Automation of Predictor™ hERG Fluorescence Polarization Assay using Precision™ Automated Pipetting System for Serial Dilution of Small Molecule Drugs for IC_{50} Determinations

BioTek
Booth #152

invitrogen™
Booth #114

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Abstract

The vast majority of drugs associated with acquired QT prolongation are known to interact with hERG. Due to the awareness of the potential danger of such QT drugs, the regulatory authorities issued recommendations for the establishment of cardiac safety testing during preclinical drug development. Traditionally, lead compounds in late stage preclinical studies were tested for hERG binding using electrophysiology. These are laborious methods, requiring significant skill in the end-user to perform a successful assay. Furthermore, many researchers wish to test lead compounds for safety earlier in the process of drug development. This requires a higher throughput type of assay. Here we describe methods for the automation of hERG screening using Invitrogen's fluorescence polarization based Predictor™ assay using the Precision™ Automated Pipetting System for the serial dilution of known inhibitors of hERG to generate IC50 data. FP-based IC50 data will be compared to electrophysiology and radiometric data.

Introduction

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. This channel is also quite sensitive to drug binding, without any predictability based on structure, resulting in prolonged QT intervals. Typically, potential drug compounds are tested using a patch clamp electrophysiology test, which is considered the gold standard, but is also slow and costly. Radiometric assays are often employed as an alternative to patch clamp, but they are labor intensive and require special treatment due to the use of radiation.

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.

Predictor™ hERG Fluorescence Polarization Assay

The Predictor™ hERG kit from Invitrogen is a homogeneous fluorescence 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.

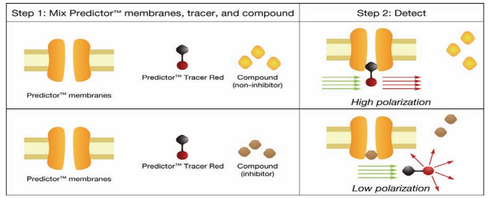

Figure 1 - Illustration of the principle of the Predictor™ hERG Fluorescence Polarization Assay.

BioTek Instrumentation

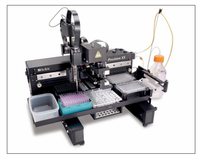

Figure 2 - Precision™ XS Microplate Sample Processor.

The Precision™ XS combines a single-channel sample processing head, an 8-channel pipetting head and an 8-channel bulk reagent dispenser in one instrument. The instrument was used to serially titrate five compounds 1:5 across a 96-well plate. 12 µL aliquots of each compound concentration were then transferred to the 384-well assay plate in quadruplicate.

Figure 3 - Synergy™ 4 Multi-Mode Microplate Reader.

The Synergy™ 4 Multi-Mode Reader 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 from the parallel and perpendicular fluorescence measurements. Its top optical head can focus up and down on the samples with a 100 µm resolution.

Precision™ XS Automated Methods

Compound Titration Method Deck Setup

1. Place 1000 µL of 10% DMSO into wells A1-D1 of a 96-well deep well microplate at position F of the deck.
2. Place 1000 µL of the positive control into well E1 and 1000 µL of the negative control into wells F1 and H1 of the same 96-well deep well microplate.
3. Place 100 µL of 200 µM compound into wells A1-D1 of a 96-well round bottom microplate at position E of the deck.
4. Place 100 µL of the positive control into well E1 and 100 µL of the negative control into wells F1 and H1 of the same 96-well round bottom microplate.
5. Place 200 µL tip boxes at positions A and D of the deck.
6. Place an empty Corning 384-well black flat bottom microplate at position B of the deck.

Compound Titration Automated Method

1. Transfer 80 µL of 10% DMSO and controls from the 96-well deep well microplate to columns 2-12 of the 96-well microplate.
2. Aspirate 20 µL of the highest concentration of test compound from column 1, dispense to column 2, and perform a 10X mix.
3. Repeat the procedure for columns 2-11, leaving column 12 as a no compound control.
4. Transfer four 12 µL aliquots from each well of the 96-well microplate to the 384-well assay plate.

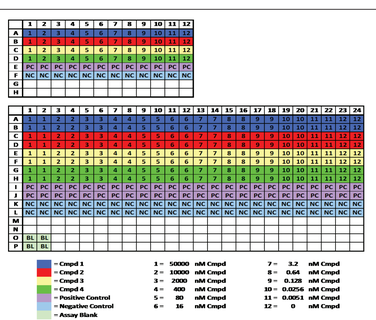

Figure 4 - Compound and control layouts for 96-Well Compound Microplate and 384-Well Assay Plate used during Precision XS automated methods.

Reagent Addition Method Deck Setup

1. Place 700 µL of Predictor™ Membrane into wells A1-F2 of a 96-well deep well microplate at position E of the deck.
2. Place 750 µL of Predictor™ Tracer Red into wells A3-F3 of the same 96-well deep well microplate.
3. Place 150 µL of Predictor™ Membrane into well A4, and 150 µL of Predictor™ hERG FP Assay Buffer into well B4 of the same 96-well deep well microplate.
4. Place 200 µL tip boxes at positions A, D and F of the deck.
5. Place the Corning 384-well black flat bottom assay microplate, containing 12 µL of compound or control, at position B of the deck.

Reagent Addition Automated Method

1. Transfer 24 µL of Predictor™ Membrane from columns 1 and 2 of the 96-well deep well microplate, to all compound and control wells of the 384-well assay microplate, using the 8-channel pipette.
2. Transfer 12 µL of Predictor™ Tracer Red from column 3 of the deep well microplate to all assay microplate wells except the blank, using the 8-channel pipette.
3. Transfer 24 µL of Predictor™ Membrane from well A4 of the deep well microplate to all assay blank wells using the single channel pipette.
4. Transfer 12 µL of Predictor™ FP Assay Buffer from well B4 of the deep well microplate to all assay blank wells using the single channel pipette.
5. Shake the microplate for 1 minute on an orbital shaker, incubate at RT for 2 hours.

Synergy™ 4 Read Parameter Optimization

The assay plates were read on Synergy™ 4 controlled by Gen5™ software using a filter set of 530/25 excitation and 590/35 emission with a 570 cutoff dichoric mirror in fluorescence polarization mode. Sensitivity was entered at 150, and the microplate was read with a number of reads per well of 150. The microplate was read from the top using the Tungsten lamp, and filter switching per well was employed. Gen5™ software automatically blanks the microplate on the designated blank wells and calculates fluorescence polarization data reported in mP. A data view of standard deviations and CV% calculations for each data set is also provided as a default within the protocol. Gen5™ software can also be used to calculate the assay window, Z'-factor, and generate a curve fit with associated IC_{50} values. For purposes of this experiment the Gen5™ generated data was exported to a Microsoft Excel® spreadsheet and then GraphPad Prism® was used to represent results of the assay.

Predictor™ hERG Assay Validation

Assay performance was validated using five established hERG channel blockers chosen for their broad range of expected IC_{50} values. Astemizole was run first to gauge accuracy and precision of the automation. The four other compounds: Dofetilide, Haloperidol, E-4031, and Thioridazine were run on a second microplate with the addition of 48 replicates of both the negative and positive controls to determine the Z' and assay window for the run. The controls were prepared according to the kit instructions using a final concentration of 2.5% DMSO. An assay window of 156 is typical for this assay at an incubation time of 2 hours (see Predictor™ kit manual). Z'—factors were calculated using the method of Zhang *et al.* [3]. Z' values in a range greater than 0.5 are generally considered good, while a value of 1 indicates a theoretically ideal assay with no variability [4].

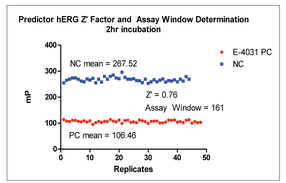

Figure 5 - Z'-Factor and assay window results.

Results of IC_{50} Determination

Experimental Setup

1. Use the Precision™ XS to titrate test compounds, add reagents, and transfer to the 384-well assay plate.
2. Read FP on Synergy™ 4 and compute IC_{50} and assay validation values.

IC_{50} Results

Compound	Automation	Patch Clamp	Radioligand
Astemizole	4.51	1.2	1
Dofetilide	9.47	12	40
E-4031	25.6	48	20
Haloperidol	194	174	90
Thioridazine	827	1250	510

Table 1 – IC_{50} values for compounds automated with Precision XS and Synergy 4.

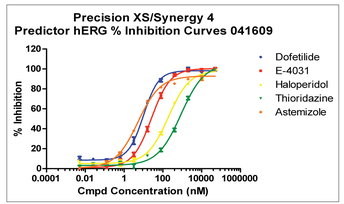

Figure 6 – hERG % inhibition curves for five compounds automated with Precision XS and Synergy 4.

Conclusions

1. The Precision™ XS provides an automated pipetting method to deliver accurate compound titrations for IC_{50} determination, and load compound and reagents to a 96-well or 384-well plate.
2. The sensitivity of the optical system in the Synergy™ 4 is able to dependably read fluorescence polarization from a 48 µL reaction in 96-well or 384-well format.
3. Automation of the Predictor assay using Precision XS and Synergy 4 provides hERG inhibition data that agrees with established literature values for the patch clamp method.

[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.

[3] Zhang, J.H., T.D. Chung, and K.R. Oldenburg. (1999) Journal of Biomolecular Screening 4(2):67-73.