Voltage Sensor Probes User Guide

Cat. no. K1016, K1019, and K1046

O-13058-r1 US 0804

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1.0 INTRODUCTION

Voltage Sensor Probes (VSPs), developed by Aurora Biosciences, are 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.

Product Name	Quantity	Cat. no.
Voltage Sensor Probes Set ($DiSBAC_2(3)$ and CC2-DMPE)	1 mg each	K1016
Voltage Sensor Probes Set ($DiSBAC_4(3)$ and CC2-DMPE)	1 mg each	K1046
VABSC-1 (**V**oltage **A**ssay **B**ackground **S**uppression **C**ompound)	1 g	K1019

CC2-DMPE, $DiSBAC_2(3)$ and $DiSBAC_4(3)$ are also available separately:

Product Name	Quantity	Cat. no.
CC2-DMPE	1 mg	K1017
	5 mg	K1020
	10 mg	K1070
$DiSBAC_2(3)$	1 mg	K1018
	5 mg	K1021
$DiSBAC_4(3)$	1 mg	K1047
	5 mg	K1022

The Voltage Sensor Probes Sets (Cat. nos. K1016 and K1046) provide sufficient reagent volumes for performing approximately 20 sets (each set is a 96-well assay plate) of 100-µL assays, each using 5 µM CC2-DMPE and 10 µM of either $DiSBAC_2(3)$ or $DiSBAC_4(3)$. VABSC-1 (Cat. no. K1019) is supplied as 1 g of powder which is sufficient for performing approximately 100 sets (each set is a 96-well assay plate) of 100-µL assays, each using 250 µM VABSC-1.

$DiSBAC_2(3)$ (molecular weight 436.0) is more water-soluble than $DiSBAC_4(3)$ and is left in the extracellular media during the assay. The majority of ion channel screening applications to date have been done with $DiSBAC_2(3)$ because it has shown the best assay stability. The time response of this oxonol is ~200 ms.

$DiSBAC_4(3)$ (molecular weight 548.2) is more hydrophobic and requires Pluronic®-127 surfactant (Sigma-Aldrich, St. Louis, MO) for cellular loading. Excess probe is washed away before the assay. The time response of this oxonol is ~20 ms. This probe is useful for applications which require faster temporal resolution or the absence of probe in the extracellular solution.

VABSC-1 dye works with CC2-DMPE to suppress background signal and provide optimal performance.

2.0 OPTIMIZING PROBE LOADING CONDITIONS

Probe loading concentrations vary with different cell types and must be empirically determined for a given cell type. Optimize the VSP loading conditions by loading cells with a matrix of probe concentrations and perform the assay. Use appropriate controls (including a cell-free Background Control) and determine optimal loading conditions by identifying the concentrations that produce a large ratio change and acceptable signal/background values (See **Section 3.0, Data Analysis for Depolarization**).

For most cell types, optimal CC2-DMPE and $DiSBAC_2(3)$ loading concentrations fall between 0.5–20 µM. However, higher CC2-DMPE concentrations (up to 40 µM) may be necessary for some cell types. $DiSBAC_2(3)$ loading concentrations should be optimized below 20 µM, due to solubility issues.

For most cell types, the optimal $DiSBAC_4(3)$ loading concentration ranges between 2–3 µM. The loading procedure for $DiSBAC_4(3)$ differs from the procedure used with $DiSBAC_2(3)$. Typically, the $DiSBAC_4(3)$ loading solution is prepared by mixing 1 µL of the 3 mM $DiSBAC_4(3)$ stock solution with 2 µL of a Pluronic®-127 surfactant•DMSO solution (10% w/v). This is added to 10 mL of a serum-free buffer with vortexing. The $DiSBAC_4(3)$ loading solution is then loaded on the cells and washed away (to remove excess dye) prior to reading.

Typically, cells are loaded first with CC2-DMPE, followed by the oxonol [either $DiSBAC_2(3)$ or $DiSBAC_4(3)$].

Test compounds should be added immediately after loading the cells with $DiSBAC_2(3)$ or $DiSBAC_4(3)$. Stimulants should be added while acquiring data.

2.1 Examples of Optimized Conditions for Common Cell Types

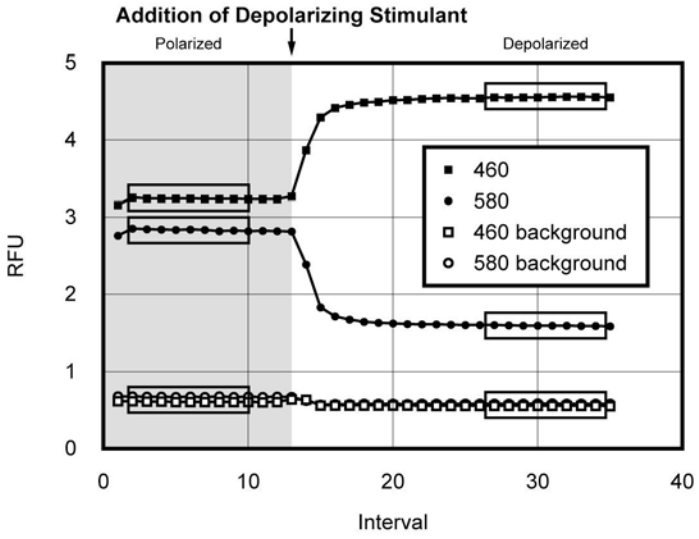
HEK293 cells: 5 µM CC2-DMPE and 10 µM $DiSBAC_2(3)$

CHO-K1 cells: 10 µM CC2-DMPE and 3 µM $DiSBAC_2(3)$

3.0 DATA ANALYSIS FOR DEPOLARIZATION

3.1 Calculating the Average $460_{initial}$ and $580_{initial}$ and 460_{final} and 580_{final} Values

1. Set the excitation setting to 405 ± 15 nm.
2. Take the initial fluorescence intensity measurements (polarized state) at 460 and 580 wavelengths before adding depolarizing stimulant.
3. Add depolarizing stimulant and continue to record fluorescence intensity readings until changes in fluorescence intensity measurements (depolarized state) have reached a plateau.
4. Select identical data windows for both the initial ($460_{initial}$ and $580_{initial}$) and final (460_{final} and 580_{final}) fluorescence intensity readings. Note that the data windows should exclude the first time point, any time points within the inflection of the curve, and the last time point. We use eight data points per data window.
5. Calculate the average of the data points within a data window to obtain Average $460_{initial}$, $580_{initial}$, 460_{final} and 580_{final} values.



3.2 Calculating the Baseline Corrected (BLC) Values

1. Obtain the baseline signals as above from control wells without cells and select data points for initial ($460_{initial}$ and $580_{initial}$) and final (460_{final} and 580_{final}) fluorescence intensity readings. Use the same data windows as in experimental wells.
2. Calculate the average of all four data windows to obtain Baseline $460_{initial}$, $580_{initial,}$ 460_{final} and 580_{final} values.
3. Calculate Baseline Corrected (BLC) values by subtracting the appropriate Average Baseline value from the Average Data value for each data window.

3.3 Calculating the Emission Ratios

1. Calculate the Emission Ratio for Depolarized and Polarized States, as shown below:

   $Emission\ Ratio_{Polarized}$ = BLC $460_{initial}$ / BLC $580_{initial}$

   $Emission\ Ratio_{Depolarized}$ = BLC 460_{final} / BLC 580_{final}

3.4 Determining the Response Ratio

1. Calculate the **Response Ratio = $Emission\ Ratio_{Depolarized}$ / $Emission\ Ratio_{Polarized}$**

4.0 RECOMMENDED DYE STOCK PREPARATION

4.1 5 mM CC2-DMPE in DMSO

1. Allow the CC2-DMPE to warm to room temperature. Keep the bottle closed.
2. Tap the bottle of CC2-DMPE to settle contents to the bottom.
3. Open the CC2-DMPE by wrapping the bottle in a wet paper towel to prevent static electricity.
4. Add 233 µL DMSO to the 1.0-mg bottle of CC2-DMPE. Tightly cap and vortex vigorously for about one minute.
5. To aid in dissolution, sonicate the solution for one minute, then incubate for one minute in a 37°C water bath.
6. Aliquot the bottle for single day uses, as repeated freeze/thaw cycles can cause variability in results.
7. Store unused portions at -20°C. Under these conditions, this stock solution is stable for at least one month.

4.2 12 mM $DiSBAC_2(3)$ in DMSO

1. Allow the $DiSBAC_2(3)$ to warm to room temperature. Keep the bottle closed.
2. Tap the bottle of $DiSBAC_2(3)$ to settle contents to the bottom.
3. Open the $DiSBAC_2(3)$ by wrapping the bottle in a wet paper towel to prevent static electricity.
4. Add 191 µL DMSO to the 1.0 mg bottle of $DiSBAC_2(3)$. Tightly cap and vortex vigorously for about one minute.
5. To aid in dissolution, sonicate the solution for one minute, then incubate for one minute in a 37°C water bath.
6. Store unused portions at -20°C. Under these conditions, this stock solution is stable for at least one month.

4.3 3 mM $DiSBAC_4(3)$ in DMSO

1. Allow the $DiSBAC_4(3)$ to warm to room temperature. Keep the bottle closed.
2. Tap the bottle of $DiSBAC_4(3)$ to settle contents to the bottom.
3. Open the $DiSBAC_4(3)$ by wrapping the bottle in a wet paper towel to prevent static electricity.
4. Add 608 µL DMSO to the 1.0 mg bottle $DiSBAC_4(3)$. Tightly cap and vortex vigorously for about one minute.
5. To aid in dissolution, sonicate the solution for one minute, then incubate for one minute in a 37°C water bath.
6. Store unused portions at -20°C. Under these conditions, this stock solution is stable for at least one month.

4.4 200 mM VABSC-1 Background Suppression Dye (9 mL)

1. Allow the VABSC-1 to warm to room temperature. Keep the bottle closed.
2. Add 8.0 mL distilled, deionized water to 1.0 g VABSC-1. Mix well to dissolve VABSC-1.
3. Filter the VABSC-1 solution into a 15-mL sterile conical tube using a 10-mL syringe with a 0.2-µm filter.
4. Adjust the pH to 7.4 with either 0.1 N NaOH or 0.1 N KOH.
5. Bring volume to 9.1 mL with distilled, deionized water.
6. Determine the concentration of the solution by a spectrophotometer at 400 nm. The extinction coefficient at 400 nm is 10,000 $M^{-1}cm^{-1}$.
7. Adjust volume as needed to a final concentration of 200 mM.
8. Store unused portions at 4°C. Stored under these conditions, this stock solution is stable at room temperature for at least six months.

4.5 100 mg/mL Pluronic®-127 surfactant stock solution in DMSO

1. Add 1.0 g Pluronic®-127 surfactant to 10.0 mL DMSO and mix well. Warm to 37°C.
2. Store the reagent at room temperature (18–22°C) protected from direct light.
3. Mix thoroughly before use.

Note: Under cold lab conditions [colder than 18°C (65°F)], the solution may freeze or a white precipitate may form. If this happens, warm and stir the solution (~37°C) until thawed and the precipitate dissolves.

5.0 SAMPLE PROTOCOL FOR KIR CHANNEL DETECTION IN RBL AND HEK CELLS

Note: Loading Dyes and washing steps utilize VSP-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).

Note: The depolarizing solution, VSP-2, provides a high K^+ concentration (164.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, pH 7.4)

5.1 Cell Preparation

1. Plate cells at a density of 50,000/well in 96-well plates (Corning #3603). Do not plate cells in Column 1 (media only).
2. Allow cells to grow ~24 hours at 37°C, 5% CO_2 to reach a confluent monolayer.

5.2 Dye Loading Solution Preparation

Prepare stock solutions as listed in **Section 4.0, Recommended Dye Stock Preparation**. Warm VSP Solution 1 (VSP-1) to room temperature. This can be done by a brief incubation at 37°C or by placing the solution at room temperature for at least one hour.

5.2.1 CC2-DMPE Loading Buffer

1. At the bottom of a 15 ml conical tube, mix 10 L of 5 mM CC2-DMPE and 10 µL of 100 mg/mL Pluronic® F-127 by pipetting up and down.
2. Add 10 mL of VSP-1 and vortex vigorously to mix.
3. Protect from light prior to use.

Note: For immediate use only. Do not save unused Loading Buffer.

5.2.2 DiSBAC$_2$(3) Loading Buffer

1. At the bottom of a 15 mL conical tube, mix 8.3 µL of 12 mM $DiSBAC_2(3)$ and 12.5 µL of 200 mM VABSC-1 by pipetting up and down.
2. Add 10 mL of VSP-1 and vigorously vortex to mix.
3. Protect from light prior to use.

Note: For immediate use only. Do not save unused Loading Buffer.

5.3 Cell Loading

1. Remove cells from incubator. Remove media from all wells of the plate.
2. Add 100 µL VSP-1 to each well.
3. Immediately remove the VSP-1. It is not necessary to shake or incubate cells with VSP-1.
4. Add 100 µL CC2-DMPE Loading Buffer to each well and incubate at room temperature for 30 minutes, with the plate covered and protected from light.
5. Remove CC2-DMPE Loading Buffer and wash briefly with 100 µL VSP-1. Remove VSP-1.
6. Add 100 µL $DiSBAC_2(3)$ Loading Buffer to each well and incubate at room temperature for 30 minutes, with the plate covered and protected from light.

5.4 Data Analysis

Note: Do not wash cells prior to analysis.

1. Read cells in plate reader. Ideally the plate reader will take a reading at resting potential, and then inject 100 µL of VSP-2 (high K^+) before taking several more readings following depolarization.

6.0 PURCHASER NOTIFICATION

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"E-stim Methods" means either of:

(A) a method of determining an electrical potential across a cellular membrane in at least one cell that comprises the steps of: (a) contacting said at least one cell with a first reagent comprising a dye capable of redistributing from a first face of the membrane to a second face of the membrane in response to changes in the membrane potential; (b) contacting said at least one cell with a second reagent selected from the group consisting of fluorophores that are capable of undergoing energy transfer with the first reagent by either (i) donating excited state energy to the first reagent, or (ii) accepting excited state energy from the first reagent, said second reagent being located adjacent to either the first face of the membrane or the second face of the membrane, wherein steps a) and b) are optionally performed in reverse order or simultaneously; (c) exposing the membrane to an electrical stimulus that is capable of altering the membrane potential; (d) exposing said at least one cell to light of a wavelength sufficient to optically excite whichever of said first or said second reagent is capable of donating excited state energy to the other of said first or second reagent; (e) measuring energy transfer between the first reagent and the second reagent; and (f) relating the energy transfer to the membrane potential; or

(B) a method of determining whether a test compound is a modulator of an electrical potential across a cellular membrane in at least one cell comprising the steps of: a) contacting said at least one cell with a first reagent comprising a dye capable of redistributing from a first face of the membrane to a second face of the membrane in response to changes in membrane potential, b) contacting said at least one cell with a second reagent selected from the group consisting of fluorophores that are capable of undergoing energy transfer with the first reagent by either (i) donating excited state energy to the first reagent, or (ii) accepting excited state energy from the first reagent, said second reagent being located adjacent to either the first face of the membrane or the second face of the membrane, wherein steps a) and b), d) exposing the membrane to an electrical stimulus that is capable of altering membrane potential, e) exposing said at least one cell to light of a wavelength sufficient to optically excite whichever of said first or said second reagent is capable of donating excited state energy to the other of said first or second reagent, f) measuring energy transfer between the first reagent and the second reagent, and g) comparing said energy transfer to a similarly determined energy transfer observed in at least one control cell that has not been contacted with said test compound to determine if said test compound modulates the electrical potential across the cellular membrane of said at least one cell.