Development of a cell-based potassium-chloride transporter assay using a new FLIPR Potassium Assay Kit

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INTRODUCTION

Potassium-chloride transporter member 5 (KCC2) is one of nine cation-chloride cotransporters (CCCs) encoded by the SLC12 family of genes, and is the only CCC transporter preferentially expressed in neurons. KCC2 plays a critical role in the correct functioning of the central nervous system where it is pivotal in maintaining neuronal intracellular Cl^- concentration ($[Cl^-]_i$). KCC2 is involved in the control of numerous neuronal processes and its impaired activity has been identified in epilepsy, neuropathic pain and spasticity following spinal cord injury[1]. This suggests that positive modulators of the activity or expression of KCC2 may provide effective therapy for neurological conditions arising from defects in KCC2 functionality.

The FLIPR® Potassium Assay Kit contains a novel, highly-sensitive thallium (Tl^+) indicator dye that produces bright fluorescence upon binding to Tl^+ introduced through potassium channels. The intensity of the signal is proportional to the number of open potassium channels on the cells; therefore it acts as a surrogate indicator of potassium ion channel activity. The kit also employs Molecular Devices proprietary masking dye to reduce background fluorescence resulting in an improved signal/background ratio. As Tl^+ is also transported by cation–chloride cotransporters, the FLIPR Potassium Assay Kit can be used to easily assess the effect of compounds on KCC2 activity by measuring the initial rate of KCC2-mediated Tl^+ transport/influx.

ASSAY PRINCIPLE

The FLIPR Potassium Assay Kit (Figure 1) contains a thallium-sensitive indicator dye. During the initial dye-loading step, the Tl^+ indicator dye enters the cells as an acetoxymethyl (AM) ester by passive diffusion across the cell membrane. Cytoplasmic esterases cleave the AM ester and release its active fluorogenic form. In addition, a proprietary extracellular masking dye is included to reduce background fluorescence. To activate the potassium-chloride transporter, the cells are stimulated with a mixture of K^+ and Tl^+ in the presence or absence of test compounds (KCC2 inhibitors). Under the assay conditions used, the increase in fluorescent signal represents the influx of Tl^+ into the cell specifically through the cotransporter, and therefore represents a functional measurement of the cotransporter activity. Modulation of the cotransporter activity is achieved by inclusion of KCC2 inhibitors.

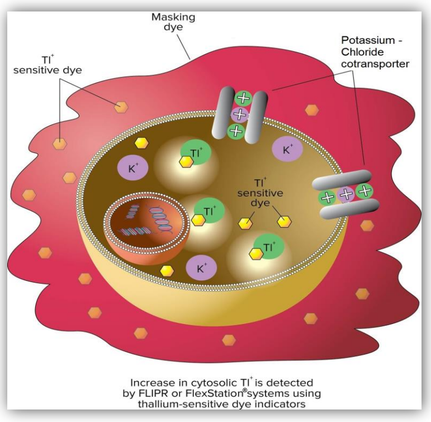

Figure 1. The FLIPR Potassium Assay Kit principle

The FLIPR Potassium Assay Explorer Kit (Molecular Devices cat. # R8222) contains a Tl^+-sensitive dye, masking dye for homogenous operation, 200 mM K_2SO_4, 50 mM Tl_2SO_4, 5X chloride-free buffer, and HBSS with 20 mM HEPES buffer. The kit is sufficient for ten 96-, 384-, or 1536-well plates.

MATERIALS & METHODS

Cell Culture: HEK293T (ECACC catalogue number 12022001) cells were grown to 80 – 90% confluence in T_{75} flasks containing growth medium (DMEM supplemented with 10% FBS and 2 mM L-Glutamine). Cultures were maintained at 37°C in the presence of 5% CO_2. Cells were passaged every 3 – 4 days at a ratio of 1:6. Two days before the assay, cells were transiently transfected with human KCC2 (hKCC2) using Lipofectamine® 2000 according to the manufacturer's instructions. The transfection mixture was mixed with growth medium, plated directly into a 96-well, black walled, clear bottom plate at 10,000 cells/well and incubated at 37°C and 5% CO_2 for 48 hours. Growth medium was removed from the plates which were then incubated with dye for one hour at 37°C in the dark. The loading buffer contained 10 µM bumetanide to reduce the endogenous NKCC Tl^+ influx/transport signal, and 0.1 mM ouabain, a Na^+/K^+-ATPase blocker, to inhibit ATP-dependent sodium-potassium exchange across the cell membrane. Test compounds were added and incubated for the last 45 minutes of the dye loading time. Optimised stimulus buffer (9 mM K^+ / 0.9 mM Tl^+) was added to the well during detection on the FLIPR® Tetra System using the 470-495 nm excitation LEDs and 515-575 nm emission filter (Figure 2).

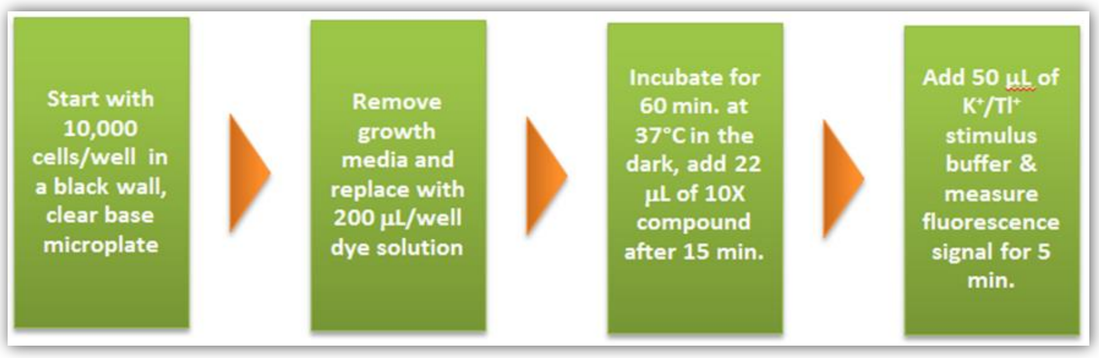

Figure 2. FLIPR Potassium Assay Kit workflow on the FLIPR Tetra High-Throughput Cellular Screening System

Data files were subsequently imported into SoftMax® Pro Data Acquisition and Analysis Software for analysis using the Import Feature (Figure 3), available for SoftMax Pro 6.4.1 or higher. The initial rate (V_{max} in units per second) was calculated from the first 10 seconds of data post stimulus addition

Figure 3. SoftMax Pro 6.4.2 import workflow

RESULTS

After loading hKCC2-expressing or mock-transfected HEK293 cells with the novel FLIPR Potassium Assay Kit, we measured the increase in fluorescence triggered by the addition of external Tl^+ during detection on the FLIPR Tetra System. The hKCC2-driven FLIPR signal exhibits a rapid increase over the first 10 seconds after K^+/Tl^+ addition followed by a slower increase and eventually a plateau phase; the increase was significantly higher in hKCC2-expressing cells, compared to mock-transfected cells as shown in Figure 4. The average Z-factor was calculated as 0.60 ± 0.05 indicating a robust assay[2]

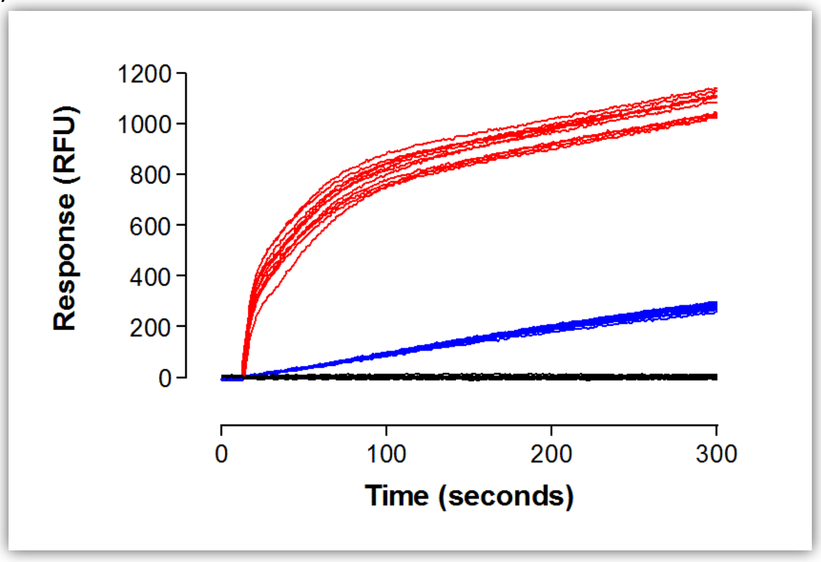

Figure 4. Representative fluorescence signal traces (8 wells per treatment) obtained on the FLIPR Tetra System in HEK293 cells loaded with the FLIPR Potassium Assay Kit. Data show the effect of buffer only addition (–) or K^+/Tl^+ addition (–) in hKCC2 transfected cells, and K^+/Tl^+ addition (–) in mock transfected cells.

A series of experiments were then carried out with hKCC2-expressing cells to assess whether the Tl^+ influx assay could be used to successfully detect modulators of hKCC2 activity. In each 96-well plate, cells were treated with either vehicle control or 30 µM R-(+)-DIOA (an alkanoic acid that has been shown to be a potent and selective inhibitor of K^+/Cl^- cotransporters). As seen in Figure 5, the resultant signals from the K^+/Tl^+ -evoked increase in fluorescence are very consistent from well to well, and the separation between the control and R-(+)-DIOA signals is significant.

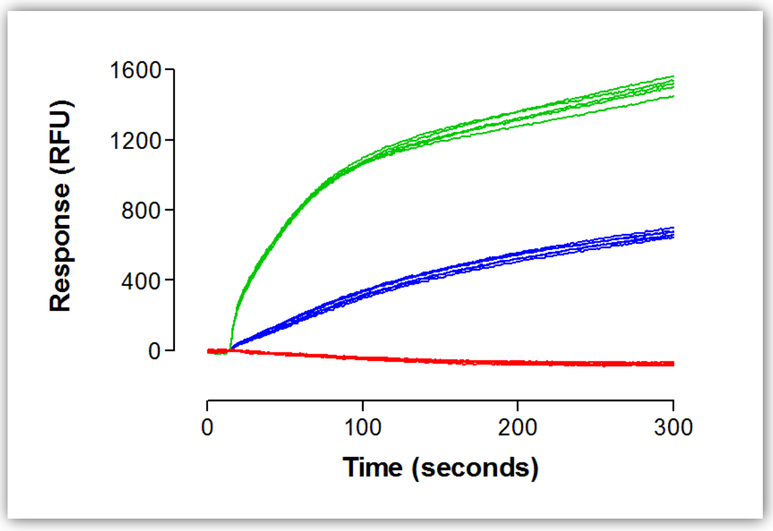

Figure 5. Representative fluorescence traces obtained on the FLIPR Tetra System in hKCC2 transfected cells loaded with the FLIPR Potassium Assay Kit. Data show the effect of buffer only addition (–) or K^+/Tl^+ addition in the absence (–) or presence (–) of 30 µM R-(+)-DIOA

In the absence of inhibitor the K^+/Tl^+ stimulus produced a V_{max} of 53.9 ± 5.1 units per second, whereas in the presence of 30 µM R-(+)-DIOA this was reduced to 17.5 ± 1.9 units per second (Figure 6) where n ≥ 30 wells over 4 independent experiments.

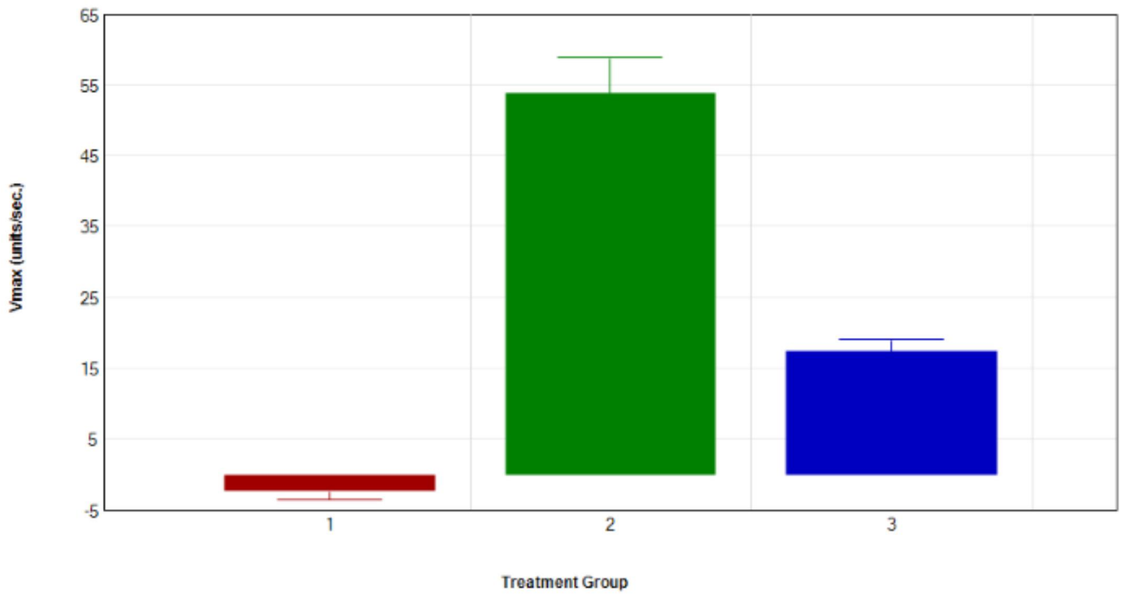

Figure 6. The effect of different challenges on hKCC2-transfected cells. Data shows the effect of three different treatment groups: 1. buffer only addition (■), and 2. Tl^+/K^+ addition in the absence (■) or 3. presence (■) of 30 µM R-(+)-DIOA. Bars represent mean V_{max} ± SEM (n ≥ 30).

CONCLUSIONS

- The FLIPR Potassium Assay Kit can be used to measure the functional activity of the hKCC2 cation-chloride cotransporter using a homogeneous, no-wash protocol.
- The FLIPR Potassium Assay Kit displayed a large assay window and excellent reproducibility, and was able to successfully detect a known modulator of hKCC2 activity.
- The simplified protocol and robust assay quality, in combination with the high-throughput capability of the FLIPR Tetra System, provides a powerful solution to screen for positive modulators of the hKCC2 cotransporter.