TECHNICAL BRIEF

ValiScreen® Stable Recombinant Ion Channel Cell Line and the EnVision Multilabel Plate Reader

Membrane Potential Assays Using the ValiScreen® Human Kv1.3 Voltage-Gated K^+ Channel Cell Line on the EnVision Multilabel Plate Reader

Introduction

Ion channels are one of the major classes of therapeutic targets due to the central role they play in numerous physiological processes. PerkinElmer's ValiScreen® cell lines provide an effective means to study compound effects on recombinant ion channels or recombinant G protein-coupled receptors in a well-characterized cell line. In this technical brief we illustrate a membrane depolarization assay performed with the EnVision® Multilabel Plate Reader, describe the optimization of important cell culture parameters, and define the EnVision optical and dispenser settings used to perform this assay.

The study was performed with the human Kv1.3 potassium channel stably expressed in CHO-DUKX cells. This cell line has been validated by both electrophysiological whole-cell voltage-clamp and fluorescent membrane potential dye assays. The Kv1.3 voltage-gated potassium channel is found in human islet cells, a specific subset of T lymphocytes, and the central nervous system. It plays an essential role in T cell proliferation and activation. The therapeutic interest around Kv1.3 include autoimmune diseases such as multiple sclerosis, rheumatoid arthritis, chronic graft-versus-host disease, cutaneous lupus, pustular psoriasis, obesity and type 2 diabetes mellitus. The Kv1.3 channel responds to membrane depolarization by opening to allow the efflux of K^+ ions as part of the process of re-polarizing the cell membrane.

In this assay, membrane depolarization is accomplished by exposing the cells to a high extracellular concentration of KCl. As the cells are loaded with a membrane potential sensitive dye, this membrane depolarization results in a change of fluorescence. This change in fluorescence occurs within seconds following addition of the KCl solution. The internal injector tip mount in the EnVision reader allows for real-time signal acquisition during the dispensing.

The EnVision is equipped with a single injector, so plates must be read one well at a time. During the early stages of assay development, or later follow up studies where high throughput is not necessarily required, the EnVision can be used to perform these assays so that high throughput screening instruments are not tied up.

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Materials and methods

The Human Kv1.3 Voltage-Gated K^+ Channel ValiScreen cell line was obtained from PerkinElmer (#AX-010-C). The assay was carried out in either black, clear bottom 384-well ViewPlate™ microplates (PerkinElmer #6007460) or the corresponding poly-D-lysine coated ViewPlate microplate (PerkinElmer #6007710). The plates were read on the PerkinElmer EnVision (#2104-0010).

Suggested suppliers for other materials used in the assay are:

Material	Supplier	Catalog Number
MEM Alpha with glutamine	Invitrogen	12571
Fetal bovine serum	HyClone	SH30071.03
Geneticin	Invitrogen	10131-027
Trypsin 0.25%	Invitrogen	25200
FLIPR MP assay kit, blue	Molecular Devices	R8034
FLIPR MP assay kit, red	Molecular Devices	R8126
Probenecid	Sigma	P8761
Margatoxin	Alomone Labs	RTM-325
Tyrode's salt solution	Sigma	T2397
KCl	Sigma	P3199

Cell culture medium: MEM alpha with glutamine, 10% FBS, and 0.4 mg/mL geneticin

Probenecid solution: 71 mg of probenecid was dissolved in 500 µL of 1N NaOH and then further diluted to 1 mL by the addition of 500 µL of Tyrode's solution.

Membrane potential dye 2x solution: The content of one bottle of dye was dissolved in 50 mL of Tyrode's buffer, aliquoted and stored at -20 ^{o}C

0.625x Membrane potential dye solution: mix 5 mL of 2x dye prepared above with 11 mL of Tyrode's buffer + 300 µL of probenecid solution prepared above.

Assay protocol: Cells were plated in 25 µL/well of culture medium without antibiotic into black, clear bottom microplates and incubated overnight at 37 ^{o}C, 5% CO_2. 25 µL/well of the 0.625x membrane potential dye solution was added and the plates were incubated an additional 1 hour at 37 ^{o}C, 5% CO_2. The plate was loaded into the EnVision and 25 µL/well of 3x KCl in Tyrode's solution was injected. The signal was acquired for 1 minute/well including a 5 second pre-injection basal reading. The data was analyzed as the maximum signal minus the basal signal. The data is plotted using GraphPad Prism® as the mean +/- SD.

EnVision dispensing and measurement settings:

Tip Mount	Real Time + Pre96 Dual Tip
Measure each (second)	0.5
Used pump	Pump 1
Dispensing speed (µL/s)	200
Dispensing volume (µL)	25
Syringe filling volume	Full
Start dispensing at measurement number	10

EnVision optical configuration:

Important note: The EnVision is capable of both top and bottom excitation and reading. For this assay it is critical to use the bottom reading mode, since top reading gives a significantly lower signal with high variability.

Excitation	Bottom
Bottom mirror	BODIPY TMR D555, Barcode 405
Excitation filter	BODIPY TMR 531, Barcode 105
Emission filter	Cy3 595, Barcode 229
Excitation light (%)	100
Detector gain	150
Number of flashes	10

Results

Comparison of the red and blue membrane potential kits

Two different membrane potential assay kits are available, which are designated “Red” and “Blue” kits. The two kits contain the same indicator dye, but have different quenchers. Depending on the particular cell line and ion channel under study, one of the kits may yield better results than the other. We therefore compared the two kits to see if one worked better with the Kv1.3 cell line on the EnVision. The cells were plated at 10,000 cells/well and the assay was performed using 130 mM KCl for depolarization. The results shown in Figure 1 indicate that the blue kit gives a slightly higher signal than the red kit. For the remainder of this work the blue kit was used.

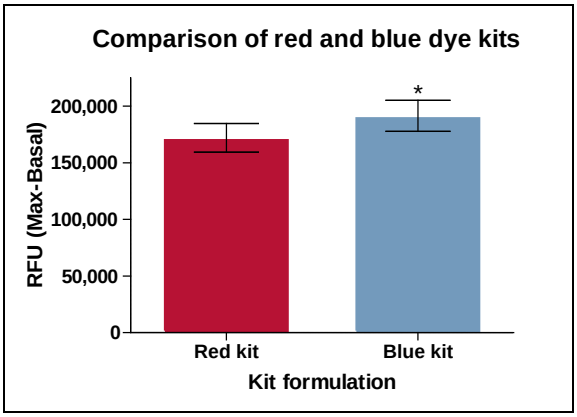

Figure 1. Comparison of the assay signal using the Red and Blue membrane potential kits. * p>0.05 (unpaired t-test)

Determination of optimal dye loading conditions

We next determined whether it was necessary to remove the culture medium prior to loading the cells with dye in order to prevent possible interference in the assay by medium or serum factors. Two different dye loading conditions were compared. In the first, 25 µL of 0.625x dye solution was added directly to the wells containing culture medium. This was compared to removing the medium first and then adding 40 µL of the dye solution directly onto the cells. Following the dye addition the plates were incubated for 1 hour at 37 ^{o}C, 5% CO_2. The data in Figure 2 shows that the assay window is better if the medium is not removed before adding the dye solution.

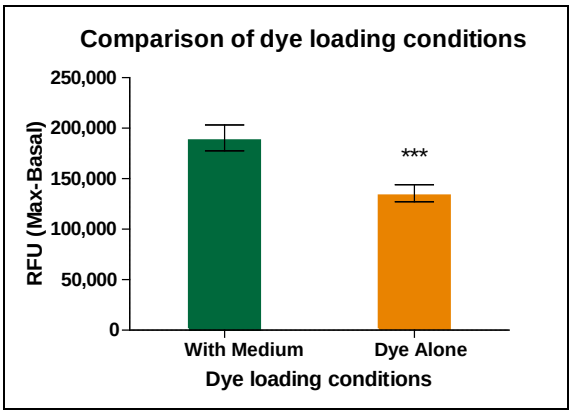

Figure 2. Comparison of effect of dye loading in the presence or absence of cell culture medium. *** p<0.0001 (unpaired t-test).

Plate selection study: poly-D-lysine coated plates compared to uncoated plates

The membrane potential assay can be performed using either adherent or non-adherent cells, although it usually works better with adherent cells. When using adherent cells it is important that the cells are not detached from the bottom of the plate when samples are injected, since that leads to variability in the signal. In cases where the cell line is loosely adherent it may be necessary it use a microplate coated with a matrix such as poly-D-lysine (PDL) to promote adherence. We tested whether this was necessary with the Kv1.3 cell line by performing the assay in both tissue culture treated ViewPlate microplates and the corresponding PDL-coated ViewPlate microplates at both 5 and 10K cells/well. The data in Figure 3 compares the assay performance with the two types of plates. The results show only a slight improvement of assay window when using PDL coating ($p<0.05$), so it is not necessary to use the more expensive PDL-coated plates.

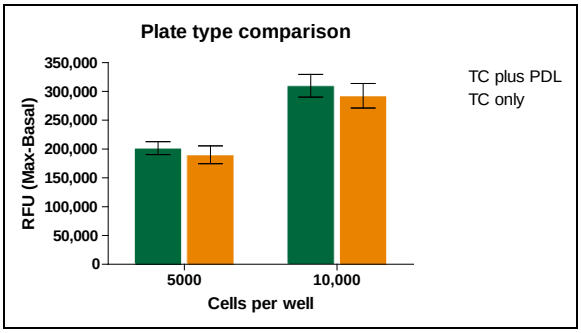

Figure 3. Assay results using cells grown in PDL-coated plates compared to uncoated plates.

Cell seeding density titration

To determine the optimal cell seeding density, plates were seeded over a range from 2,500 to 10,000 cells/well. Following an overnight incubation the cells were stimulated with either 60 or 130 mM KCl. A higher signal was obtained when the cell density was from 5,000 to 10,000 cells/well as plotted in Figure 4. However, the magnitude of the signal increase was not enough to warrant using twice the number of cells, so subsequent work was done at 5,000 cells/well.

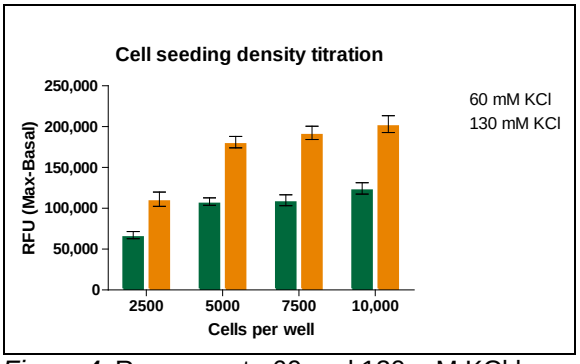

Figure 4. Response to 60 and 130 mM KCl by cells seeded from of 2,500 to 10,000 cells/well.

KCl dose-response curve

We next performed an agonist dose-response experiment. Plates were seeded at 5,000 cells/well and incubated overnight. Exposure of the cells to a KCl solution at concentrations ranging from 15 to 130 mM yielded the dose-response results shown in Figure 5.

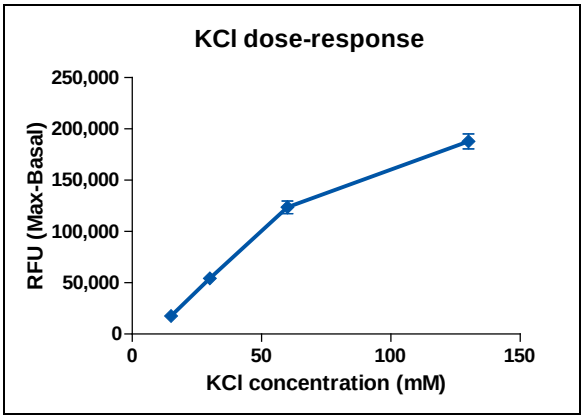

Figure 5. Dose-response of KCl induced membrane depolarization.

Margatoxin inhibition of KCl stimulation

The scorpion toxin, Margatoxin, is a selective blocker of the Kv1.3 ion channel. In order to demonstrate that the membrane depolarization response triggered by KCl is mediated by the Kv1.3 channel, cells were pre-treated with

either Tyrode's buffer or 1 mM Margatoxin for 3 minutes prior to the addition of 100 mM KCl. Those cells that had been exposed to margatoxin showed a greater than 90% decrease in response compared to cells which had been pre-treated with buffer (Figure 6).

Intra-assay reproducibility

In order to assess the assay reproducibility, 36 wells of cells which were plated at 5,000 cells/well were stimulated with 130 mM KCl. The results presented in Figure 7 and tabulated in Table 1 for the average maximum and basal signal show that the response is highly reproducible, and that the cells loaded with dye are not adversely impacted by the 40 minute lapsed time between reading the first and last well.

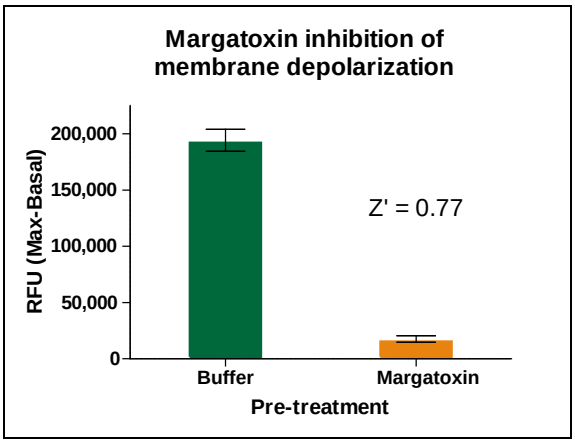

Figure 6. Margatoxin inhibition of KCl-induced membrane depolarization.

Conclusion

The human Kv1.3 voltage-gated K^+ channel expressed in CHO-DUKX cells was used to perform a membrane potential assay. Membrane depolarization was accomplished by exposing the cells to a KCl solution. The KCl was injected and the plates were read on an EnVision Multilabel Reader equipped with an injector and operating in the bottom-reading mode. The assay gave excellent intra-assay reproducibility when the cells had been plated at 5,000 cells/well. The membrane potential assay platform is an excellent alternative to much more time consuming electrophysio-logical experiments, at least for a first examinations of a compounds effect on the channel. The EnVision multilabel plate reader with a built-in dispenser is a sensitive and robust instrument for performing this assay.

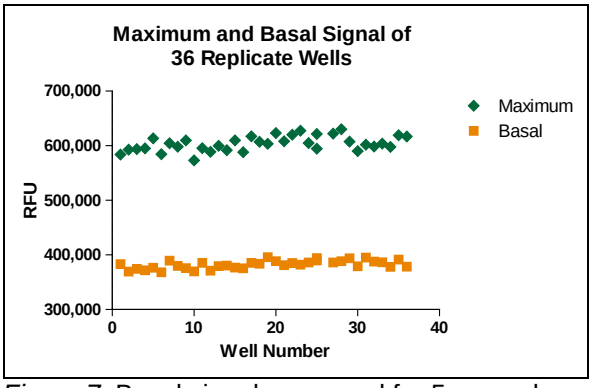

Figure 7. Basal signal measured for 5 seconds before dispensing and maximum signal acquired after dispensing of 130 mM KCl.

	Basal	Maximum
Average	382,389	604,727
SD	7,600	15,177
%CV	2.0	2.5
Z'	0.69	

Table 1. Intra-assay reproducibility of consecutive reading of 36 replicate wells.

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