Analysis of a New High Throughput Screening Detection Technology for Rapid hERG Safety Testing using a Fluorescence Polarization Assay

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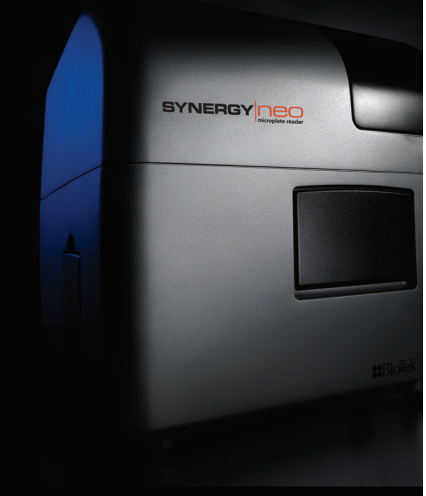

The vast majority of drugs associated with acquired QT prolongation are known to interact with hERG. Due to the awareness of the potential danger of such QT drugs, regulatory authorities issued guidelines for cardiac safety testing during preclinical drug development. The gold standard test method, traditionally done on lead compounds in late stage preclinical studies, is done by manual electrophysiology as approved within the regulatory guidelines. This laborious method requires 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 a new detection platform suitable for High Throughput Screening (HTS) applications using a fluorescence polarization based hERG inhibition assay as a model. By means of assay controls and a panel of multiple hERG inhibitors, analysis of pharmacology endpoints for instrument validation include Z', assay window, precision and plate read times. Additionally, FP-based IC_{50} data is generated and compared to electrophysiology and radiometric data.

Key Words:

Fluorescence Polarization

Drug Discovery

Drug Development

HTS

Drug Safety Testing

hERG

Small Molecule Binding

Cardio Toxicity

ADME/Tox

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Introduction

A manual patch-clamp electrophysiology platform is the gold standard method used for reporting GLP compliant hERG safety testing as part of an Investigational New Drug (IND) submission. Recent advances in automated patch-clamp technology (APC) have begun to show promise of becoming an alternative compliant test to the gold standard manual method. Although APC technologies provide the higher throughput advantages of automation and somewhat lower cost per test, patch-clamp technologies remain slow, costly, and difficult to perform.

Radioligand binding assays have also been introduced for hERG liability assessment with the intent of introducing greater flexibility for predicting hERG inhibition of drug compounds along the drug development pipeline. Able to provide results comparable to the gold standard, and having benefits over patch clamp for higher throughput requirements, these assays are viable choices for non-compliant hERG screening, but they require special skill and treatment due to the use of radiation.

In response, the Life Technologies Predictor hERG Fluorescence Polarization assay was developed to introduce a homogenous mix and read biochemical assay that is faster, easier to use, nonradioactive, amenable to automation, and with a lower cost per test. This assay, performed in low volume high density microplates, has shown to provide results comparable to both the patch clamp and radioligand methods [1].

Using the Predictor hERG assay as a model, we demonstrate here the sensitive and fast fluorescence polarization detection capabilities of a new multi-mode microplate reader conducive to high throughput environments.

Detection and Assay Principle

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 its size (molecular volume), solution viscosity, absolute temperature and the gas constant [3].

The Predictor™ hERG Fluorescence Polarization Assay Kit from Life Technologies provides validated components to perform hERG channel biochemical binding studies in the absence of radioligand. The assay is based on the principle of fluorescence polarization where a red-shifted fluorescent tracer is displaced from the hERG channel by compounds that bind to the channel. Lower polarization values correlate to greater displacement of the tracer, and therefore indicate greater proclivity for hERG binding [4].

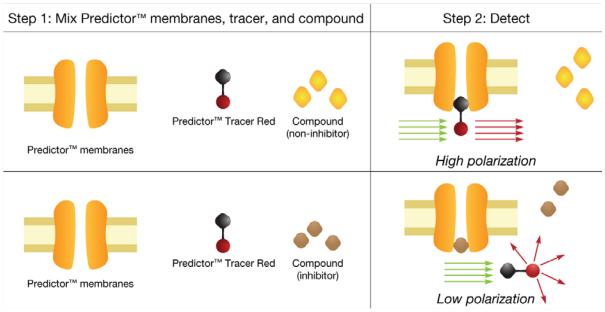

Figure 1. Predictor™ hERG Fluorescence Polarization Assay Principle.

The Synergy™ Neo is a new HTS Multi-Mode Microplate Reader equipped with multiple parallel top and bottom monochromators and filter-based detectors. Ease of use and user confidence are key to the design of Synergy Neo's unique filter cubes that are barcoded for positive filter ID – eliminating the possibility of errors and streamlining the workflow. Specialized optics impart ultra-fast measurements conducive to high throughput requirements and include a FP cube beam splitter and non-standard high-transmission filters.

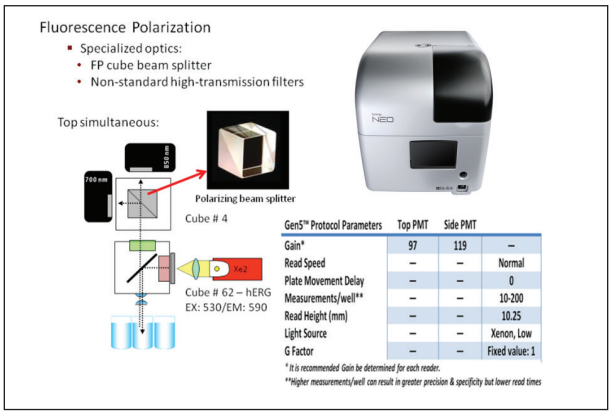

Figure 2. Synergy™ Neo Fluorescence Polarization Detection Principle and hERG Assay Parameters.

Materials and Methods

Materials

- Life Technologies Predictor™ hERG Fluorescence Polarization Assay Kit (catalog #PV5365)
- Astemizole (Sigma-Aldrich, A2861)
- Pimozide (Tocris Biosciences Catalog No. 0937
- Terfenadine (Sigma-Aldrich, T9652)
- E-4031 (supplied with kit)
- Corning 384-well low volume black microplate #3677
- BioTek Synergy™ Neo HTS Multi-mode Microplate Reader (P/N NEOB):
  - Filter cube 4 (P/N 1035004)
  - Filter cube 62 (P/N 1035062)
- BioTek Gen 5 v2.01.14 Software

Methods

Optimization of Instrument Setting

Run 1 was an incubation time course performed to determine optimal assay run times and detector parameters by assaying 16 replicates of positive and negative control following the kit insert procedure. Data is shown by Figure 5, 6, and Table 1.

Instrument Performance Validation

In Run 2, following the procedure in the kit insert, and as shown by the Plate Map and Workflow in Figures 3 and 4, two plates of data were run with two sets of compounds on each plate to validate pharmacology data endpoints. Compounds were titrated as 16-point 3-fold serial dilutions in replicates of four with a starting concentration of $1x10^4$ nM. Positive and negative controls were again run in replicates of 16 on both plates for calculation of Z' and assay window. Blank wells were assayed for optional data correction, but were not used in the final results analysis.

Each plate was read every hour starting at 2 hours of incubation up to 5 hours of incubation to compare data over a range of incubation times given by the kit. Data was generated for 6 individual measurement/well settings (10, 25, 50, 100, 150, and 200) to correlate detection sensitivity to read speed. Data corrections for this assay can be done using one of 2 methods described by the kit insert. Correction Method B was assayed by adding 10 µM E-4031 to columns 6-9 and 18-21 shown by Figure 3, but no constraint of the data was done for results herein.

nM	1	2-5	6-9	10	11	12	13	14-17	18-21	22-24
A	Empty	Cmpd 10000	Cmpd+E4031	NC	PC	Free	Blk	Cmpd 10000	Cmpd+E4031	Empty
B	Empty	Cmpd 3330	Cmpd+E4031	NC	PC	Free	Blk	Cmpd 3330	Cmpd+E4031	Empty
C	Empty	Cmpd 1109	Cmpd+E4031	NC	PC	Free	Blk	Cmpd 1109	Cmpd+E4031	Empty
D	Empty	Cmpd 369.3	Cmpd+E4031	NC	PC	Free	Blk	Cmpd 369.3	Cmpd+E4031	Empty
E	Empty	Cmpd 123	Cmpd+E4031	NC	PC	Free	Blk	Cmpd 123	Cmpd+E4031	Empty
F	Empty	Cmpd 41	Cmpd+E4031	NC	PC	Free	Blk	Cmpd 41	Cmpd+E4031	Empty
G	Empty	Cmpd 13.64	Cmpd+E4031	NC	PC	Free	Blk	Cmpd 13.64	Cmpd+E4031	Empty
H	Empty	Cmpd 4.54	Cmpd+E4031	NC	PC	Free	Blk	Cmpd 4.54	Cmpd+E4031	Empty
I	Empty	Cmpd 1.512	Cmpd+E4031	NC	PC	Free	Blk	Cmpd 1.512	Cmpd+E4031	Empty
J	Empty	Cmpd 0.504	Cmpd+E4031	NC	PC	Free	Blk	Cmpd 0.504	Cmpd+E4031	Empty
K	Empty	Cmpd 0.168	Cmpd+E4031	NC	PC	Free	Blk	Cmpd 0.168	Cmpd+E4031	Empty
L	Empty	Cmpd 0.056	Cmpd+E4031	NC	PC	Free	Blk	Cmpd 0.056	Cmpd+E4031	Empty
M	Empty	Cmpd 0.02	Cmpd+E4031	NC	PC	Free	Blk	Cmpd 0.02	Cmpd+E4031	Empty
N	Empty	Cmpd 0.006	Cmpd+E4031	NC	PC	Free	Blk	Cmpd 0.006	Cmpd+E4031	Empty
O	Empty	Cmpd 0.002	Cmpd+E4031	NC	PC	Free	Blk	Cmpd 0.002	Cmpd+E4031	Empty
P	Empty	Cmpd .0007	Cmpd+E4031	NC	PC	Free	Blk	Cmpd .0007	Cmpd+E4031	Empty

Figure 3. Assay Map showing placement detail of compound serial dilutions, bound (NC) and displaced (PC) controls, free tracer, and blank wells. No blank correction was performed.

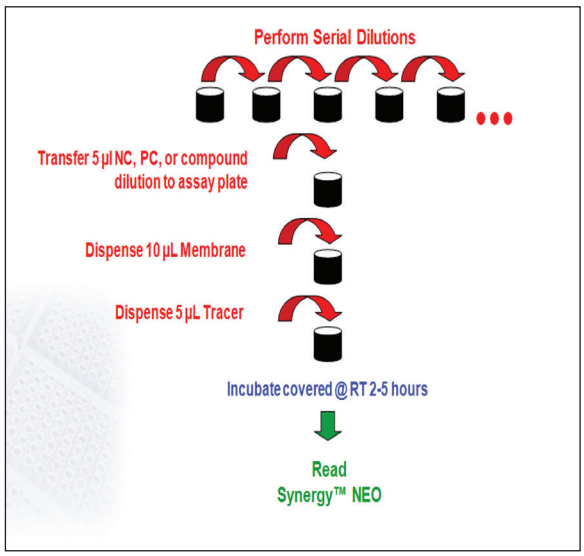

Figure 4. Instrument Performance Validation Workflow for Predictor™ hERG Fluorescence Polarization Assay on Synergy™ Neo.

Results

Polarization data auto generated by Synergy Neo under Gen5™ software control was exported to Microsoft Office Excel 2007 and GraphPad Prism® for data analysis.

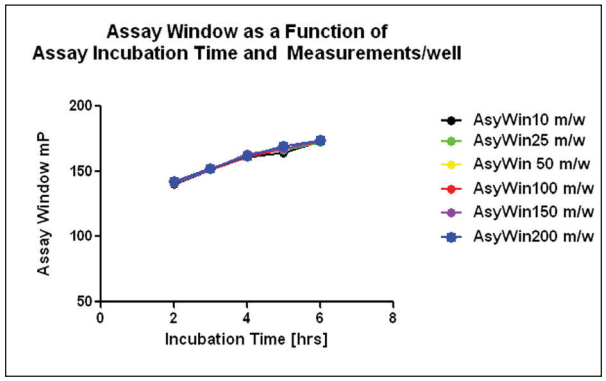

Figure 5. Representative data from Run 1 shows a trend up in assay window as a function of incubation time independent of detection settings.

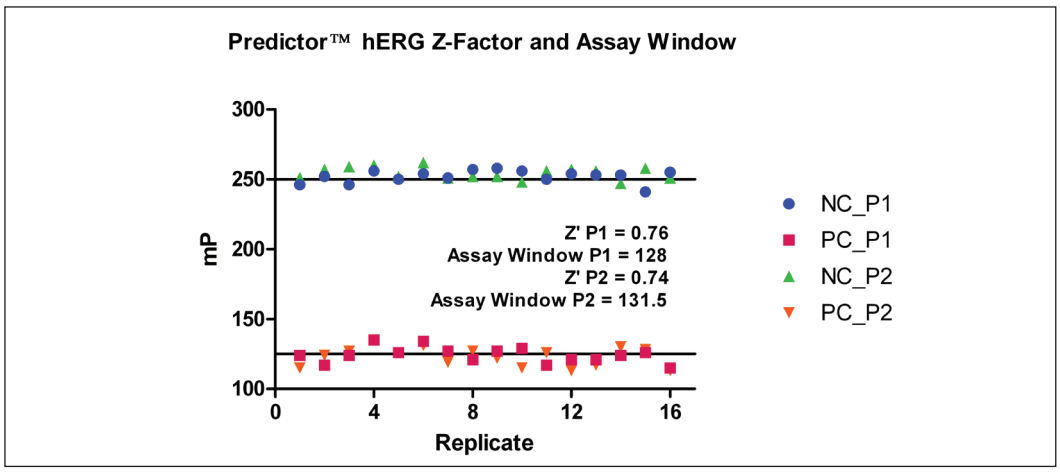

Figure 6. Run 2 assay window and Z′ results at 2 hour incubation and 10 m/w with a read time of 1 minute 17 seconds for each of 2 plates of data. Values of 111-153 are an expected range for assay windows calculated at incubation times of 1-4 hours, with corresponding Z′ values of 0.56 – 0.77 respectively. Z′ factors were calculated using the method of Zhang *et al.* (1999).

Data Measurements per Well (m/w)	Read Speed 384-well low volume microplate		Z'	Assay Window	CV%		IC50			
					NC	PC	Ast	Terf	Pim	E-4031
10	77 seconds	Plate1	0.76	128	4.58	5.64	3.59	39.85		
		Plate2	0.74	133	4.39	7.09			6.00	30.61
25	103 seconds	Plate1	0.82	129	3.44	4.4	3.54	39.42		
		Plate2	0.80	129	4.75	3.67			6.76	29.30
50	149 seconds	Plate1	0.81	131	4.13	3.95	3.52	43.72		
		Plate2	0.80	131	4.72	3.98			6.38	28.94
100	240 seconds	Plate1	0.83	130	3.84	3.46	3.54	39.99		
		Plate2	0.80	132	4.68	4.28			6.19	28.91
150	326 seconds	Plate1	0.84	132	3.79	3.16	3.55	42.69		
		Plate2	0.81	133	3.89	4.38			6.16	30.02
200	418 seconds	Plate1	0.84	134	3.21	4.04	3.63	43.66		
		Plate2	0.82	132	4.05	3.81			6.44	29.72

Table 1. Sensitivity and read speed data summary for Run 2 at the 2-hour assay incubation point. Data shows very little variability between detection measurements/well, and the corresponding decrease in speed at lower m/w offers enhanced flexibility for HTS workflows.

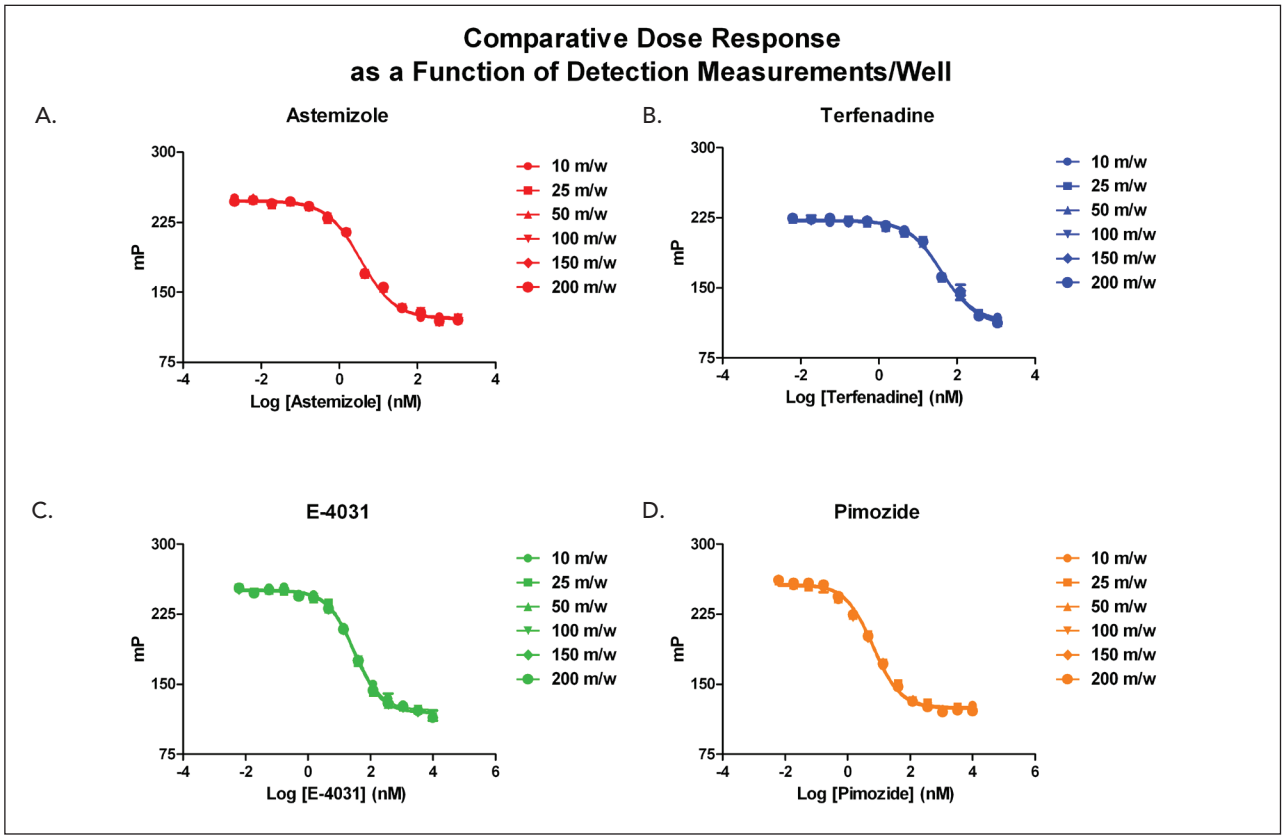

Figure 7. Comparative dose response data of 4 known hERG channel blockers for a variety of measurements/well shows high correlation within each compound regardless of detection setting. Table 1 shows comparative IC_{50} data for each compound.

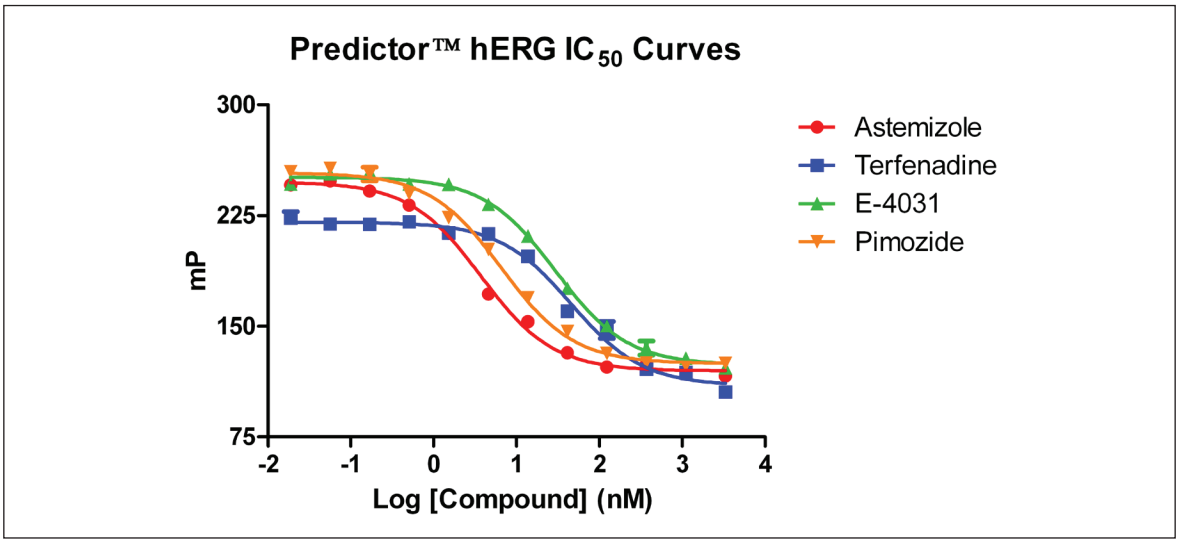

Figure 8. Representative dose response curves. Assay performance was validated using four established hERG channel blockers to show rank order and potency of their IC_{50} values. Comparative data is shown in Table 2.

Compound	Assay specific IC_{50} (nM)			
	NEO_Predictor™	Patch Clamp	Radioligand	
			[^{3}H]-astemizole	[^{3}H]-dofetilide
Astemizole	3.6	1	4	1
Pimozide	6.0	18	19	6
E-4031	30.6	48	75	20
Terfenadine	39.9	16	127	30

Table 2. Representative IC_{50} values for Predictor™ hERG validation data from Synergy Neo vs comparative methods [5]. Data shown for 2 hr assay incubation and 10 m/w with a read time of 1 minute 17 seconds.

Discussion

The sensitivity of the Synergy™ Neo optical system reliably detects fluorescence polarization from a 20 µL reaction in a 384-well low volume (30 µL) microplate format with a total read time of less than 2 minutes.

Synergy Neo offers enhanced flexibility for HTS workflows with a broad range of performance settings that produce sensitive data without sacrificing read speed. Even a 2.5-fold increase in the lowest measurements/well setting to correct for slight variations in data results in only a 1.4-fold increase in plate read time as shown by Table 1.

Data generated by Synergy Neo has tight precision and produces Z' and Assay Window values consistent with those expected for the assay over a range of incubation times, measurement/well settings, and read speeds as shown by Figures 5,6 and Table 1.

Data presented on four known hERG inhibition compounds in Figure 7 and Table 1 demonstrate highly correlative pharmacology results for a variety of measurements/well and read speeds.

Synergy Neo provides hERG inhibition data that agrees with established literature values for both the Patch Clamp and Radioligand methods as shown by Figure 8 and Table 2.

hERG safety testing is recommended by regulatory guidance before drug compounds are administered for human use. Typically, results from gold standard manual patch-clamp electrophysiology are submitted as part of the Investigational New Drug process. Screening assay technologies that are easier, faster, and less expensive to perform, while still producing comparative data to the gold standard method, can be useful in enhancing the investigational workflow within higher throughput drug discovery/development environments. In turn, this can help reduce demands on slower, more expensive confirmatory testing. As shown here, the Synergy™ Neo multi-mode microplate reader can support the demands of higher throughput safety testing by producing sensitive detection results in as little as 1 minute 17 seconds for a 384-well plate as modeled by data generated using the Predictor™ hERG Fluorescence Polarization assay technology.

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