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Application Note

# Invitrogen's Predictor™ hERG Fluorescence Polarization Assay Using BioTek's Synergy™ 4 Hybrid Microplate Reader

## Non-Radioactive High Throughput Screening Assay for hERG Binding

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*The hERG channel is one of a family of ion channels shown to be important in the regulation of cardiac rhythm. Compounds that block this ion channel have been shown to increase the QT interval and lead to cardiac arrhythmias. Because there is no way to predict from a compound's structure whether or not it will block the hERG ion channel, potential drug compounds are screened as early as possible in the R&D process. Here we describe measuring Invitrogen's fluorescence polarization based Predictor™ assay with a Synergy 4 Hybrid Microplate Reader from BioTek.*

## Introduction

The hERG is the human homolog of the Ether-a-go-go gene found in Drosophila in the 1960s by William Kaplan [1]. This potassium channel consists of 4 identical subunits, each containing six transmembrane domains. Abnormalities in this channel have been associated with both Long QT and Short QT syndrome, both potentially fatal cardiac arrhythmias, depending on the abnormality. The hERG channel has been shown to be the target for class III antiarrhythmic drugs such as amiodarone, which reduce the risk of re-entrant arrhythmias by prolonging the AP duration and refractory period without slowing conduction velocity in the myocardium. This channel is also quite sensitive to drug binding, without any predictability based on structure, resulting in elongated QT intervals. Due to the potential for complications with increased QT intervals, regulatory agencies have issued recommendations for the evaluation of potential drugs in the preclinical stages of development. Typically, potential drug compounds are tested using a patch clamp electrophysiology test.

Fluorescence polarization (FP) is a fluorescence detection technique first described in 1926 by Perrin [2]. It is based on the observation that fluorescent molecules in solution, when excited by polarized light, emit polarized light, albeit the plane of emitted light will be different than that of the excitatory light due to molecular rotation. A molecule's polarization is inversely proportional to the molecule's rotational speed, which is influenced by solution viscosity, absolute temperature, molecular volume and the gas constant. If one keeps viscosity and temperature constant, then the key variable for rotational speed differences is molecular volume or molecular weight.

The Predictor™ hERG kit from Invitrogen is a homogeneous fluorescent assay that uses a simple add-and-read format.

The assay is based on the principle of fluorescence polarization where a red fluorescent tracer is displaced from the hERG channel by compounds that bind to the channel. Assay performance is validated using established hERG channel blockers.

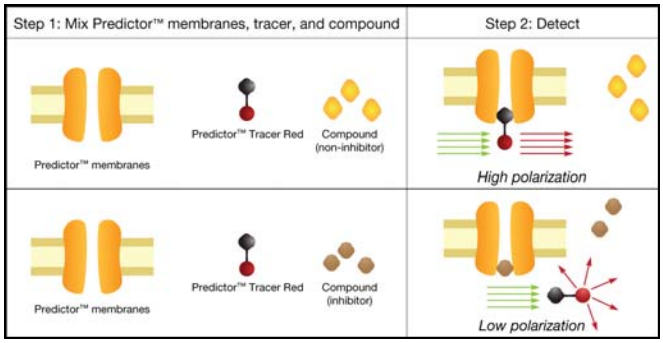


**Figure 1. Illustration of the Principle of the Predictor™ hERG Fluorescence Polarization Assay.**

## Materials and Methods

Predictor hERG Assay test kits were obtained from Invitrogen Carlsbad, CA). The binding assay was carried out according to the kit instructions. Briefly, reagents were thawed and Predictor hERG membrane preparations were sonicated. Working 4X tracer was prepared by diluting the 250 nM stock provided in the test kit to 4 nM with hERG FP assay buffer also provided. Dilutions of test compounds were prepared as a 4X stocks from their final intended concentrations. Assays were performed in 384-well microplates. Aliquots (5 µL) of each concentration of test compound were pipetted into the appropriate wells of the microplate. As required, 10 µL of (2X) membrane preparation was then dispensed. Working Tracer solution (5 µL) was then added and the plate was allowed to incubate at room temperature for at least one hour


 

prior to measuring fluorescence polarization. Fluorescence polarization measurements were made using a Synergy™ 4 Hybrid Microplate Reader from BioTek Instruments (Winooski VT). Measurements were made from the top using the tungsten light source. Both parallel and perpendicular fluorescence were measured using the same 530/25-excitation and a 590/35-emission filters along with a 570 nm cut off dichroic mirror. The PMT sensitivity setting was set automatically such that the positive control well had a raw fluorescence value for the parallel signal of 50,000 relative fluorescent units (RFU). Polarization values were calculated automatically using Gen5™ Data Analysis Software (BioTek Instruments).

## Results

Increasing concentrations of the hERG-binding compound, astemizole, demonstrates a marked change in fluorescence polarization (Figure 2). Astemizole binds to the hERG ion channel and displaces the fluorescent tracer molecule. As more tracer is displaced and free to rotate freely in solution the polarization decreases. Using a semilog plot a sigmoidal shaped response curve is observed (Figure 2).

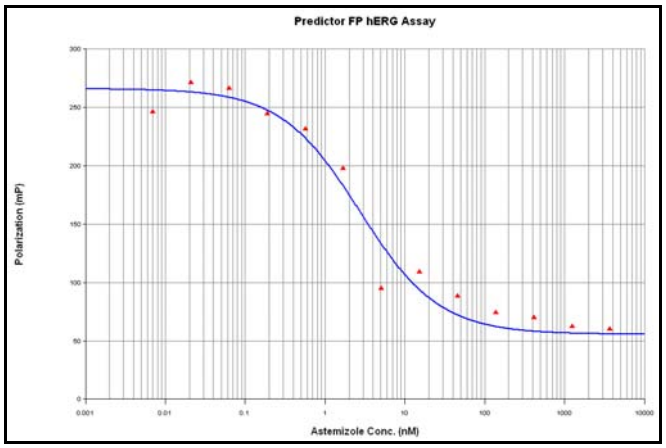


**Figure 2. Astemizole Titration Curve.** Following incubation of test compound and Predictor™ kit constituents, fluorescence polarization is measured and plotted against compound concentration. Data points represent the mean of three determinations.

Comparison of compounds to known hERG binding molecules can provide information as to whether or not prospective drugs will have this side effect. As demonstrated in Figure 3 a dose comparison of E410 with astemizole, a known hERG binding molecule, has a 10 fold greater EC50. While this indicates that this compound is less likely to bind than astemizole, the fact that it displaces the tracer indicates that it is a hERG-binding molecule.

## Discussion

These data demonstrate the ability of the Synergy 4 to provide the fluorescence polarization measurements necessary for the Predictor™ hERG Binding assay. The Synergy 4 is a modular reader that combines a highly sensitive fluorescence capability with light polarizers. All of the necessary Gen5 Data Analysis Software automatically subtracts the blank wells and calculated polarization values form the parallel and perpendicular fluorescence measurements.

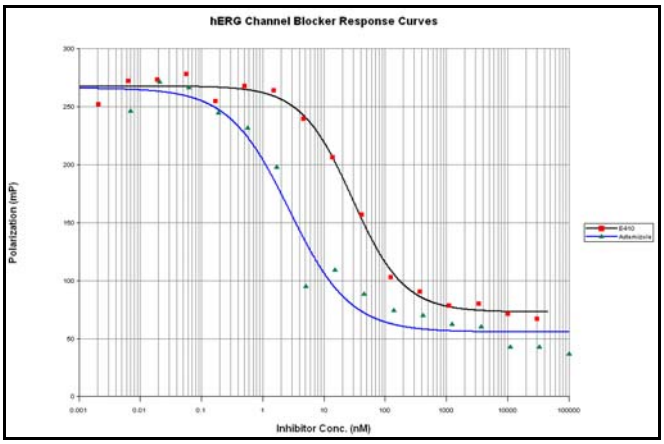


**Figure 3. Comparison of potential drug compound to Astemizole.** Following incubation of test compounds and Predictor™ kit constituents, fluorescence polarization is measured and plotted against compound concentration. Data points represent the mean of three determinations.

Traditionally hERG screening is performed with a whole cell voltage patch clamp apparatus. This method is considered the "gold standard" in regards to testing ion channels. Comparison of the Predictor™ hERG FP assay to the patch clamp method has previously been shown to correlate very closely. While the patch clamp method works very well for identifying compounds that block potassium ion flow through hERG channels, it requires a high resistance seal be made with a single cell at a time. In the presence of different amounts of drug, a voltage step is applied and the current recorded. This procedure is repeated for different drug concentrations in order to determine what drug concentration results in a half-maximal response (EC50). This makes screening numerous compounds tedious, expensive and almost impractical. The Predictor™ hERG Fluorescence Polarization test kit provides the ability to screen numerous compounds easily. Its use of the 384-well microplate format allows multiple replicates at each drug concentration for multiple drugs to be tested on a single plate.

The Predictor hERG kit consists of all of the necessary components to run the assay including: validated hERG membrane preparations; hERG red tracer; a positive control hERG channel blocker and the necessary associated buffers. The Synergy 4 Hybrid Microplate Reader and Gen5 Data Analysis Software provide superior fluorescence polarization detection performance, as well as data analysis and storage.

## References

[1] Jan, L.Y. and Jan, Y.N. (1990). A superfamily of Ion Channels. Nature, 345:672.

[2] Perrin, F. (1926) Polarization de la Lumiere de Fluorescence. Vie Moyenne de Molecules dans L'etat Excite. J. Phys Radium 7:390.