# Ion Channel Assay Development using Invitrogen's FRET-based Voltage Sensor Probes

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## Introduction

Ion channels are important drug targets because of their critical role in nerve, cardiac, endocrine and skeletal muscle tissues. The lack of sufficiently sensitive screening systems has hampered research in this area. This application note focuses on Voltage Sensor Probes (VSP), a Fluorescence Resonance Energy Transfer (FRET)-based voltage-sensing assay technology from Invitrogen for measuring changes in cellular membrane electrical potential.

This technology enables detection and measurement of rapid changes in membrane voltage and quickly reports them as fluorescence signals from living cells. This technology can be used with any target that changes membrane potential, and is therefore well suited for sodium, potassium, calcium, chloride and ligand-gated ion channel research. The ratiometric method used to detect and quantify changes in cellular membrane potential significantly reduces errors arising from well-to-well variations in cell number, dye loading and signal intensities, plate inconsistencies and temperature fluctuations. These combined features make VSP technology highly amenable for high-throughput screening (HTS) applications.

In order to develop and validate an ion channel assay prior to HTS, it would be helpful for assay development and therapeutic groups to have access to less expensive equipment amenable to VSP technology. This poster demonstrates the use of the POLARstar OPTIMA and NOVOstar plate readers from BMG LABTECH as suitable platforms for development of VSP ion channel assays. These plate readers are much less expensive than HTS ion channel readers and can be used for multiple detection technologies.

## Assay Principles

Voltage Sensor Probes (VSP) is a Fluorescence Resonance Energy Transfer (FRET)-based assay technology used for high-throughput ion channel drug discovery. The FRET donor is a membrane-bound, coumarin-phospholipid (CC2-DMPE), which binds only to the exterior of the cell membrane. The FRET acceptor is a mobile, negatively charged, hydrophobic oxonol [either $DiSBAC_2(3)$ or $DiSBAC_4(3)$], which will bind to either side of the plasma membrane in response to changes in membrane potential.

Resting cells have a relatively negative potential, so the two probes associate with the exterior of the cell membrane, resulting in efficient FRET (Figure 1). Exciting the CC2-DMPE donor probe (at 400 nm) generates a strong red fluorescence signal (at 580 nm) from the oxonol acceptor probe. When the membrane potential becomes more positive, as occurs with cell depolarization, the oxonol probe rapidly translocates (on a subsecond time scale) to the other face of the membrane. Thus, each oxonol probe "senses" and responds to voltage changes in the cell. This translocation separates the FRET pair, so exciting the CC2-DMPE donor probe now generates a strong blue fluorescence signal (at 460 nm) from the CC2-DMPE probe.

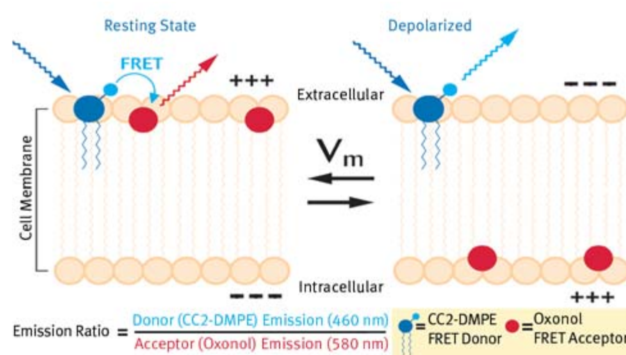


**Fig. 1:** Schematic illustration of the mechanism of voltage-dependent, FRET-based VSPs

During high-throughput ion channel assay development, a simple model is often first tested for proof of principle for both the assay technology and the instrumentation. In this poster, we demonstrate the compatibility of two instruments from BMG LABTECH for developing ion channel assays using VSP technology from Invitrogen.

The ion channel model we have used is endogenously expressed inward rectifying potassium (Kir) channels present in rat basophilic leukemia cells. These channels are opened when high potassium is added to the cell's medium. This causes a change in the cell's membrane potential which can be modulated by barium chloride. In the assay, the cells are manually loaded with the dyes as described below. Barium chloride is then immediately added at various concentrations.

After incubation, the plated cells are placed in the reader and in the case of the NOVOstar, the barium chloride is added by the pipettor inside the reader. The reader begins to take baseline readings at the two wavelengths, injects the high potassium buffer (VSP-2 buffer, below), and continues to take readings for a specified time.

## Materials and Methods

*Cell Culture*

RBL-2H3 (Rat Basophilic Leukemia, ATCC #CRL2256) cells were plated at 50,000 cells/well in Corning® 3603 96-well plates 18-24 hours prior to experimental procedure.

Preparation of VSP Loading Buffers

A. 5 µM CC2-DMPE Loading Buffer: Premix 10 µL of 5 mM CC2-DMPE (Invitrogen) and 10 µL of 100 mg/mL Pluronic® F-127 (Sigma). Add 10 mL of VSP Solution 1 (160 mM NaCl, 4.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, pH 7.4) and vortex vigorously to mix. The solution was protected from light until use.

B. 10 µM $DiSBAC_2(3)$ Loading Buffer: Premix 8.3 µL of 12 mM $DiSBAC_2(3)$ (Invitrogen) and 12.5 µL of 200 mM VABSC-1 (Invitrogen). Add 10 mL of VSP Solution 1 and vigorously vortex to mix. The solution was protected from light until use.

*Loading Cells*

Media was removed from all wells of the 96-well plates and replaced with 100 µL VSP Solution 1 (VSP-1). The VSP-1 was immediately removed and replaced with 100 µL CC2-DMPE Loading Buffer and incubated at room temperature for 30 minutes, covered and protected from light. The CC2-DMPE Loading Buffer was removed after 30 minutes and the plates washed once with 100 µL VSP-1. The VSP-1 was immediately replaced with 100 µL $DiSBAC_2(3)$ Loading Buffer and incubated at room temperature for 30 minutes, covered and protected from light.

$BaCl_2$ (Sigma), when appropriate, was added either immediately following addition of $DiSBAC_2(3)$ Loading Buffer or immediately prior to VSP-2 high $K^+$ buffer (164.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, pH 7.4).

*Instrumentation*

The plates were analysed in real-time with either the POLARstar OPTIMA or NOVOstar.

**Fig. 2:** NOVOstar microplate reader with plate-to-plate transfer, $CO_2$ environment and temperature control feature

## Results and Discussion

Immediately following the $DiSBAC_2(3)$ Loading Buffer 30 minutes incubation, the plates where analyzed with either the POLARstar OPTIMA or NOVOstar. Both instruments basically follow the same protocol of initially reading the plate with two "simultaneous" emissions before VSP-2 addition and continuing after VSP-2 addition.

Data is analyzed by comparing the baseline subtracted ratio of donor (460 nm) to acceptor (580 nm) before (Ro) and after (Rf) addition of VSP-2: the final normalized assay ratio = Rf/Ro.

The POLARstar OPTIMA can inject VSP-2 and collect data at two wavelengths once per second. The software enables real-time visual readout in both plate and well views. Data is automatically exported to the BMG LABTECH evaluation software package for analysis. A POLARstar OPTIMA plate readout view is shown in Figure 3A) for a "positive control" assay with a 96-well plate with 50,000 RBL cells/well. Column 1 is the no cells control.

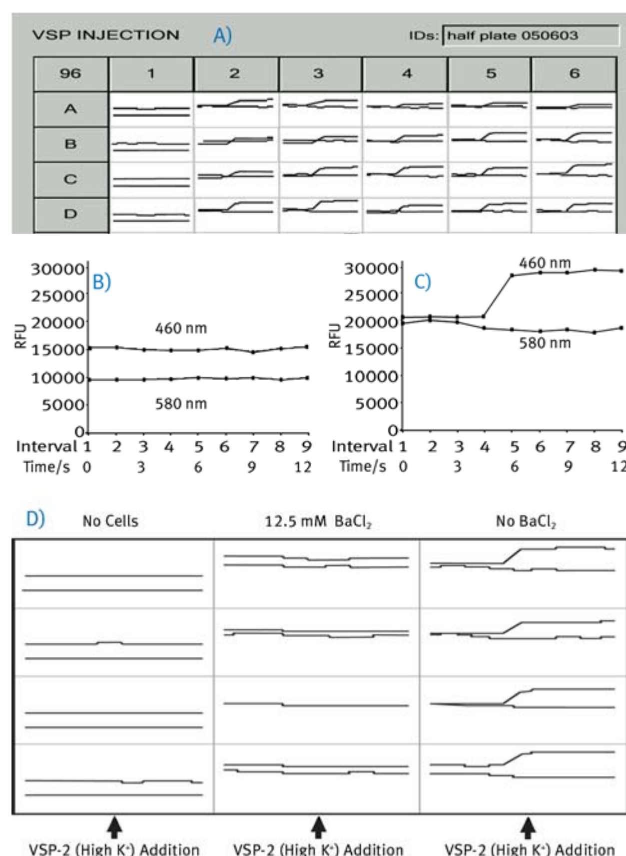


**Fig. 3:** VSP data on the BMG LABTECH POLARstar OPTIMA

In Figure 3B) a current state window view of a no cell control shows no change in 460 nm upon VSP-2 addition, whereas in Figure 3C) an increase in the 460 nm signal upon injection of VSP-2 is detected. Figure 3D) shows enlarged views of current state windows from 12 wells of a $BaCl_2$ dose response. Note the lack of 460 nm emission increase in both the no cell control and 12.5 mM $BaCl_2$ wells compared to untreated wells.

The NOVOstar was programmed to use its auto pipettor to add $BaCl_2$ dilutions directly from one 96-well plate via plate-to-plate transfer to the assay plate with RBL cells. It then injected VSP-2 using one of its two additional injectors and collected data at two wavelengths once per second (Figure 4).

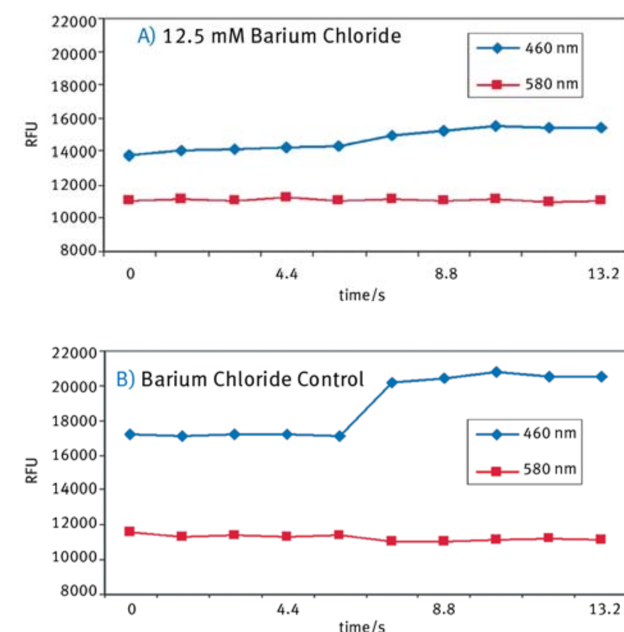


**Fig. 4:** VSP data on the BMG LABTECH NOVOstar

The raw fluorescence readout was evaluated in Excel and the addition of 12.5 mM $BaCl_2$ avoids a significant change in 460 nm emission (Figure 4A), whereas an untreated control well shows as expected an increase in 460 nm fluorescence signal upon VSP-2 addition (Figure 4B).

## Conclusion

This poster demonstrates the compatibility of Voltage Sensor Probe technology with two plate readers from BMG LABTECH. The key to the technology is having instrumentation that can measure two wavelengths in rapid succession. This takes advantage of the ratiometric FRET readout, which greatly reduces variation common to many cell based assays. Our model for ion channel assay development is endogenously expressed inward rectifying Kir channels present in rat basophilic leukemia cells. With both instruments we showed real-time readouts of the ion channel following $K^+$ addition and blockage of the Kir channel with $BaCl_2$. The NOVOstar injectors were able to inject both the $BaCl_2$ inhibitor and the $K^+$ stimulant. The ability of both the POLARstar OPTIMA and NOVOstar to inject ion channel stimulant, switch filters at approximately 1 Hz, and continue to take readings during the kinetic event make these suitable instruments for therapeutic groups in developing ion channel assays for lower throughput applications and screening.

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