Thermo Fisher SCIENTIFIC

Screening Protocol and Assay Conditions

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Overview and Assay Theory

The SelectScreen Biochemical hERG Screening Service utilizes the Predictor™ hERG Fluorescence Polarization (FP) assay to provide you with a reliable, rapid and sensitive method for identifying compounds that bind to the hERG channel protein. The Predictor hERG FP assay is a homogenous, fluorescence polarization biochemical-based format utilizing a membrane fraction containing hERG channel protein (Predictor hERG Membrane) and a high affinity, red-fluorescent hERG channel ligand (Predictor hERG Tracer Red). When the Predictor hERG Tracer ligand is bound to the hERG channel it produces high fluorescence polarization values. Compounds that bind to the hERG channel protein (competitors) will displace the Predictor hERG Tracer Red resulting in decreased fluorescence polarization values.

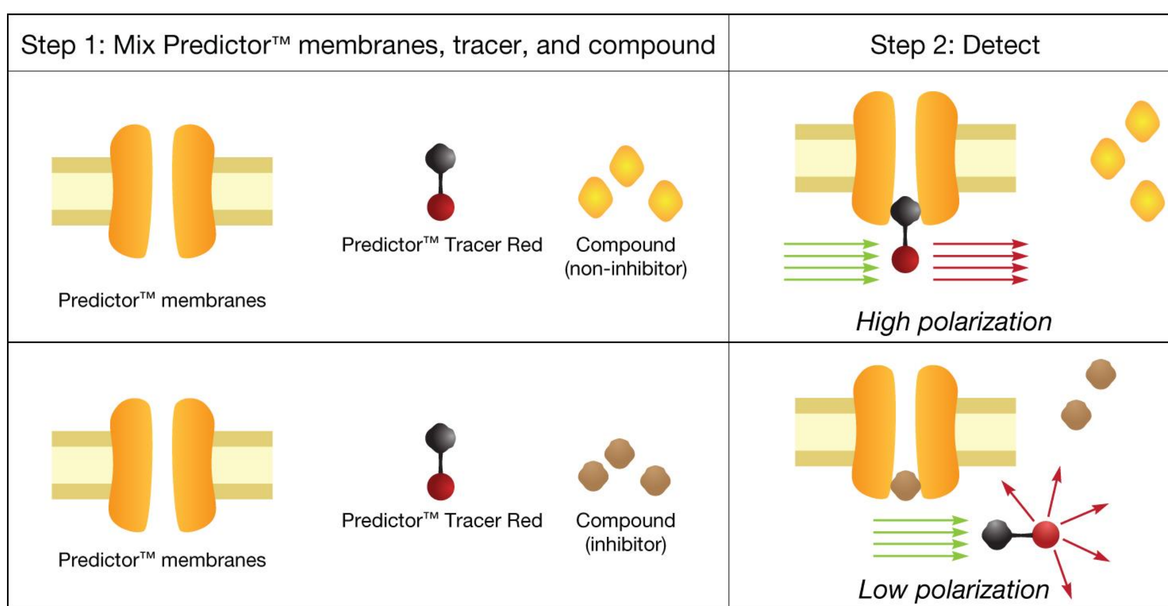

Figure 1: Predictor hERG Fluorescence Polarization Assay Principle.

A reference for the development of the Predictor hERG FP Assay is provided below (Piper *et al.*).

The Predictor hERG FP assay used in the Service is tested and documented to show a high level of performance. These assays must meet the following specifications:

Z′-factor > 0.5

IC_{50} of the known inhibitor, E-4031, is within ± ½ log of the IC_{50} spec

Any assay results not meeting these specifications are automatically repeated until the results pass our QC criteria.

SelectScreen Assay Conditions

Test Compounds

Test compounds are received at 100X (or greater) of the desired starting concentration in 100% DMSO. If compounds are supplied at greater than 100X concentration, an initial dilution is made in 100% DMSO to bring the compounds to 100X concentration. The test compounds are screened with a final concentration of 1% DMSO. For 10-point titrations, 3-fold serial dilutions are performed from the starting concentrations of the client's specifications.

Assay Buffer

25 mM HEPES (pH 7.5), 15 mM KCl, 1 mM $MgCl_2$, and 0.05% Pluronic F-127

Predictor hERG Membranes

Membranes are stored as a 2X stock at -80˚C. Each membrane lot is titrated to identify the concentration of membranes required to obtain ~70% bound tracer. Before setting up the assay the membranes are thawed in a 37˚C water bath and stored at room temperature. To avoid light scatter caused by large membrane particulates the membranes are sonicated until a homogeneous solution free of particulates is obtained. Thawed membranes are sonicated with a Branson Sonifer® 450 for 10 pulses (Duty cycle: 20%, Output Control: 3 out of 10, Timer: hold). The sample is returned to ice for 30 seconds followed by another 5-10 pulses until homogeneous. After sonication the membranes are stored at room temperature.

Predictor hERG Tracer Red

The tracer is stored as a 250X stock at -20˚C. Prior to the assay the tracer is thawed and stored at room temperature. The tracer is diluted in Assay Buffer to 4X with a final assay concentration of 1 nM.

Assay Protocol

Barcoded 384 well untreated low-volume polystyrene microplates (Corning Cat. #4511)

1. 5 µL of 4X test compound diluted in Assay Buffer (4% DMSO) or 200 nL 100X test compound in 100% DMSO plus 4.8 µL of Assay Buffer is added to the assay plate. Each compound titration is performed in the absence and presence of E-4031.
2. 10 µL of 2X Predictor hERG Membranes is added to the appropriate wells of the assay plate.
3. 5 µL of 4X Predictor hERG Tracer Red is added to the appropriate assay wells.
4. The assay plate is mixed on an orbital shaker for 20-30 seconds.
5. The assay plate is covered and incubated for 3 hours at room temperature.
6. The assay plate is read on a fluorescence plate reader (Tecan Safire^2) and the data is analyzed.

SelectScreen Assay Controls

The following controls are run on each plate:

100% Inhibition Control

Represents 100% tracer displacement (the minimum polarization value) as identified by 30 µM E-4031 displacement of Predictor hERG Tracer Red from Predictor hERG Membranes. Assay wells consist of 30 µM E-4031, Predictor hERG Membranes and Predictor hERG Tracer Red with 1% DMSO.

0% Inhibition Control

Represents 0% tracer displacement from the Predictor hERG Membranes (the maximum polarization value). Assay wells consist of Assay Buffer, Predictor hERG Membranes, and Predictor hERG Tracer Red with 1% DMSO.

Assay Blanks

Assay blank control wells consist of Predictor hERG Membranes and Assay Buffer. The blank wells are used for background subtraction of raw parallel and perpendicular fluorescence values prior to the calculation of polarization values.

Known Inhibitor

An 8-point titration of the known inhibitor, E-4031, in duplicate is included on each assay plate to ensure the assay is performing within the expected IC_{50} range previously determined.

The following controls are prepared for each concentration of test compound:

1. ***Test Compound Polarization Interference (TCPI)***

At higher concentrations some test compounds may exhibit an additional non-hERG specific reduction in polarization values producing data that appears to be affecting a one-site binding model. This phenomenon is observed with membranes lacking the hERG channel protein suggesting the presence of a non-hERG component in the membrane prep that is binding the tracer. This non-specific interaction is displaced by certain compounds at higher concentrations resulting in a reduction in polarization values. The effect of the test compound on this non-specific interaction is tested in the presence of saturating concentrations of E-4031. Assay wells utilized to test for Test Compound Polarization Interference will contain test compound, 30 µM E-4031, Predictor hERG Membranes, and Predictor hERG Tracer Red with 1% DMSO. Calculated TCPI values outside ± 25% are flagged.

2. ***Test Compound Fluorescence Interference (TCFI)***

Test Compound Fluorescence Interference is determined by comparing the total fluorescence for test compound wells with the total fluorescence values from the 0% and 100% inhibition control wells. TCFI values outside ± 20% the controls are flagged.

SelectScreen Data Analysis

The following equations are used for each set of data points:

	Equation
Background-Subtracted Perpendicular Fluorescence Intensity (FL⊥)	$\mathbf{RFU}_{\text{Test Compound}} - \mathbf{RFU}_{\text{Assay Blank}}$
Background- Subtracted Parallel Fluorescence Intensity (FI‖)	$\text{G-Factor} * (\mathbf{RFU}_{\text{Test Compound}} - \mathbf{RFU}_{\text{Assay Blank}})$
Total Fluorescence Intensity (TFI)	$FI_{\parallel} + (2 * FL_{\perp})$
Test Compound Fluorescence Interference (TCFI)	
IF $TFI_{\text{0\% Inhibition}} > TFI_{\text{100\% Inhibition}}$	
If $TFI_{\text{Test Compound}} > [Avg\ (TFI_{\text{0\% Inhibition}}) * 1.2]$	$\left\{ \frac{Avg\ TFI_{\text{Test Compound}}}{Avg\ TFI_{\text{0\% Inhibition}}} - 1 \right\} * 100$
If $TFI_{\text{Test Compound}} < [(Avg\ (TFI_{\text{0\% Inhibition}}) * 1.2]$ *And* If $TFI_{\text{Test Compound}} > [Avg\ (TFI_{\text{100\% Inhibition}}) * 0.8]$	**0**
If $TFI_{\text{Test Compound}} < [Avg\ (TFI_{\text{100\% Inhibition}}) * 0.8]$	$\left\{ \frac{Avg\ TFI_{\text{Test Compound}}}{Avg\ TFI_{\text{100\% Inhibition}}} - 1 \right\} * 100$
IF $TFI_{\text{100\% Inhibition}} > TFI_{\text{0\% Inhibition}}$	
If $TFI_{\text{Test Compound}} > [Avg\ (TFI_{\text{100\% Inhibition}}) * 1.2]$	$\left\{ \frac{Avg\ TFI_{\text{Test compound}}}{Avg\ TFI_{\text{100\% Inhibition}}} - 1 \right\} * 100$
If $TFI_{\text{Test Compound}} < [(Avg\ (TFI_{\text{100\% Inhibition}}) * 1.2]$ *And* If $TFI_{\text{Test Compound}} > [Avg\ (TFI_{\text{0\% Inhibition}}) * 0.8]$	**0**
If $TFI_{\text{Test Compound}} < [Avg\ (TFI_{\text{0\% Inhibition}}) * 0.8]$	$\left\{ \frac{Avg\ TFI_{\text{Test Compound}}}{Avg\ TFI_{\text{0\% Inhibition}}} - 1 \right\} * 100$
mP	$\frac{FI_{\parallel} - FI_{\perp}}{FI_{\parallel} + FI_{\perp}} * 1000$

Test Compound Polarization Interference (TCPI)	$\left\{ \frac{\mathbf{mP}_{\text{Test Compound + E-4031}} - \mathbf{mP}_{\text{100\% Inhibition}}}{\mathbf{mP}_{\text{0\% Inhibition}} - \mathbf{mP}_{\text{100\% Inhibition}}} \right\} * 100$
% Inhibition	$\left\{ 1 - \frac{\mathbf{mP}_{\text{Test Compound}} - \mathbf{mP}_{\text{Test Compound + E-4031}}}{\mathbf{mP}_{\text{0\% Inhibition}} - \mathbf{mP}_{\text{100\% Inhibition}}} \right\} * 100$
Z'* (using mP values)	$1 - \frac{3{*}\mathbf{Std\ Dev}_{\text{0\% Inhibition}} + 3{*}\mathbf{Std\ Dev}_{\text{100\% Inhibition}}}{\mathbf{Mean}_{\text{0\% Inhibition}} - \mathbf{Mean}_{\text{100\% Inhibition}}}$

*Zhang et al.

Graphing Software

SelectScreen Biochemical hERG Screening Service uses XL*fit* from IDBS. The dose response curve is curve fit to model number 205 (sigmoidal dose-response model). If the bottom of the curve does not fit between -20% and 20% inhibition, it is set to 0% inhibition. If the top of the curve does not fit between 70% and 130% inhibition, it is set to 100% inhibition.

FP hERG Assay Validation

Table 1. Comparison of IC_{50} values generated with the Predictor hERG Fluorescence Polarization assay with reported IC_{50} values from patch-clamp and radioligand displacment assays.

Compound	Patch-Clamp*	Radioligand*	FP
	IC_{50} (nM)		
Astemizole	1.2	1	1.3
Dofetilide	12	40	6.9
Terfenadine	16	30	23
E-4031	48	20	34
Bepridil	550	170	210
Thioridazine	1250	510	708
Fluoxetine	990	2230	4310
Amitripyline	10000	†2440	11200

*Chouabe et al (1998), Diaz et al (2004), Kongsamut et al (2002), Snyders & Chaudhary (1996), Tie et al (2000), and Wang et al (2003).
†[3H]-dofetilide binding (Diaz, et al 2004) with 60 mM K^+, except amitryptiline with 5 mM K^+.

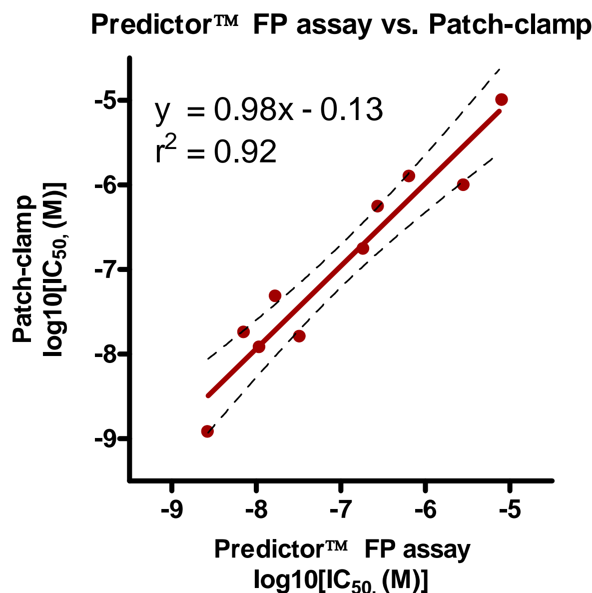

Figure 2. Correlation graph of IC_{50} values generated with the Predictor hERG FP assay and reported IC_{50} values generated from patch-clamp.

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