Table of Contents

1. Kit Contents

The Predictor™ hERG Fluorescence Polarization Assay Kit (catalog no. PV5365) contains the components listed below. This kit contains enough reagents for 400 assays of 20 µl each. The potent hERG ligand, E-4031, is supplied as a positive control.

Component	Composition	Amount	Storage Temp.	Individual Catalog no.
Predictor™ hERG Tracer Red	250 nM (250X) in 100% DMSO	40 µl	–20°C	PV5363
Predictor™ hERG Membrane	hERG channel supplied at 2X; see Certificate of Analysis for lot specific titration with E-4031.	5 ml	–80°C	K1785
Predictor™ hERG FP Assay Buffer	Proprietary buffer (pH 7.5)	20 ml	Room temp.	PV5364
E-4031	3 mM in water	80 µl	–20°C	PV5366

2. Materials Required but Not Supplied

The following materials are required but not supplied in the kit:

- A plate reader capable of measuring fluorescence polarization in the red spectrum (see **Section 6** for instrument requirements).
- Pipetting devices for 1–1000 µl volumes, suitable repeater pipettors, or multi-channel pipettors.
- Black 384-well assay plates. We recommend untreated low-volume polystyrene plates (*e.g.*, Corning #3677) or polypropylene plates (*e.g.*, Matrical #MP101-1-PP). ***Important***: Plates treated with a non-binding coating (*e.g.*, Corning #3676) should *not* be used. Predictor™ hERG Tracer Red binds to the coating on these plates, and this results in a lower assay window and increased assay variability.
- 384-well polypropylene plates (*e.g.*, Corning #3657). These plates are used for preparing serial dilutions of test compounds.
- High Purity DMSO for compound stocks and serial dilutions. We recommend Fluka 41647.

3. Introduction

The cardiotoxic effects that may accompany undesired blockage of the human ether-a-go-go-related gene (hERG) potassium channel continue to present challenges and obstacles to the development of safe small-molecule therapies. Invitrogen's Predictor™ hERG Fluorescence Polarization Assay provides an efficient method for determining whether test compounds block the hERG channel. The assay can be used at the early stages of drug discovery to help identify or triage compounds from larger collections.

The assay kit contains all of the reagents necessary to test your compounds of interest for hERG channel binding. The assay uses a membrane fraction containing hERG channel protein (Predictor™ hERG Membrane) and a high-affinity red fluorescent hERG channel ligand, or "tracer" (Predictor™ hERG Tracer Red), in a homogenous, fluorescence polarization (FP)-based format. FP is based on the observation that when a small fluorescent molecule (the tracer) is excited with plane-polarized light, the emitted light is largely depolarized because the molecule tumbles rapidly in solution during its fluorescence lifetime. If the tracer is bound to a large molecule, then the tracer's rotation is slowed and the light remains more highly polarized. When Predictor™ hERG Tracer Red is bound by the hERG channel protein, the tracer produces a high fluorescence polarization. Compounds that bind to the hERG channel protein (competitors) are identified by their ability to displace the tracer, resulting in a lower fluorescence polarization (see **Figure 1**).

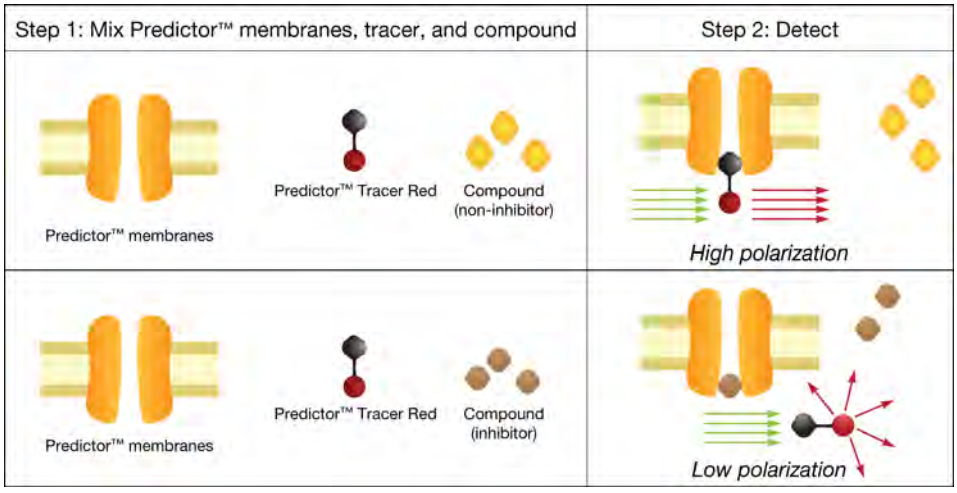


Figure 1. Competitors displace the tracer from the hERG channel protein, lowering its fluorescence polarization.

The Predictor™ assay is:

- Homogeneous (mix and read)
- Efficient (provides data in as little as 2 hours)
- Stable (can be read for up to 24 hours after preparation)
- Tolerant of common organic solvents (up to 5% DMSO, methanol, or ethanol)
- Validated on multiple instrument platforms

4. Reagent Handling, Thawing, and Storage

Important: **Do not use water baths for thawing!** Certain antifungal and antialgae products commonly used in water baths appear to act as hERG blockers and can reduce assay performance or cause complete assay failure.

4.1 Predictor™ hERG Tracer Red

Store Predictor™ hERG Tracer Red at –20°C. Thaw at room temperature and vortex briefly before use. The tracer is stable for at least six freeze-thaw cycles.

4.2 Predictor™ hERG FP Assay Buffer

Thaw and store Predictor™ hERG FP Assay Buffer at room temperature upon receipt.

4.3 E-4031

Store E-4031 at –20°C. Thaw at room temperature before setting up the assay. The E-4031 is stable for at least six freeze-thaw cycles.

4.4 Predictor™ hERG Membrane

Store Predictor™ hERG Membrane at –80°C.

Thaw the Predictor™ hERG Membrane at room temperature (do not use a water bath; see **Important** note above). Mix by pipetting ~20X with a small-bore pipette or pipette tip (*e.g.*, P1000 or 5 mL pipette), being careful to include all of the material in the vial. Continue to pipette if filaments/precipitates persist.

Store the dispersed Predictor™ hERG Membranes at room temperature until use.

Note: The Predictor™ hERG Membrane retains >90% activity after up to three freeze-thaw cycles. If desired, the pipetted, homogenous membrane preparation can be divided up into smaller aliquots (*e.g.*, 1-ml portions stored in 1.5-ml plastic vials) to avoid loss of activity.

***Important*:** The membrane preparation must be pipetted after each thaw to avoid light-scatter caused by large membrane particles and to deliver a consistent amount of hERG receptor to each assay well. A failure to do so will negatively impact the Z′ value, a measure of assay variability.

Alternative membrane preparation methods have been tested. If desired, please contact technical support for more information.

5. Assay Procedure

5.1 Controls

The following table summarizes the controls to be included on each assay plate.

Buffer Blank	Assay Blank	Free Tracer Control	Negative Control	Positive Control (E-4031)
Used only when determining the G-factor with Free Tracer	Used to subtract the background fluorescence from raw emission values to calculate polarization values.	Used for normalization of polarization values by setting the instrument G-factor. See **Section 6.3** for more information.	Represents 0% tracer displacement (maximum polarization value). Important to set the top of the assay curve.	Represents 100% tracer displacement (minimum assay polarization value) by a known hERG channel ligand. Important to set bottom of the assay curve.

We highly recommend running the Positive (E-4031) and Negative controls on every assay plate to clearly establish the top and bottom of the assay window for data analysis. The Positive and Negative controls can be used set the top and the bottom of the curve correctly. Three log units above and below the IC_{50} of a test compound are needed to define the curve in an FP assay (see Appendix for further information).

Special note for comparison to electrophysiology data: The control tail current amplitude from a patch clamp assay is analogous to the Negative Control value in the Predictor™ hERG FP assay. Therefore, unless very low concentrations of test compounds are included in the compound titrations, the full assay window will not be properly defined, resulting in curves that have unusual shape and IC_{50} estimates that will not be accurate. Fixing the top and the bottom of the assay window to the Negative and Positive Control values for E-4031 will increase the accuracy of IC_{50} estimates.

See **Section 8.3.1, Number of Data Points and Data Analysis,** for a more complete discussion.

5.2 Initial Instrument Setup

First-time users should run the following controls on the instrument to establish correct instrument setup prior to running dose response curves. Volumes are provided per well.

The instrument setup run is designed to determine the G factor, observe the mP shift and calculate the Z′ (n=8).

	Buffer Blank	Assay Blank	Free Tracer Control	Negative Control	Positive Control
E-4031					5 µL
Buffer	20 µL	10 µL	15 µL	5 µL	
Membrane		10 µL		10 µL	10 µL
Tracer			5 µL	5 µL	5 µL

5.3 Reagent Preparation

1. Thaw the reagents and mix the Predictor™ hERG Membrane by pipetting up and down ~20 times as described in **Section 4.4**. As noted in **Section 4**, use of water baths for thawing is *not* recommended.

2. Prepare the following reagents before plate loading. Volumes are provided for one-quarter of a 384-well plate. Volumes can be scaled for different numbers of wells using the same dilution ratio. Add reagents to buffer.

4 nM Tracer (4X)		E-4031 in buffer (4X = 120 µM)		2X Membrane
Buffer	615 µl	Buffer	480 µl	No buffer
Tracer	10 µl	E-4031	20 µl	Total volume = 1.25 mL
Total volume	625 µl (1:62.5)	Total volume	500 µl (1:25)	

5.4 Plate Loading Summary

Load the appropriate well with the volume shown below. The order of addition should be:

a. Buffer or compound
b. Membrane
c. Tracer

	Buffer Blank	Assay Blank	Free Tracer Control	Negative Control	Positive Control	Reference Compound (*e.g.*, E-4031) Titration	Test Compounds
Test Compound							5 µL
E-4031					5 µL	5 µL	
Buffer	20 µL	10 µL	15 µL	5 µL			
Membrane		10 µL		10 µL	10 µL	10 µL	10 µL
Tracer			5 µL	5 µL	5 µL	5 µL	5 µL

5.5 Dilutions of Reference and Test Compounds

We highly recommend that you evaluate at least 10 concentrations of reference or test compounds in initial experiments—16-point, 3-fold dilutions are preferred.

E-4031 is a compound that is known to bind to the hERG channel, and is typically used as a reference compound in such experiments. It is used as a typical reference compound in the following protocol.

Note: We recommend running compound titrations in at least duplicate.

Note: We recommend using a 384-well polypropylene plate for the dilutions (*e.g.*, Corning 3657).

5.6 Plate Loading

The diagram below shows a example layout for a 384-well assay plate for a Z′ experiment, E-4031 (reference compound) titration, and test compound titrations. You can reference this layout during the plate loading steps in this section.

Note: For the assay plate, we recommend untreated low-volume polystyrene plates (*e.g.*, Corning #3677) or polypropylene plates (*e.g.*, Matrical #MP101-1-PP).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	Buffer Blank	Negative Control	E-4031 Titration	E-4031 Titration	Test Compound Titration	Test Compound Titration	Test Compound Titration	Test Compound Titration	Test Compound Titration	Test Compound Titration	Test Compound Titration	Test Compound Titration	Test Compound Titration	Test Compound Titration	Test Compound Titration	Test Compound Titration	Test Compound Titration	Test Compound Titration	Test Compound Titration	Test Compound Titration	Test Compound Titration	Test Compound Titration	Test Compound Titration	Test Compound Titration
B																								
C																								
D																								
E																								
F																								
G	Assay Blank																							
H																								
I																								
J																								
K																								
L	Free Tracer	Positive Control																						
M																								
N																								
O																								
P																								

Important: Many compounds and the tracer used in the hERG assay are quite hydrophobic and easily carried from one well to the next on pipette tips. When adding membrane or tracer, if additions are made with a repeater pipette without tip change, it is critical to start at the lowest compound concentration and work toward higher compound concentration.

Note: We recommend adding the test compound, membrane, and tracer sequentially for optimal assay performance. While a master mix of membrane and tracer may be used, it will result in a preformed membrane-tracer complex, which will typically lengthen the amount of time required for complete competition with the test compounds and result in a lower assay window. The separate addition of membrane and tracer provides more consistent results.

Note: The solvents DMSO, methanol, and ethanol are well tolerated in the assay up to 5% of the reaction volume. If you are using other solvents or higher concentrations to prepare the test compound stock solution and master dilutions, add appropriate amounts to the Assay Buffer when preparing the Negative and Positive Controls.

1. Buffer Blanks: Transfer 20 µL per well of Predictor™ hERG FP Assay Buffer to wells A1–F1 in column 1 of the assay plate.
2. Assay Blanks: Transfer 10 µL per well of Predictor™ hERG FP Assay Buffer to wells G1–K1 in column 2 of the assay plate. Add 10 µL of Predictor™ hERG Membrane per well to wells G1–K1 in column 1 of the assay plate.
3. Free Tracer Control: Transfer 15 µL per well of Predictor™ hERG FP Assay Buffer to wells L1–P1 in column 1 of the assay plate.
4. Negative Control: Transfer 5 µL per well of Predictor™ hERG FP Assay Buffer to wells A2–H2 in column 2 of the assay plate. Add 10 µL of Predictor™ hERG Membrane per well to wells A2–H2 in column 2 of the assay plate.
5. Positive Control: Transfer 5 µL of the 4X stock of E-4031 prepared in Step 2 to wells I2–P2 in column 2 of the assay plate. Add 10 µL of Predictor™ hERG Membrane to wells I2–P2 in column 2 of the assay plate.
6. For initial instrument setup, we strongly recommend skipping to Step 11 (adding the Tracer), as noted in **Section 5.2**, and optimizing assay and instrument conditions prior to running a separate experiment in which compound titrations are included. The instrument setup run is designed to determine the G factor, observe the mP shift and calculate the Z′ (n=8).
7. Prepare a dilution series of reference compounds (*e.g.*, E-4031) and test compounds at 100X (see **Section 5.5, Dilutions of Reference and Test Compounds**). We recommend using a 384-well polypropylene plate for the dilutions (*e.g.*, Corning 3657).

   Example: For example, a dilution series of E-4031 is prepared in a 384-well dilution plate in Assay Buffer at 100X the concentrations to be assayed. This dilution series is further diluted to 4X in a separate 4X dilution plate using Predictor™ hERG FP Assay Buffer, and then transferred to the 384-well assay plate. A similar method is recommended for hydrophobic, DMSO-soluble test compounds, performing the initial dilutions in 100% DMSO at 100X. Performing the serial dilutions in 100% DSMO stabilizes IC_{50} values by minimizing problems with compounds coming out of solution while preparing the dilutions.

   a. Add 20 µl of assay buffer (or 100% DMSO in the case of test compounds dissolved in DMSO) to wells B1–P1 in a 384-well plate.

   b. Add 30 µl of 3-mM E-4031 (or a stock solution of the desired test compound at 100X) to well A1 of the same 384-well plate.

   c. Transfer 10 µl from well A1 to well B1 and mix several times by pipetting up and down.

   d. Transfer 10 µl from well B1 to well C1 and mix several times by pipetting up and down.

   e. Repeat this process through well P1.
8. Dilute compound dilution series to 4X as follows:

a. Add 48 µL of Predictor™ hERG FP Assay Buffer to each well of column 1 of a separate 4X dilution plate.

b. Transfer 2 µL of the master inhibitor dilution series from column 1 of the 100X dilution plate to column 1 of the 4X dilution plate, and mix well by pipetting up and down.

c. Repeat for all compounds to be tested.

9. E-4031 Titration: Transfer 5 µL of the 4X intermediate dilution from column 1 of the 4X dilution plate to columns 3–4 of the assay plate. Add 10 µL of Predictor™ hERG Membrane to all wells in column 3–4 of the assay plate.

10. Test compounds: Transfer 5 µL of the compounds in the 4X intermediate dilution plate into the test compound wells on the assay plate (columns 5–24, as determined by user). Repeat for as many compounds as have been prepared. Alternatively, add the test compounds to the plate using your preferred dilution method. Add 10 µL of Predictor™ hERG Membrane to all wells containing test compounds.

11. Tracer: Dispense 5 µL of the 4X Predictor™ hERG Tracer prepared in step 2 to wells L1–P1 in column 1 and to all other wells utilized on the plate. Do not dispense this reagent into wells designated for the buffer blank or the assay blank (wells A1–K1 in column 1).

12. Cover the assay plate to protect the reagents from light and evaporation, and incubate at room temperature (20–25°C) for at least 2 hours prior to measuring fluorescence polarization. See the Appendix for guidelines on the effect of incubation time on assay performance. You may wish to take multiple reads over a period of time to ensure that binding of the test compound has reached equilibrium.

5.7 Quick Reference Guide

Table 5: Quick Reference Guide of Step-by-Step Instructions

Step 1 Thaw reagents	Thaw Predictor™ hERG tracer, E-4031, and membranes at room temperature.						
Step 2 Pipette membrane	Mix Predictor™ hERG membranes by pipetting ~20X before use.						
Step 3 Prepare tracer dilution	Prepare 4X tracer. For ¼ of a 384–well plate, add 10 µL of Predictor™ hERG tracer to 615 µL of Assay Buffer (1:62.5 dilution).						
Step 4 Prepare E-4031 Positive Control Dilution	Prepare 4X (120 µM) E-4031 for positive control. For ¼ of a 384-well plate, add 20 µL of E-4031 to 480 µL of Assay Buffer (1:25 dilution).						
Step 5 Prepare test compound and E-4031 dilutions	Note: We recommend running an E-4031 titration curve and test compounds only after instrument set-up and assay performance have been verified.						
	Buffer Blank	**Assay Blank**	**Free Tracer Control**	**Negative Control**	**Positive Control**	**E-4031 Titration**	**Test Compounds**
Step 6 Transfer assay buffer, test compound, or E-4031 dilutions	20 µL Assay Buffer	10 µL Assay Buffer	15 µL Assay Buffer	5 µL Assay Buffer	5 µL 4X E-4031 (120 µM)	5 µL 4X E-4031 Titration	5 µL 4X Test Compound Titration
Step 7 Transfer hERG membrane		10 µL hERG membrane		10 µL hERG membrane	10 µL hERG membrane	10 µL hERG membrane	10 µL hERG membrane
Step 8 Transfer tracer			5 µL 4X Tracer	5 µL 4X Tracer	5 µL 4X Tracer	5 µL 4X Tracer	5 µL 4X Tracer
Step 9 Develop assay plate 2+ hours	Cover the assay plate to protect the reagents from light and evaporation, and incubate at room temperature (20–25°C) for at least two hours (see appendix for more information on assay performance and incubation time) prior to measuring fluorescence polarization						
Step 10 Read Assay	Read fluorescence polarization as indicated in **Section 5.2**.						

6. Fluorescence Polarization Measurements

6.1 Filter and Mirror Requirements

The excitation and emission spectra of Predictor™ hERG Tracer Red in assay buffer are shown in **Figure 6**. The tracer is characterized by an excitation peak at 540 nm and an emission peak at 573 nm. Polarized filter sets that are commonly used for measuring fluorescence polarization of tetramethylrhodamine or Cy3-type ("red") fluorophores will work well, such as those with excitation and emission centered at approximately 530 nm and 590 nm, respectively. Filters must be designated as FP filters. Non-polarized filters are not suitable for reading the Predictor™ hERG assay.

Some monochromator (non-filter based) readers such as the Tecan Safire²™ or Infinite® M1000 can be configured as an option or upgraded to perform FP assays. If you are uncertain of the ability of your plate reader to read the Predictor™ hERG assay, please contact Invitrogen Technical Support at 1-800-955-6288, option 3 and enter 40266, or e-mail us directly at drugdiscoverytech@invitrogen.com.

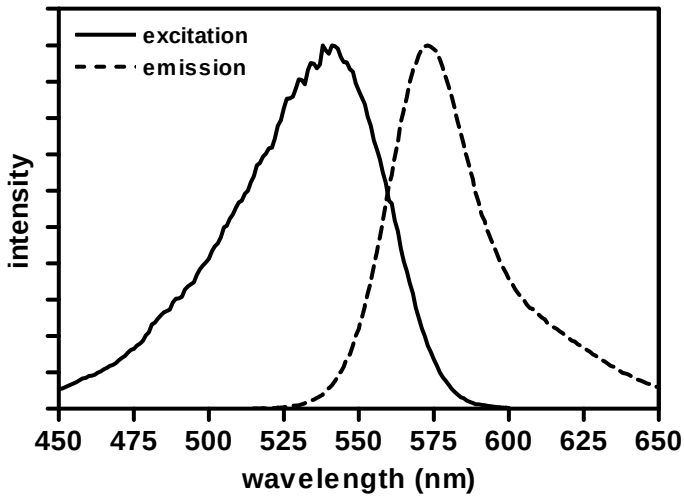


Figure 3. Excitation and emission spectra of Predictor™ hERG Tracer Red.

6.2 Recommended Settings for Common Plate Readers

The Predictor™ hERG fluorescence polarization assay has been performed successfully on a range of commonly-used plate readers from Tecan (Safire²™, Infinite® F500, Infinite® M1000), PerkinElmer (EnVision®), BMG LABTECH (PHERAstar), BioTek Instruments (Synergy™ 2, Synergy™ 4) and Molecular Devices (Analyst®). For instrument specific set-up guides visit our instrument web portal at http://www.invitrogen.com/instrumentsetup. See the table below for recommended instrument settings for these plate readers. The dichroic mirror required for filter-based instruments is the stock mirror recommended and supplied by the instrument manufacturer. All of the data presented in the customer protocol was generated using a Tecan Safire²™.

Instrument	Type	Flashes or Reads/well	Excitation Center/ Bandwidth (nm)	Emission Center/ Bandwidth (nm)
Safire²™	Monochromator	10	530/20	585/20
Infinite® F500	Filter-based	10	535/25	590/20
Infinite® M1000	Monochromator	10	530/5	585/20
EnVision®	Filter-based	≥60	531/25	595/60
PHERAstar	Filter-based	200	540/20	590/20
Synergy™ 2/ Synergy™ 4	Filter-based	≥150	530/25	590/35
Analyst®	Filter-based	10	530/25	580/10

Table 6. Recommended instrument settings for plate readers available from Tecan (Safire²™, Infinite® F500, Infinite® M1000), PerkinElmer (EnVision®), BMG LABTECH (PHERAstar), BioTek Instruments (Synergy™ 2, Synergy™ 4) and Molecular Devices (Analyst®).

6.3 Gain Settings and Z-Position

An appropriate instrument gain setting and Z-position (the optimum distance between the assay plate and instrument optics, adjustable on most instruments) can be determined from any assay well containing tracer. We recommend using a Negative Control well (A2–H2 of column 2) for this purpose following the instrument manufacturer's recommendations.

6.4 G-Factor Calibration

Polarization values are standardized by setting the instrument G-factor. To do this, assign a value of 50 mP to wells containing tracer in the absence of membrane, Free Tracer Control (wells L1–P1 of column 1). The Buffer Blank (wells A1–F1 in the first column) is defined as the blank or reference for G-Factor Calibration.

Often the instrument G-factor can be determined once on a particular instrument for a given combination of tracer and filter sets, and then applied to future measurements. Redetermine the G-factor if the instrument has been serviced or moved. In general, the G-factor shifts the mP values up or down the Y-axis without large effects on IC_{50} value calculations. Negative mP readings indicate that the G-factor needs to be set.

Note: Once the G-factor is set, the Buffer Blank wells (wells A1-F1 of column 1) are no longer used. For subsequent reads using the plate, use the Assay Blank (wells G1–K1 of column 1) as the "blank" and redefine the Buffer Blanks and Free Tracer wells as "samples". The mP values from wells without tracer should be disregarded.

6.5 Reading the Assay Plate

After the gain, Z-position, and G-factor have been set, reread the assay plate to obtain your mP values. The Assay Blanks (wells G1-K1 of column 1) should be used to subtract the background fluorescent intensity prior to calculating polarization values. On most instruments this can be done via the accompanying software by designating the appropriate assay wells as Assay Blanks.

Note: The Buffer Blank (wells with assay buffer only) should not be substituted for the Assay Blank.

Note: Because the membrane adds viscosity to the solutions in the assay wells, the mP value of displaced tracer in the Positive Control wells will be above that of Free Tracer alone in buffer.

For step-by-step instrument set-up guides, go to, www.invitrogen.com/instrumentsetup and contact our support for Drug Discovery Products at 760-603-7200, option 3, extension 40266 or e-mail us directly at drugdiscoverytech@invitrogen.com.

Using the data obtained from the readings in column 2, the product performance specification is an average shift of 120 mP and Z′ greater than 0.5 in five hours after adding tracer and mixing.

7. Appendix

7.1 Reagent Order of Addition and Incubation Time

See the table below for sample equilibration data generated using separate reagent additions as outlined in the procedure in **Section 5**. Binding of the tracer to the hERG channel typically requires approximately 4 hours to reach equilibrium at room temperature (20–25°C), though at earlier timepoints the assay window and Z′-factor are still robust. We recommend an incubation time of at least 2 hours.

Z′-factors and assay windows (ΔmP) listed in the table below were determined using the polarization values from 16 wells of negative and positive controls. Z′-factors were calculated using the method of Zhang *et al.* (1999), and are an indication of the robustness of the assay. Values of greater than 0.5 are generally considered good, while a value of 1 indicates a theoretically ideal assay with no variability. The small IC_{50} value changes seen with longer incubation times likely indicate a complex equilibrium between tracer, specific binding sites, and membrane that we have not studied further. We recommend that you determine the optimal incubation time for your workflow; data consistently collected at a given timepoint will yield a solid basis for comparison of compounds across experiments.

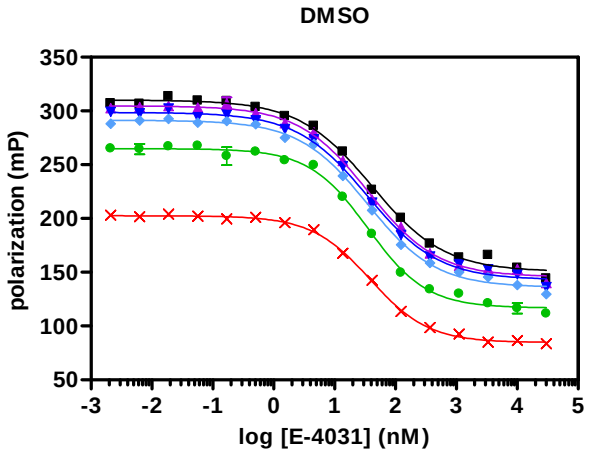
Incubation Time (hrs)	E-4031 IC_{50} (nM)	Assay Window (ΔmP)	Z′-Factor
1	23	111	0.56
2	32	138	0.68
3	35	147	0.74
4	39	153	0.77

Table 7. Performance of the assay with varying incubation times.

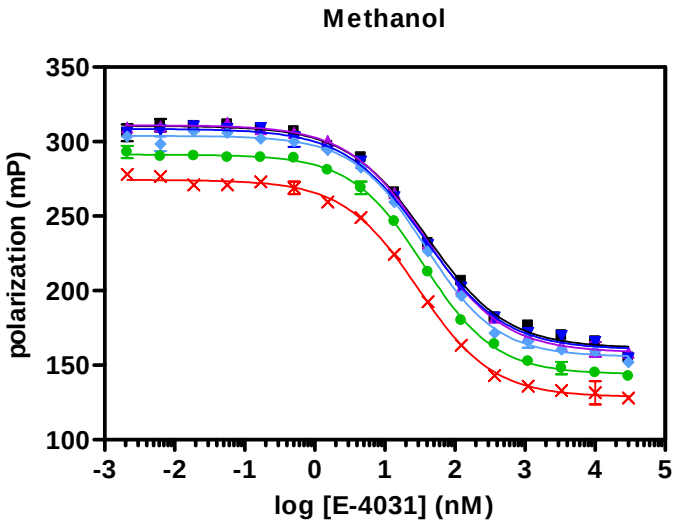
7.2 Solvent Tolerance

The Predictor™ hERG Fluorescence Polarization Assay is compatible with organic solvents commonly used to dissolve test compounds. The assay will tolerate a final concentration of DMSO, methanol, or ethanol of up to 5% (v/v) (see **Figure 2**). Above 5%, the assay window and Z′-factor may suffer.

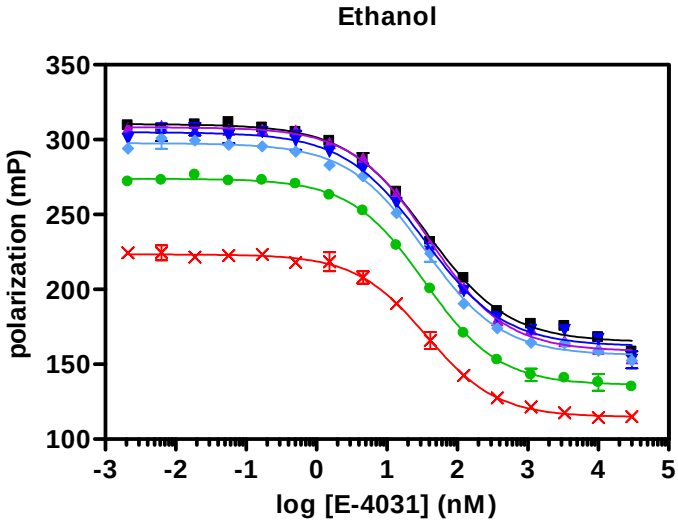
Note that while parameters such as IC_{50}, ΔmP, and Z′-factor may not change appreciably in the presence of organic solvents, the exact polarization values (the upper and lower bounds of the assay) may be affected. In the case of the solvents tested here, at 10% of the reaction volume, a reduction in polarization values was observed.



	IC_{50} (nM)	ΔmP	Z'-factor
no DMSO	41	164±3	0.92
0.5% DMSO	35	157±4	0.89
1% DMSO	35	161±4	0.90
2% DMSO	35	157±5	0.88
5% DMSO	35	157±3	0.92
10% DMSO	38	116±7	0.75



	IC_{50} (nM)	ΔmP	Z'-factor
no MeOH	40	157±4	0.90
0.5% MeOH	38	154±5	0.86
1% MeOH	37	157±4	0.90
2% MeOH	37	158±4	0.88
5% MeOH	35	161±4	0.89
10% MeOH	30	151±7	0.83



	IC_{50} (nM)	ΔmP	Z'-factor
no EtOH	37	155±4	0.90
0.5% EtOH	39	150±4	0.89
1% EtOH	33	151±5	0.87
2% EtOH	34	150±5	0.87
5% EtOH	34	144±2	0.93
10% EtOH	36	110±8	0.73

Figure 4. Effect of commonly used organic solvents on assay performance.

7.3 Concentration Response Curves

7.3.1 Number of Data Points and Data Analysis

We recommend 16-point, half-log interval concentration-response curves with 3 points at the top and bottom of the curves to fully define the curves. See **Figure 5**, E-4031, Full Data, No Constraints. We also recommend that the top and bottom of the curves be constrained to the values obtained from E-4031. See for example amitryptyline, which as a weak hERG blocker, does not have a fully defined bottom of the curve with a 16-point titration.

Constraining the bottom and the top and bottom of the curve to the E-4031 value defines the curve and allows for accurate IC_{50} determination, as shown in **Figure 5**. A small number of data points over only a few logs of concentration yields poor data, even with the top and bottom of the curves constrained to the E-4031 values (Partial Data, **Figure 5**). With Partial Data, some IC_{50} values will be unaffected while others will be significantly shifted (see the comparison of E4031 and amitriptyline in **Figure 5**).

A small number of data points spread out over 6 logs of concentration followed by constraint of the top and bottom of the curves to the E-4031 values allows good determination of IC_{50} values (Spread

Data in **Figure 5**). Constraint is done in the curve-fitting software by forcing the top and bottom of the curve to equal the mP values for the positive control E4031.

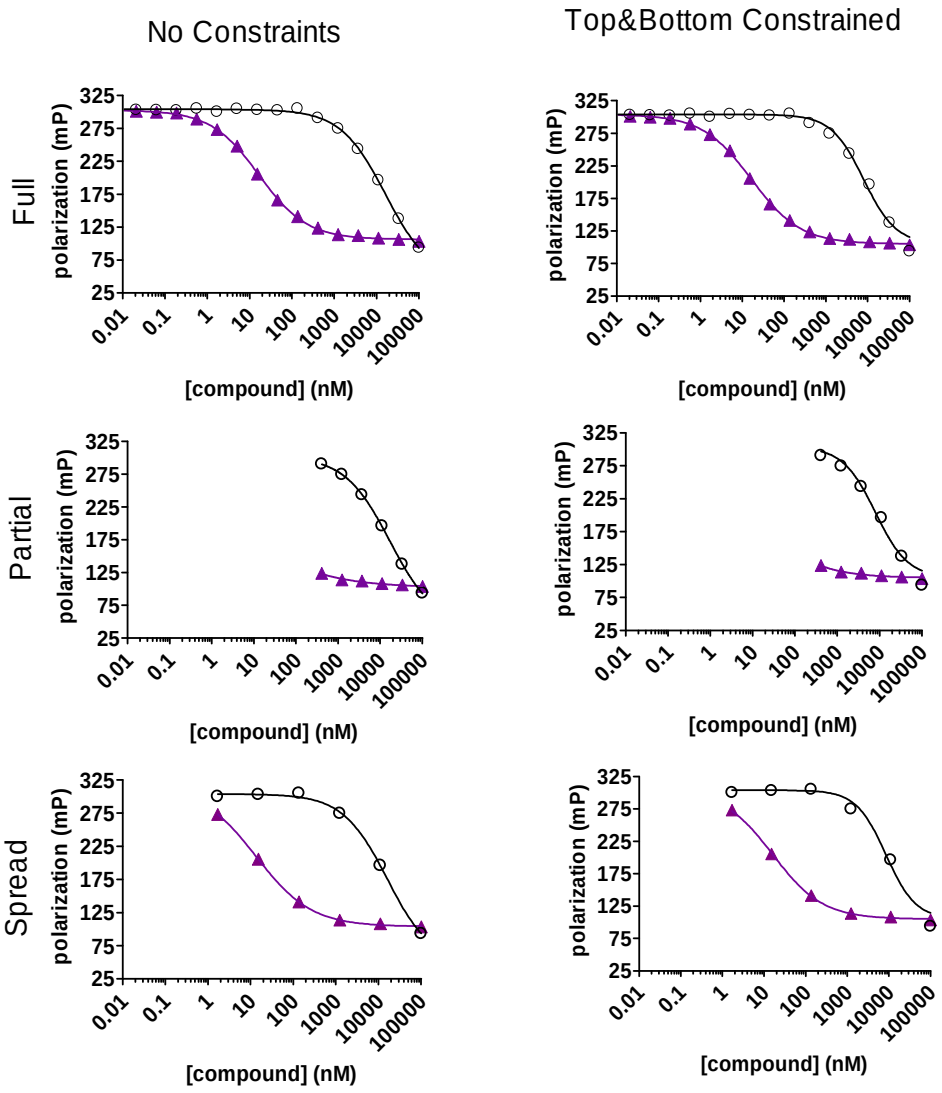


Figure 5. Graphs of concentration-response curves obtained with differing numbers of data points over varying orders of magnitude. We recommend that the titrations cover 6 orders of magnitude, 16-point ½-log intervals preferred, and that the top and bottom of the curves be constrained to the high and low mP values obtained from E-4031. Triangles represent E-4031, open circles represent amitriptyline.

E-4031 IC_{50} (nM)		
Titration	**No Constraints**	**Top and Bottom Constrained**
Full	16.5	16.5
Partial	0.04	9.7
Spread	13.6	16.3
Amitriptyline IC_{50} (nM)		
Titration	**No Constraints**	**Top and Bottom Constrained**
Full	16220	7942
Partial	16891	7931
Spread	16164	8472

Table 8: Summary of the IC_{50} values obtained from the experiment shown in **Figure 5** with the constraints listed.

7.3.2 Data Corrections

Precipitates from certain test compounds at higher concentrations can cause light scatter in FP experiments. Light scatter is highly polarized. In a dose response curve, one observes the usual decline in mP values for compounds that displace tracer, but then the mP values rise again at higher compound concentrations (see **Figure 6**).

Higher concentrations (>1 µM) of certain compounds such as astemizole (see **Figure 7**) exhibit an additional, non-hERG-specific reduction in the observed polarization value of the tracer, resulting in polarization values below those values seen for the highest concentrations of E-4031. The mP values can approach that of the Free Tracer control. This reduction in the mP reading is a non-hERG specific effect, since membranes lacking the hERG channel protein show this same reduction in the mP value.

Either case—precipitates causing light scatter and non-hERG specific increase in the polarization values or compounds causing non-hERG specific reductions in polarization values—can be corrected by one of two methods.

Correction Method A

The first method for correcting polarization values is to "fix" the top and the bottom of the dose-response curve fit to the polarization value of the positive control wells. Since in general we recommend fixing both the top and bottom of the curve to the mP values obtained for E4031, the effect of compounds that show non-hERG effects has already been corrected.

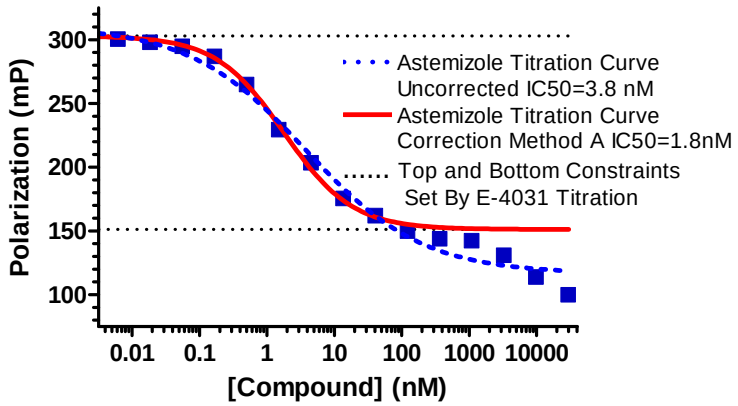


Figure 6: Concentration response curve for astemizole showing non-hERG specific effects beginning at ~500 nM. Uncorrected curve and a curve corrected by Method A are plotted.

Correction Method B

The second option is to set up two dose-response curves for a test compound—one in the presence of saturating control inhibitor (*e.g.*, 30 µM E-4031) and one in the absence of control inhibitor. This can be accomplished by adding 60 µM E-4031 to a portion of the 2X membrane prior to its addition to test compound. This concentration of E-4031 provides a saturating amount of inhibitor, but does not cause an additional non-hERG specific effect on tracer polarization. Any non-specific reductions in polarization due to test compound can then be observed.

This type of correction is analogous to the subtraction of non-specific binding as is done in a radioligand binding assay.

For more information on this method, see Piper, DR, et al., or contact our technical support department for further information.

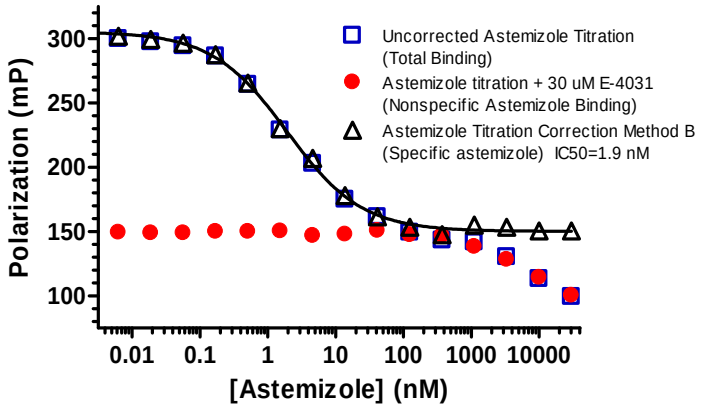


Figure 7. Concentration response curve for astemizole showing non-hERG specific effects beginning at ~500 nM. Uncorrected curve and a curve corrected by Method B are plotted.

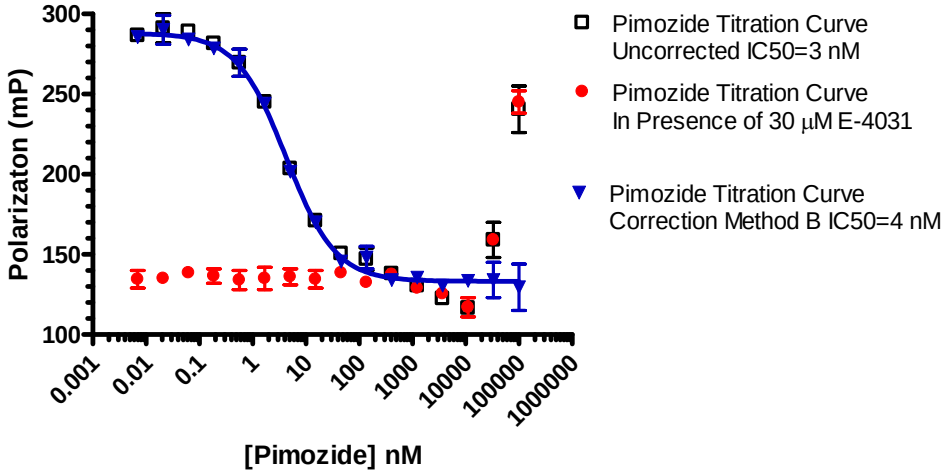


Figure 8: Concentration response curve for pimozide showing non-hERG specific effects due to precipitation at higher concentrations beginning at ~500 nM. Uncorrected curve and a curve corrected by Method B are plotted.

8. References

Piper, D.R., Duff, S.R., Eliason, H.C., Frazee, W.J., Frey, E.A., Fuerstenau-Sharp, M., Jachec, C., Marks, B.D., Pollok, B.A., Shekhani, M.S., Thompson, D.V., Whitney, P., Vogel, K.W., and Hess, S.D. (2008) Development of the Predictor hERG fluorescence polarization assay using a membrane protein enrichment approach. *Assay Drug Dev Technol*. 6(2): 213–23.

Zhang, J.H., Chung, T.D., and Oldenburg, K.R. (1999) A simple statistical parameter for use in evaluation and validation of high-throughput screening assays. *J. Biomol. Screen.* 4: 67–73.

9. Limited Use Label Licenses

Limited Use Label License No: 5 Invitrogen Technology

The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for-profit entity). The buyer cannot sell or otherwise transfer (a) this product (b) its components or (c) materials made using this product or its components to a third party or otherwise use this product or its components or materials made using this product or its components for Commercial Purposes. The buyer may transfer information or materials made through the use of this product to a scientific collaborator, provided that such transfer is not for any Commercial Purpose, and that such collaborator agrees in writing (a) not to transfer such materials to any third party, and (b) to use such transferred materials and/or information solely for research and not for Commercial Purposes. Commercial Purposes means any activity by a party for consideration and may include, but is not limited to: (1) use of the product or its components in manufacturing; (2) use of the product or its components to provide a service, information, or data; (3) use of the product or its components for therapeutic, diagnostic or prophylactic purposes; or (4) resale of the product or its components, whether or not such product or its components are resold for use in research. For products that are subject to multiple limited use label licenses, the most restrictive terms apply. Invitrogen Corporation will not assert a claim against the buyer of infringement of patents owned or controlled by Invitrogen Corporation which cover this product based upon the manufacture, use or sale of a therapeutic, clinical diagnostic, vaccine or prophylactic product developed in research by the buyer in which this product or its components was employed, provided that neither this product nor any of its components was used in the manufacture of such product. If the purchaser is not willing to accept the limitations of this limited use statement, Invitrogen is willing to accept return of the product with a full refund. For information on purchasing a license to this product for purposes other than research, contact Licensing Department, Life Technologies, 5791 Van Allen Way, Carlsbad, California 92008. Phone (760) 603-7200. Fax (760) 602-6500. Email: outlicensing@invitrogen.com.