

Multimode Detection

Authors:**Ebru Betül Yurtseven****Maria Kuzikov**

Fraunhofer IME

ScreeningPort, Germany

Measuring KCNT1 Ion Channel Activities Using the FluxOR Potassium Ion Channel Assay Kit on the VICTOR Nivo Multimode Reader

Introduction

Potassium channels are membrane proteins expressed in most cell types including excitable cells such as neurons. They comprise the largest class of ion channels. With more than 75 members, the family of human potassium channels

is highly diverse. The KCNT1 gene, also known as Slack, encodes potassium channels expressed in neurons of the central nervous system. Mutations in this gene can lead to neurological disorders such as ADFLE (Autosomal Dominant Nocturnal Frontal Lobe Epilepsy) or MPSI (Migrating Partial Seizures of Infancy). Furthermore, like other ion channels, potassium ion channels underlie numerous physiological processes in different stages of development, and therefore present several challenges in drug discovery and validation of new channel targets.¹ The thallium-based FluxOR™ potassium ion channel assay is specially developed for screening of potassium ion channels and can be used to measure the ion conduction activities of potassium ion channels. In this application note we describe the adaptation of the FluxOR™ potassium ion channel assay for detection of compound-related effects on KCNT1-expressing HEK-293 cells on the VICTOR Nivo™ multimode reader.

Principle of the Assay

The FluxOR™ potassium ion channel assay takes advantage of the permeability of potassium channels for thallium and uses the thallium influx to measure channel activity. The fluorogenic FluxOR™ dye, which is transported into cells by the PowerLoad™ reagent, serves as a thallium indicator. The FluxOR™ dye is a non-fluorescent AM-ester, which is converted by esterases to a negatively charged, thallium-sensitive dye inside the cytoplasm. Its extrusion is also inhibited by Probenecid, which blocks organic anion pumps. Fluorescence of extracellular FluxOR™ dye can be blocked with use of a background suppressor. Therefore, no wash steps are needed, and it is possible to incubate cells with the loading buffer and cell culture medium at the same time. Compounds can be added to cells at the end of this incubation period. The effect on channel opening propensity is observed after addition of the stimulus buffer containing thallium. Thallium permeates through open potassium ion channels and binds to the sensitive FluxOR™ dye. The deesterified dye form is capable of fluorescence with a peak emission at 530 nm when excited at 480 nm. Although thallium can be transported by open potassium channels, it is sometimes required to incubate cells with an agonist before adding the buffer containing thallium to increase the signal window. Kinetic measurement of fluorescence intensity (FI) allows the detection of time-dependent transport of thallium across the membrane. The signal increase is proportional to the number of open potassium channels.

Material and Method

The protocol of commercially available FluxOR™ Potassium Ion Channel Assay (# F10016, Thermo Fisher Scientific) was modified according to required assay conditions. By using the background suppressor Red Back Drop™ (# B10512, Thermo Fisher Scientific) in addition to the assay kit, the wash steps could be omitted and were not performed. The assay buffer was prepared as a stock solution and stored at 4 °C. Assay Buffer, loading buffer and stimulus buffer were prepared as indicated in the table 1 A-B.

Cell Line

HEK-293 cells stably transfected with KCNT1 were kindly provided by the cooperation partners Prof. Dr. Dirk Isbrandt (Deutsches Zentrum für Neurodegenerative Erkrankungen e. V. (DZNE)) and Dr. Axel Neu (Universitätsklinikum Hamburg-Eppendorf (UKE)). HEK-293/KCNT1 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (# 733-1731, VWR) supplemented with 10 % FBS (# FBS-12A, Capricorn Scientific), 2 mM glutamine (# GLN-B, Capricorn Scientific) 1 % penicillin (100 U/mL)/streptomycin (100 µg/mL) (# PS-B, Capricorn Scientific) and 150 µg/mL hygromycin (# CP12.2, Carl Roth) at 37 °C in a humidified 5 % CO₂ atmosphere. HEK-293 transfected with an empty vector were cultured under the same conditions but supplemented with 100 µg/mL Zeocin (# R25001, ThermoFisher Scientific GmbH) in the culture medium. Cells without KCNT1 served as a negative control for KCNT1 inhibition.

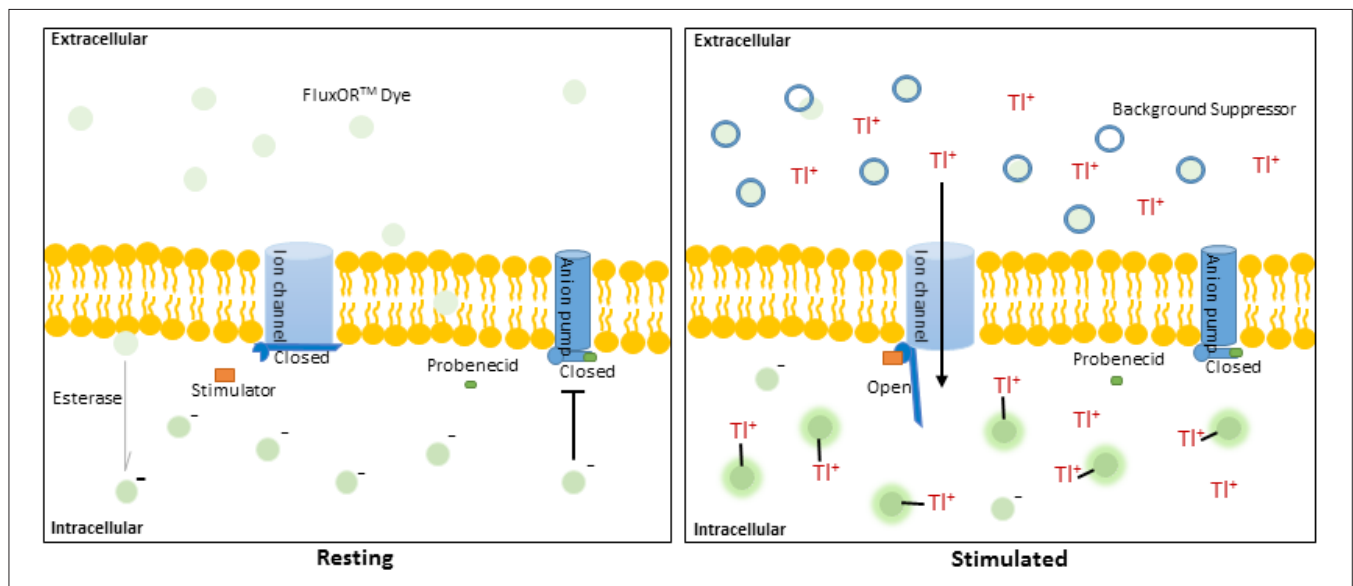


Figure 1. Principles of the modified, no wash step FluxOR™ Potassium Ion Channel Assay. (left) The non-fluorescent FluxOR™ dye diffuses over the cell membrane into the cytosol and is converted by esterases into a thallium sensitive dye. (right) Thallium permits through open potassium ion channels and binds to the sensitive FluxOR™ dye. The fluorogenic thallium-sensitive dye can be excited at 480 nm and detected with an emission peak of 530 nm.

Table 1-A. Assay Buffer.

Component	Dilution	Stock Solution: 300 mL	Prepare Assay Buffer: IMPORTANT Adjust pH to 7.4 with NaOH Store at 4 °C
FluxOR™ Assay Buffer (10X)	1:10	30 mL	
HEPES (1 M)	1:50	6 mL	
Deionized Water	Rest	264 mL	

Table 1-B. Loading Buffer.

Component	Dilution	Total Volume: 3 mL (One 384-well Microplate)		<p><i>Prepare Loading Buffer:</i></p> <p>Step One: Prepare Vial 1</p> <p>Step Two: Prepare Vial 2</p> <p>Step Three: Transfer Solution in Vial 2 to Vial 1</p>
PowerLoad™	1:25	120 µL	Vial 1	
FluxOR™	1:750	4 µL		
Assay Buffer	Rest	2051 µL	Vial 2	
Red BackDrop™	1:4	750 µL		
Probenecid	1:40	75 µL		

Table 1-C. Stimulus Buffer.

Component	Dilution	Total Volume: 3 mL (One 384-well Microplate)	Prepare Stimulus Buffer: Mix in Indicated Order
Thallium Sulfate (50 mM)	1:10	300 µL	
FluxOR™ Chloride-free Buffer (5X)	1:5	600 µL	
Deionized Water	Rest	2100 µL	

Test Compounds

KCNT1 channel activator loxapine (# 20760, Cayman Chemicals) and unspecific KCNT1 inhibitor quinidine (# 3625-5G, Sigma Aldrich) were solved in 99.9 % DMSO and transferred in the microtiter plates using the Echo® 550 Liquid Handling System (Labcyte, Inc.) Throughout this study the final DMSO concentrations did not exceed 0.5 % v/v.

Assay Procedure

One day before the assay, cells were trypsinized and seeded in DMEM, supplemented with 1 % FBS, 2 mM glutamine and 1 % penicillin (100 U/mL)/streptomycin (100 µg/mL). Then cells were seeded at 15,000 cells per well in a final volume of 20 µL per well in a 384-well black clear bottom microtiter plate (# 3764, Corning) and incubated at 37 °C in a humidified 5 % CO₂ atmosphere. On the following day, cells were loaded with FluxOR™ dye (5 µL loading buffer per well) for 60 min in the dark at room temperature. Test compounds were transferred to each well using the Echo® 550 Liquid Handling System and incubated in the dark at room temperature for additional 30 min. Prior to injection, a baseline was recorded and thallium was added automatically using the dispenser system of the VICTOR Nivo multimode reader or with a multichannel pipette. FluxOR™ dye was excited at 480/30 nm and detected at 530/30 nm with the VICTOR Nivo multimode reader. The pre-installed protocols for fluorescence and dispenser kinetics were combined and adjusted to assay requirements. When using the built-in instrument dispenser, baseline fluorescence was measured for 15 s prior to thallium addition, and for a period of 125 s after injection. The measurement was performed every second. For manual addition of thallium without the dispenser, fluorescence was measured every one min.

Data Analysis

The values after thallium addition were normalized to baseline values ($\Delta F/F$) using Microsoft® Excel® by

$$\frac{\Delta F}{F} = \frac{RFU(t)}{RFU(t_0)}$$

Data was analyzed with GraphPad® Prism (GraphPad Software). For the EC₅₀, IC₅₀ and Z' factor calculations, data points at 60 s after thallium injection were used in order to be in the sensitive range of the assay. Data are presented as means ± SD. An effect was considered significant if p ≤ 0.05.

Results

Cell number titration

In order to examine the effect of cell density, KCNT1-expressing and empty vector containing HEK-293 cells were seeded at different cell concentrations in a 384-well plate (Fig. 2) and potassium channel activity was measured on the following day as described in the assay procedure. Fig. 2 shows the time-dependent change of normalized fluorescence intensity in KCNT1-expressing and non-expressing cells stimulated with 25 µM loxapine (red curve) and in unstimulated cells using DMSO as a control (grey curve). As expected, non-expressing cells did not show an increase in signal after loxapine addition compared to cells incubated with DMSO (Fig. 2B). The time-dependent increase of signal in non-expressing cells is most likely due to an unspecific transport of thallium ions across the cell membrane. On the other hand, the signals of loxapine-stimulated KCNT1-expressing cells were also increasing in a time-dependent manner (Fig. 2A). The optimal assay window was observed when using 15,000 and 30,000 cells per well, the signal window decreased with 7,500 cells per well. On this basis, 15,000 cells per well were selected as parameter for further experiments.

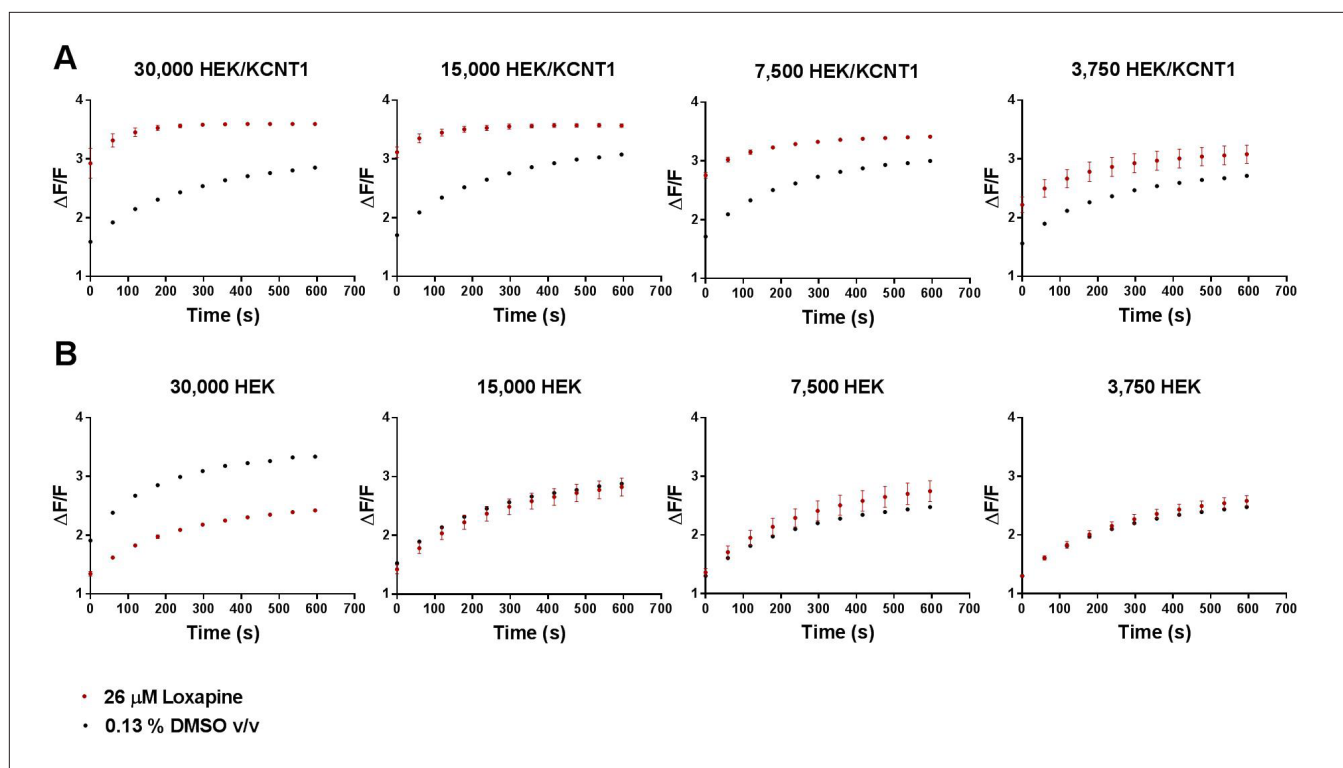


Figure 2. Cell number titration with transfected HEK-293 cells, expressing KCNT1 and mock-transfected HEK-293 cells using the FluxOR™ potassium ion channel assay. (A) Expressing (HEK/KCNT1) or (B) non-expressing (HEK) cells were seeded in a 1:2 serial dilution, starting at 30,000 cell per well and a final volume of 20 μL , in a 384-well plate and incubated overnight at 37 °C in a humidified 5 % CO_2 atmosphere. After one hour incubation with Loading Buffer, cells were incubated with 26 μM loxapine and 0.13 % DMSO v/v for 30 min. Thallium was added and KCNT1 activity was measured for 10 min as described in the assay protocol.

Optimizing Agonist Concentration

To validate agonist concentration, dose-response upon loxapine stimulation was recorded using HEK/KCNT1 expressing and non-expressing (HEK) cells and DMSO (0.5 % v/v) as a negative control. Cells were incubated with loxapine between 100 μM and 1.6 μM . For a better overview, only three loxapine concentrations were used to assemble time-dependent graphs (Fig. 3A-B). As shown in Fig. 3, KCNT1-expressing cells show an increasing signal window with growing loxapine concentrations, while non-expressing cells remained unaffected. In KCNT1 expressing HEK-293 cells an EC_{50} of 14.6 μM was calculated for loxapine. A similar value (4.4 μM) was reported by Biton et al.² for CHO cells expressing this channel. In further experiments, KCNT1 channels were stimulated with loxapine at EC_{80} of 25 μM .

Optimizing Antagonist Concentration

The antiarrhythmic drug quinidine was selected as a reference compound to test inhibition of thallium transport in KCNT1-expressing HEK-293 cells. Quinidine is a small molecule that blocks sodium and potassium channels non-specifically and was first reported by Yang et al. in 2006 as a potent KCNT1 blocker with a reported IC_{50} of 100 μM .³ In order to determine an IC_{50} for quinidine, KCNT1 expressing cells were incubated with quinidine at concentrations between 0 μM and 500 μM (Fig. 4A). In addition, cells were incubated with quinidine and loxapine (Fig. 4B) to analyze the effects of quinidine on stimulated KCNT1 channels. In both cases quinidine inhibited the thallium influx by blocking KCNT1 channels, leading to a concentration-dependent decrease of the signal with an IC_{50} of 434.5 μM in non-stimulated cells (Fig. 4C) and an IC_{50} of 113.2 μM in loxapine-stimulated cells (Fig. 4D).

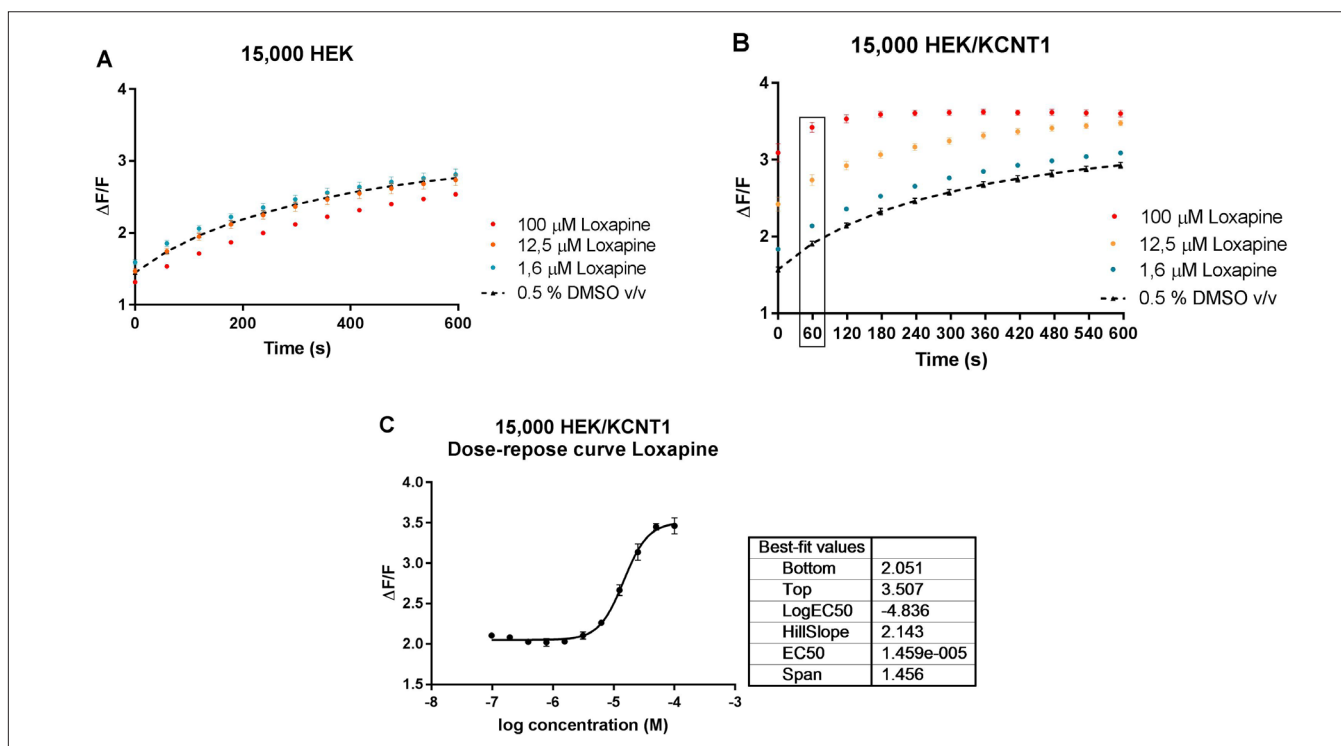


Figure 3. Loxapine titration in HEK293 cells using the FluxOR™ potassium ion channel assay. (A) KCNT1 expressing (HEK/KCNT1) and (B) non-expressing cells (HEK) were seeded at 15,000 cells/well in a 384-well plate and incubated overnight at 37 °C in 5 % CO₂. After 1 h incubation with Loading Buffer, cells were incubated with different loxapine concentrations or 0.5 % DMSO v/v for 30 min. (C) Dose-response curve for loxapine. EC₅₀ values were calculated using data obtained at 60 s after thallium injection.

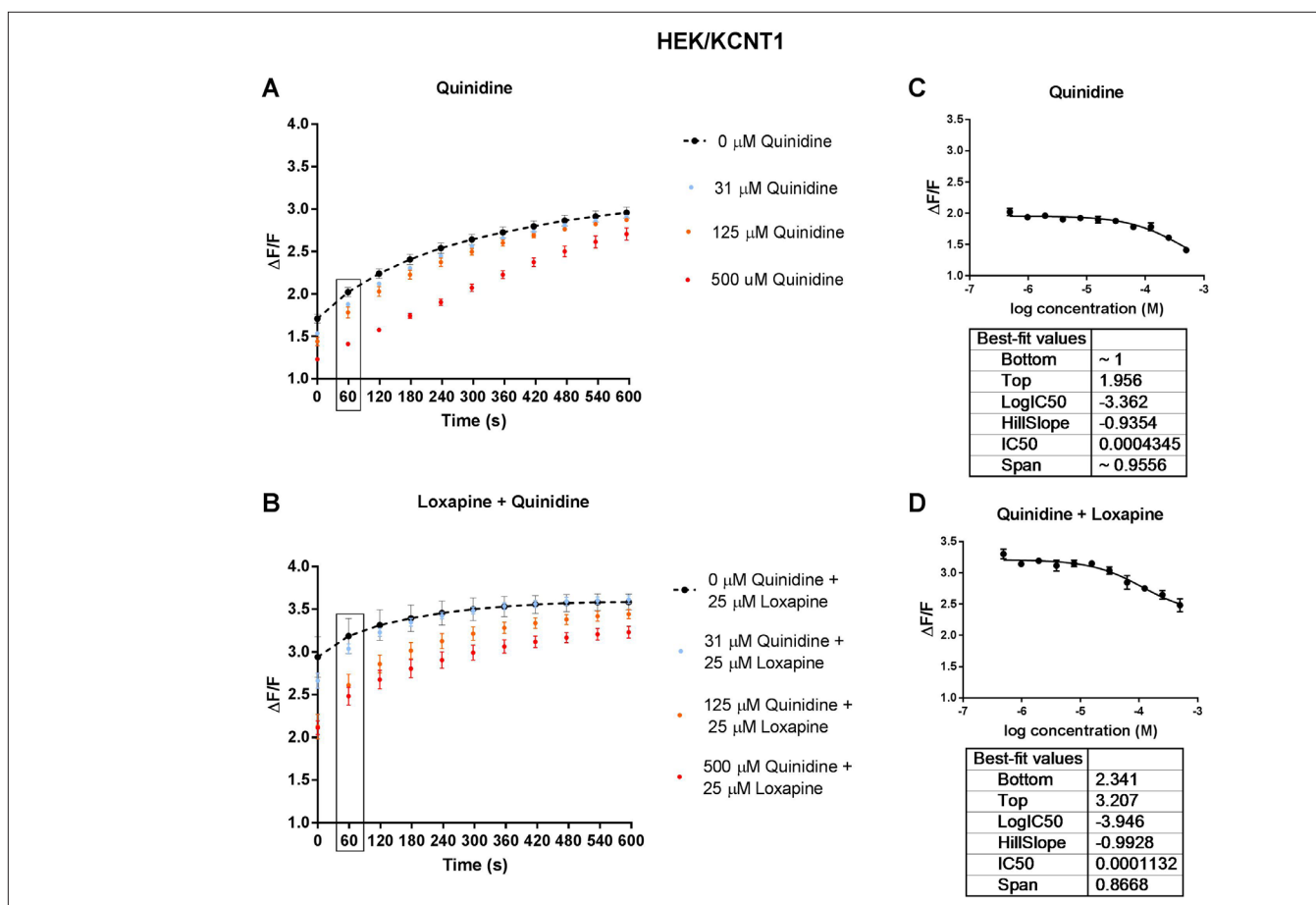


Figure 4. Cytotoxicity tested on transfected HEK-293 cells (expressing KCNT1) using the FluxOR™ potassium ion channel assay. Expressing cells (HEK/KCNT1) were seeded at 15,000 cells/well in a 384-well plate and incubated overnight at 37 °C in 5 % CO₂. After one hour incubation with Loading Buffer, cells were incubated with quinidine and loxapine at EC₅₀ ~ 25 μ M (A) or with quinidine only (B). Prior to loxapine incubation (25 μ M), cells were pre-incubated with different quinidine concentrations for 15 min. (C-D) Dose-response curve for quinidine. IC₅₀ were calculated using data obtained at 60 s after thallium injection.

Z' determination

To evaluate assay quality and robustness Z' analysis was performed. Quinidine at 400 µM served as a positive control and DMSO at 0.5 % v/v as a negative control. Both conditions were stimulated with loxapine at EC₈₀ ~ 25 µM at a total of 16 replicates (Fig. 5). The Z' factor was calculated by using data obtained at 60 s after thallium injection and the equation⁴:

$$Z' = 1 - \frac{3 \times (\text{STD}_{\text{pos control}} + \text{STD}_{\text{neg control}})}{|\text{AV}_{\text{pos control}} - \text{AV}_{\text{neg control}}|}$$

Assays with Z' > 0.5 represent good performance characteristics. The calculated Z' factor of the FluxOR™ potassium ion channel assay using KCNT1 expressing HEK-293 cells is 0.61 ± 0.5, therefore the assay is suitable for high-throughput screening approaches.

Conclusion

This application note demonstrates a successful approach to adapt the FluxOR™ potassium channel assay on the Victor Nivo multi-mode reader to monitor compound-related effects on potassium channel functionality. The low standard deviation observed in the experiments and the calculated Z' value are indicative of a good suitability of the FluxOR™ assay for studies interrogating multiple compounds. The user-friendly protocols of the VICTOR Nivo provide a good basis for an easy adaptation of those to the needs of the assay. Software configurations can easily be stored for further experiments.

References

1. Beacham, D., Blackmer, T., O'Grady, M. & Hanson, G., 2010. Cell-based potassium ion channel screening using the FluxOR assay, s.l.: J Biomol Screen.
2. Biton, B. et al., 2012. The Antipsychotic Drug Loxapine Is an Opener of the sodium-Activated Potassium Channel Slack (Slc2.2), s.l.: The Journal of Pharmacology and Experimental Therapeutics.
3. Yang, B. et al., 2006. Pharmacological activation and inhibition of Slack (Slc2.2) channels, s.l.: s.n.
4. Zhang, J.-H., Chung, T. D. & Oldenburg, K. R., 1999. A simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. Journal of Biomolecular Screening.

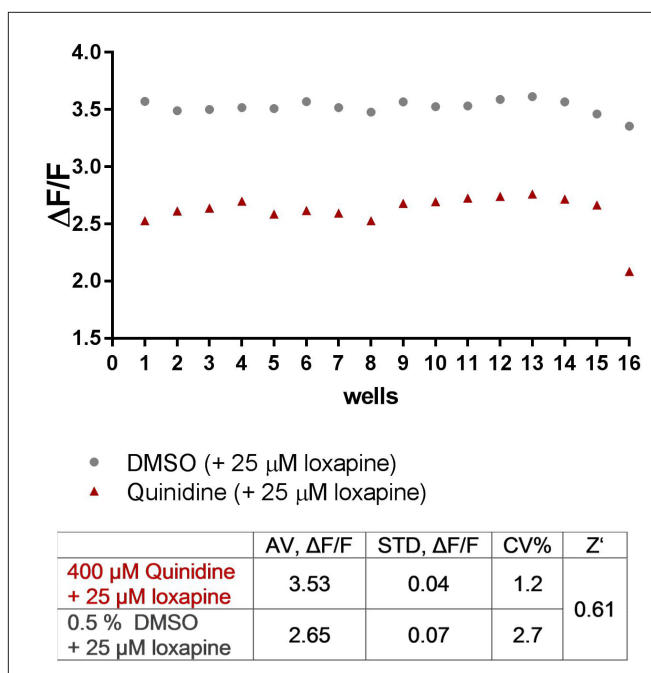


Figure 5. Z' analysis on the FluxOR™ potassium ion channel assay using HEK293 cells (expressing KCNT1). Expressing cells (HEK/KCNT1) were seeded at 15,000 cells/well in a 384-well plate and incubated overnight at 37 °C in 5 % CO₂. After one hour incubation with Loading Buffer, cells were incubated with quinidine and loxapine (positive control) and DMSO and loxapine together (negative control). Before loxapine incubation (25 µM), cells were pre-incubated with quinidine for 15 min.