the RFU of each timepoint after drug addition. (b) Veratridine dose-response curve using normalized fluorescence at 150s were fitted in Prism using log(agonist) vs. response – variable slope (four parameters), generating an EC_{50} of 101.8 µM. (c) Fluorescence images were collected by BioTek Cytation 5 using RFP filters after the 150s readout on Flexstation 3.

impractical [5]. Fluorescent Na^+ indicators that measure the intracellular ion concentrations haven been successfully used to assess veratridine pharmacological functions on $Na_V1.1$-$Na_V1.7$ channels [6]. In our assay, a dose-dependent increase of intracellular fluorescence is detected after veratridine treatment induces an influx of Na^+ (Fig 2a). Dose-response curve yield an EC_{50} of 101.8 µM (Fig 2b), which is comparable to previously reported values for veratridine [6,7]. Fluorescence images acquired after the kinetic read also show a sustained elevation in fluorescence intensity at higher veratridine concentrations (Fig 2c). Collectively, these data demonstrate that a sodium flux assay using CHO-NaV1.5-DUO™ instaCELLs is an accessible and rapid approach to detect $Na_V1.5$ channel activators.

Tetracaine is an amino-ester class local anesthetic used to numb the eyes, nose, or throat. Tetracaine inhibits Na_V function by blocking the channel at the resting state [8]. To measure tetracaine inhibition, CHO-NaV1.5-DUO™ instaCELLs were first incubated with different concentrations of tetracaine for 15 min at 37°C. Then 300 µM veratridine was added to wells during kinetic data acquisition. A tetracaine concentration-dependent reduction in fluorescence (F/F_0) was observed in the normalized kinetic data when compared to a veratridine only control (Fig 3a). A tetracaine IC_{50} of 84.4 µM was calculated (Fig 3b), which is comparable to previously reported values [6]. Fluorescence images acquired after the kinetic read showed cellular fluorescence was lower in the presence of higher concentrations of tetracaine (>333 µM) (Fig 3c). These data demonstrate that CHO-NaV1.5-DUO™ cell-based sodium flux assay can also be used to identify inhibitors of $Na_V1.5$ channels.

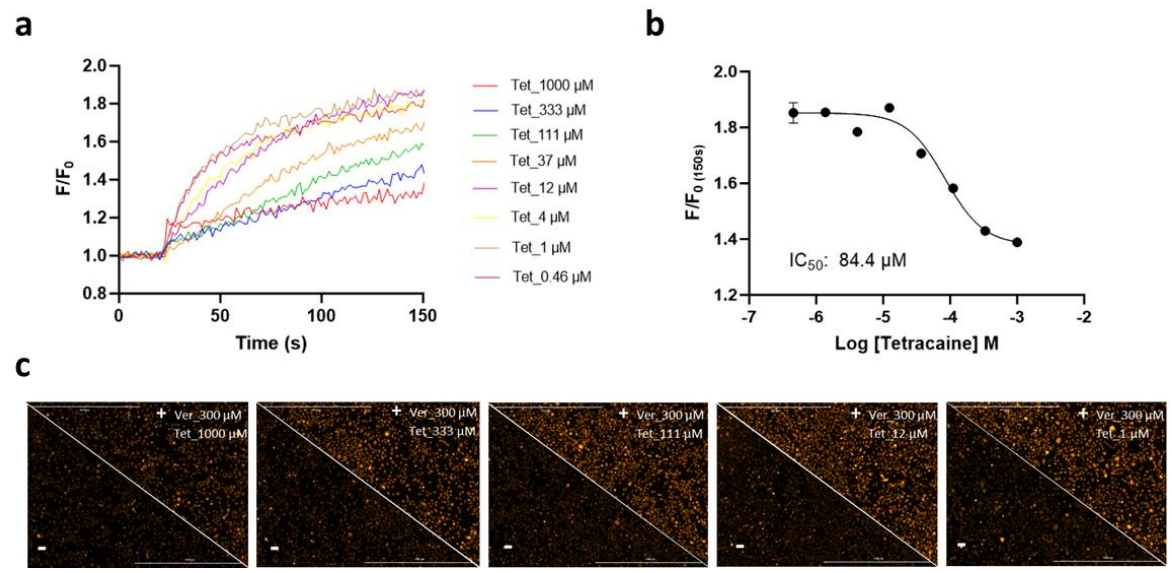

Figure 3. $Na_V1.5$ inhibitor assay. (a) Kinetic traces of a 96-well CHO-$Na_V1.5$-DUO sodium flux assay conducted on Flexstaton3. The Na_V inhibitor, tetracaine, was added 15 min prior to data acquisition. A fluorescence baseline was acquired for 20s prior to the addition of 300µM veratridine. Data are presented as normalized fluorescence (F/F_0), where F_0 is the mean RFU of the initial 20s baseline and F is the RFU of each timepoint after drug addition. (b) Tetracaine dose-response curve plotted as Log tetracaine concentration vs. normalized fluorescence at 150s. Data were fitted in Prism using log(antagonist) vs. response – variable slope (four parameters), generating IC_{50} of 84.4 µM. (c) Fluorescence images were collected by BioTek Cytation 5 using RFP filters before veratridine addition (-) and after the 150s readout on Flexstation 3 (+).

The 96-well assay was miniaturized to a 384-well format using Wavefront Biosciences Panoptic kinetic plate reader. The assay was tested for screening both $Na_V1.5$ channel activators and $Na_V1.5$ inhibitors. For activator screening, a quarter of the 384-well plate was treated with 300 µM veratridine as the positive control group and another quarter of the 384-well plate was a buffer only negative control group. Normalized kinetic data shows a large dynamic window of 1.9-fold at 300s after veratridine addition (Fig 3a). The calculated Z' factor of 0.83 indicates a high-quality screening assay to identify $Na_V1.5$ channel activators (Fig 3b). For inhibitor screening, a quarter of the 384-well plate was pre-treated with 1 mM tetracaine as the negative control group and another quarter of the 384-well plate was pre-treated with buffer only as the positive control group. High concentration (300 µM) veratridine was added to all wells after the 20s baseline data collection during the kinetic readout. Normalized kinetic data showed a significant dynamic window at 320s timepoint (Fig 3c). The calculated Z' factor of 0.70 indicates a high-quality screening assay to identify $Na_V1.5$ channel inhibitors (Fig 3d).

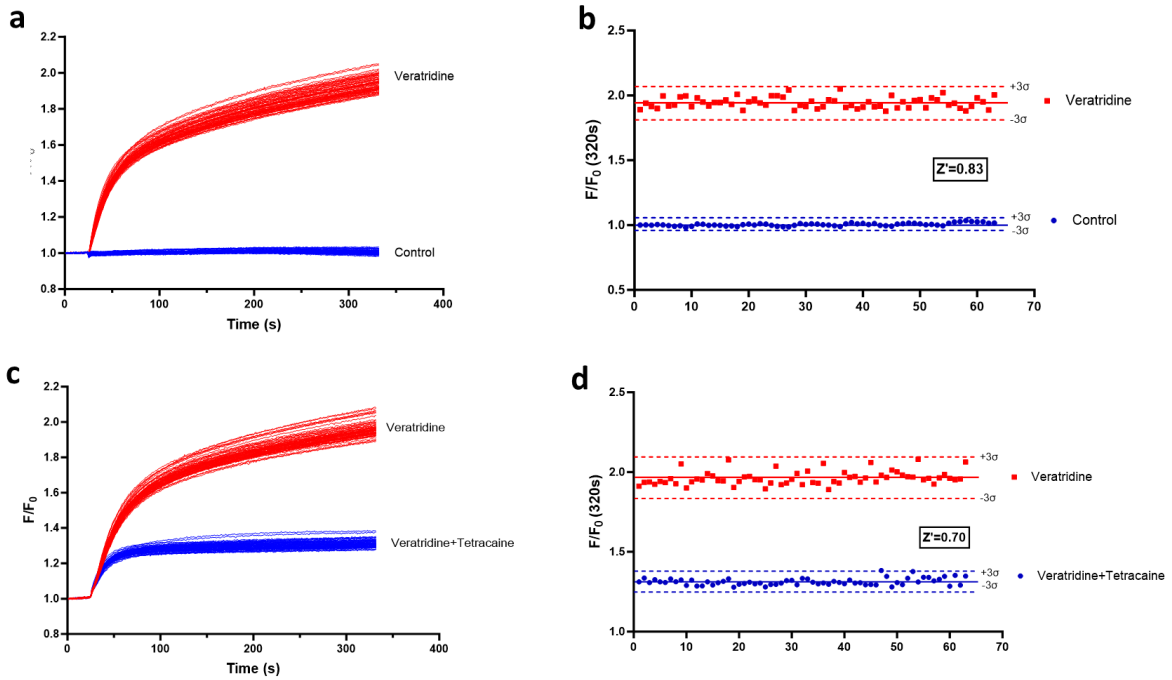

Figure 4. 384-well sodium flux assays for $Na_V1.5$. (a) Normalized kinetic data of positive control group (veratridine 300 µM) and negative control group (veratridine 0 µM). Data are presented as normalized fluorescence (F/F_0), where F_0 is the mean RFU of the initial 20s baseline and F is the RFU of each timepoint after drug addiction. (b) Dot plot of normalized fluorescence at 320s of both positive (veratridine 300 µM) and negative (veratridine 0 µM) groups. The Z' factor is calculated using equation: $Z' = 1 - \frac{3\sigma_{C+} + 3\sigma_{C-}}{|\mu_{C+} - \mu_{C-}|}$ [9]. A Z' factor of 0.83 (>0.5) indicates a high-quality screening assay. (c) Normalized kinetic data of positive control group (veratridine 300 µM) and negative control group (veratridine 300 µM + tetracaine 1 mM). (d) Dot plot of normalized fluorescence at 320s of both positive (veratridine 300 µM) and negative (veratridine 300 µM + tetracaine 1 mM) groups. A Z' factor of 0.70 (>0.5) indicates a high-quality screening assay.

Conclusion

High-throughput sodium flux assays for $Na_V1.5$ channels using assay ready instaCELLs is a high-quality assay that can be used to identify $Na_V1.5$ modulators and assess potency of $Na_V1.5$ activators and inhibitors. Veratridine EC_{50} and tetracaine IC_{50} are comparable to reported values, and both activator and inhibitor screens generated Z' factors larger than 0.5. In this assay, veratridine is used instead of membrane depolarization to open sodium channels as part of a high-throughput intracellular Na^+ concentration screening strategy to identify modulating compounds. The combination of ION Biosciences' Brilliant Sodium Assay and acCELLerate's assay ready cells provides a robust method for testing $Na_V1.5$ and can be extended to identify other Na_V channel modulators as well.

Acknowledgements

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We thank acCELLerate for providing CHO-NaV1.5-DUO™ instaCELLs for this work. CHO-NaV1.5-DUO™ instaCELLs are qualified frozen aliquots of cells from a recombinant cell line which stably express $Na_V1.5$. The cell line had been developed and validated by B'SYS GmbH, Switzerland and is distributed by acCELLerate in the assay ready instaCELL format.

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