

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

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## Introduction

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 requirements 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 laborious methods required 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 HTS detection platform suitable for hERG screening via fluorescence polarization based assay. Using 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<sub>50</sub> data is generated and compared to electrophysiology and radiometric data.

## Predictor™ hERG Fluorescence Polarization Assay

Fluorescence polarization (FP) is a fluorescence detection technique first described in 1926 by Perrin<sup>1</sup>. 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<sup>2</sup>.

The Predictor™ hERG Fluorescence Polarization Assay Kit 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<sup>3</sup>.

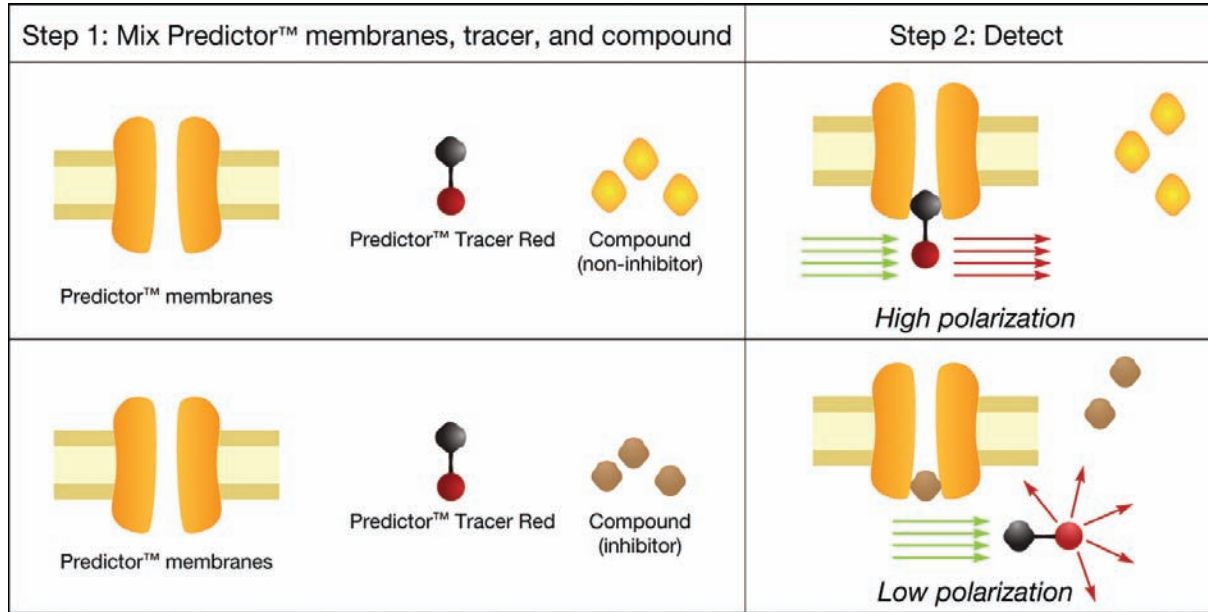


Figure 1 – Principle of the Predictor hERG Fluorescence Polarization Assay.

## BioTek Instrumentation



Figure 2 – Synergy™ NEO HTS Multi-Mode Microplate Reader.

The new Synergy™ NEO is an HTS Multi-Mode Microplate Reader designed for screening and core laboratories. Synergy NEO has multiple parallel detectors for ultra-fast measurements, laser-based excitation, plate stacker and high sensitivity on low volume assays. The Synergy NEO also incorporates BioTek's unique patented Hybrid Technology™ for wavelength flexibility.

## Materials and Methods

### Materials

- Predictor hERG Fluorescence Polarization Assay Kit (catalog #PV5365)
- Astemizole (Sigma-Aldrich, A2861)
- Pimozide (Tocris Bioscience 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 Instruments Gen5 v2.01.14 Data Analysis Software

## Materials and Methods (Continued)

### Methods

Following the procedure in the kit insert, and as shown by the Plate Map and Workflow illustrated by Figures 3 and 4, two plates of data were run comprised of 2 sets of compounds on each plate. Compounds were titrated as 16-point, 3-fold serial dilutions in replicates of four with a starting concentration of 1x10<sup>4</sup> nM. Bound (NC) and displaced (PC) controls were run in replicates of 16 on both plates. 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. Data was generated for 6 individual measurement/well settings (10, 25, 50, 100, 150, and 200) to correlate detection sensitivity to read speed. For purposes of this experiment the Gen5 generated data was exported to Microsoft® Excel® and then GraphPad Prism was used to represent results of the assay. Data corrections for this assay can be done using one of 2 methods described by the kit insert. Although Correction Method B was assayed by adding 10 µM E-4031 to columns 6-9 and 18-21 as shown on Figure 3, no constraint to the top and bottom of the curve using the NC and PC was done on results presented here.

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	BA	Cmpd 10000	Cmpd+E4031	Empty
B	Empty	Cmpd 3330	Cmpd+E4031	NC	PC	Free	BA	Cmpd 3330	Cmpd+E4031	Empty
C	Empty	Cmpd 1109	Cmpd+E4031	NC	PC	Free	BA	Cmpd 1109	Cmpd+E4031	Empty
D	Empty	Cmpd 369.3	Cmpd+E4031	NC	PC	Free	BA	Cmpd 369.3	Cmpd+E4031	Empty
E	Empty	Cmpd 123	Cmpd+E4031	NC	PC	Free	BA	Cmpd 123	Cmpd+E4031	Empty
F	Empty	Cmpd 41	Cmpd+E4031	NC	PC	Free	BA	Cmpd 41	Cmpd+E4031	Empty
G	Empty	Cmpd 13.64	Cmpd+E4031	NC	PC	Free	BA	Cmpd 13.64	Cmpd+E4031	Empty
H	Empty	Cmpd 4.54	Cmpd+E4031	NC	PC	Free	BA	Cmpd 4.54	Cmpd+E4031	Empty
I	Empty	Cmpd 1.512	Cmpd+E4031	NC	PC	Free	BA	Cmpd 1.512	Cmpd+E4031	Empty
J	Empty	Cmpd 0.504	Cmpd+E4031	NC	PC	Free	BA	Cmpd 0.504	Cmpd+E4031	Empty
K	Empty	Cmpd 0.168	Cmpd+E4031	NC	PC	Free	BA	Cmpd 0.168	Cmpd+E4031	Empty
L	Empty	Cmpd 0.056	Cmpd+E4031	NC	PC	Free	BA	Cmpd 0.056	Cmpd+E4031	Empty
M	Empty	Cmpd 0.02	Cmpd+E4031	NC	PC	Free	BA	Cmpd 0.02	Cmpd+E4031	Empty
N	Empty	Cmpd 0.006	Cmpd+E4031	NC	PC	Free	BA	Cmpd 0.006	Cmpd+E4031	Empty
O	Empty	Cmpd 0.002	Cmpd+E4031	NC	PC	Free	BA	Cmpd 0.002	Cmpd+E4031	Empty
P	Empty	Cmpd .0007	Cmpd+E4031	NC	PC	Free	BA	Cmpd .0007	Cmpd+E4031	Empty

Figure 3 – Assay Map showing detail of compound serial dilutions. Correction Method B was assayed in columns 6-9 and 18-21 (plate 1 only) respectively, but no constraint of the dose response curves was implemented. Placement of Bound (NC) and Displaced (PC) controls, Free Tracer, and blank wells are also shown. No blank correction was performed.

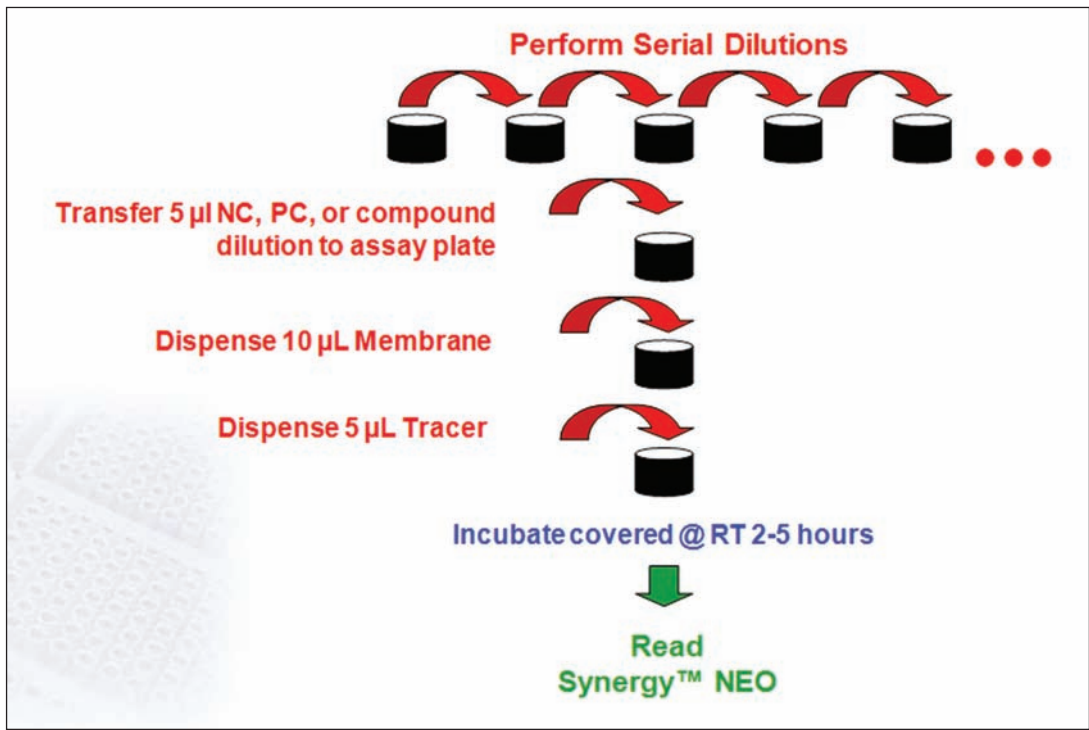


Figure 4 – Assay Validation Workflow for Predictor hERG Fluorescence Polarization Assay on Synergy NEO.

## Results

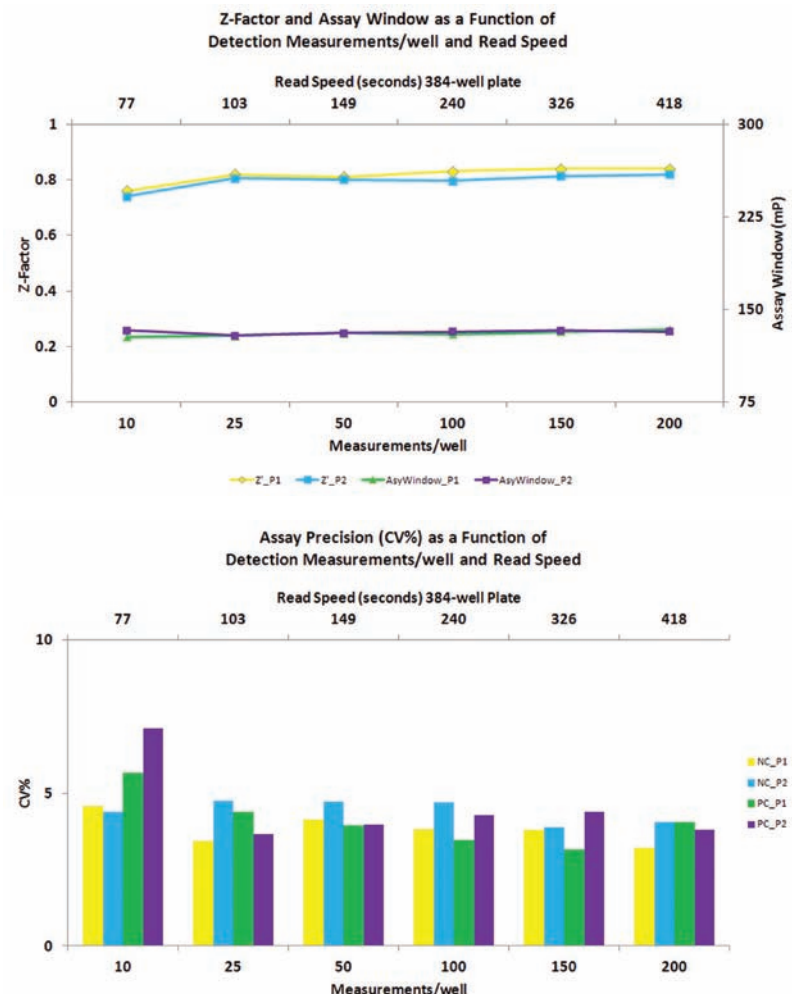


Figure 5 – Assay performance data correlated to detection measurements/well and read speed at 2 hr incubation. Data shows a slight increase in Z' >10 m/w, but generally the data remains constant for both test variables (top). Z' factors were calculated using the method of Zhang et al. (1999)<sup>3</sup>. At bottom, precision data remains consistent among Bound (NC) controls, with slightly lower CV% on Displaced (PC) controls read >10 m/w. See Table 1 for detail.

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 – Summary of total time course data for a 2 hour assay incubation. Data shows very little variability between detection measurements/well. With little appreciable difference in data quality, the corresponding decrease in speed at lower m/w offers enhanced flexibility for HTS workflows.

## Results (Continued)

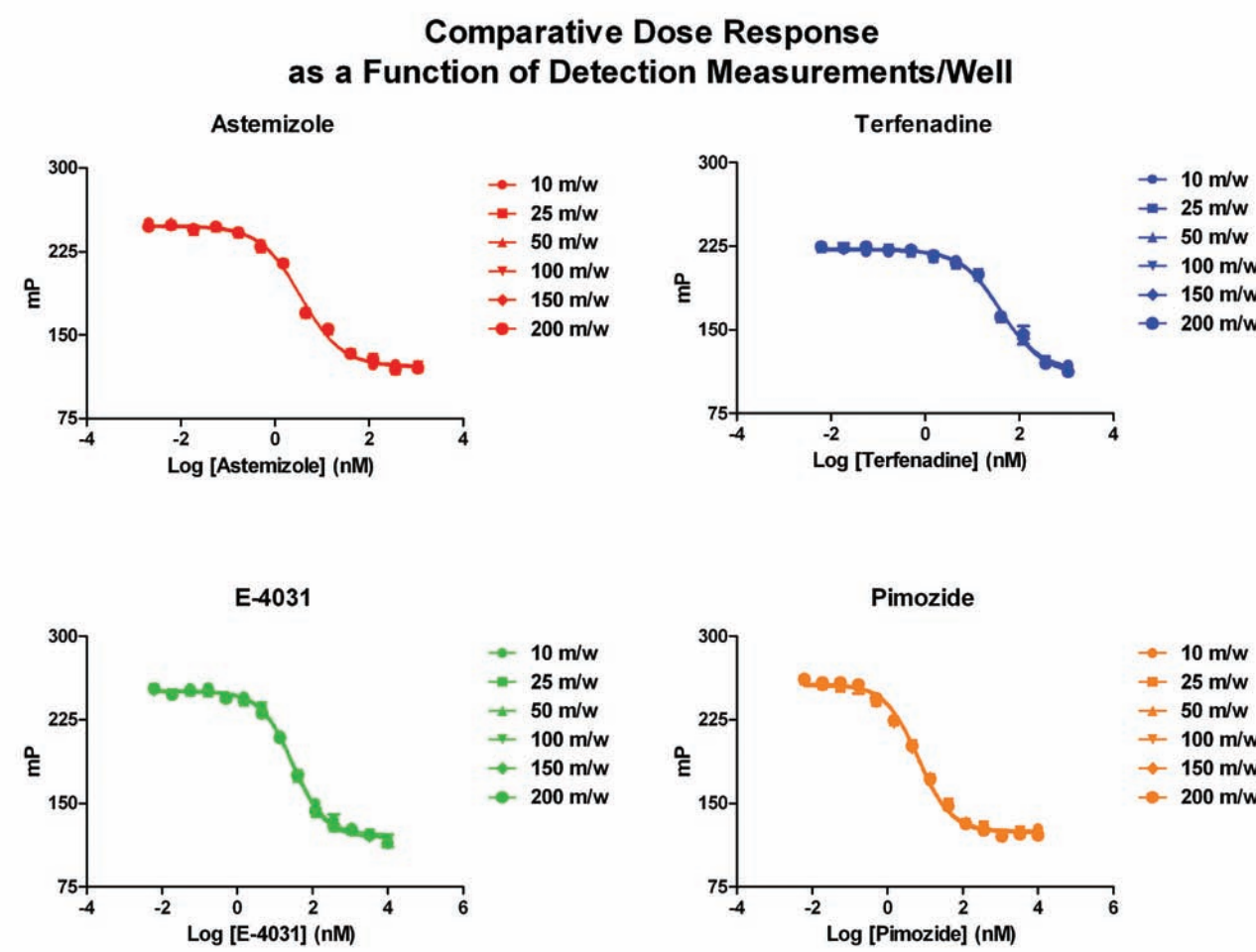


Figure 6 – Comparative dose response data of 4 known hERG channel blockers for a variety of detection measurements/well shows high correlation within each compound. IC<sub>50</sub> comparative data for each compound is given in Table 1. Two hour incubation data shown.

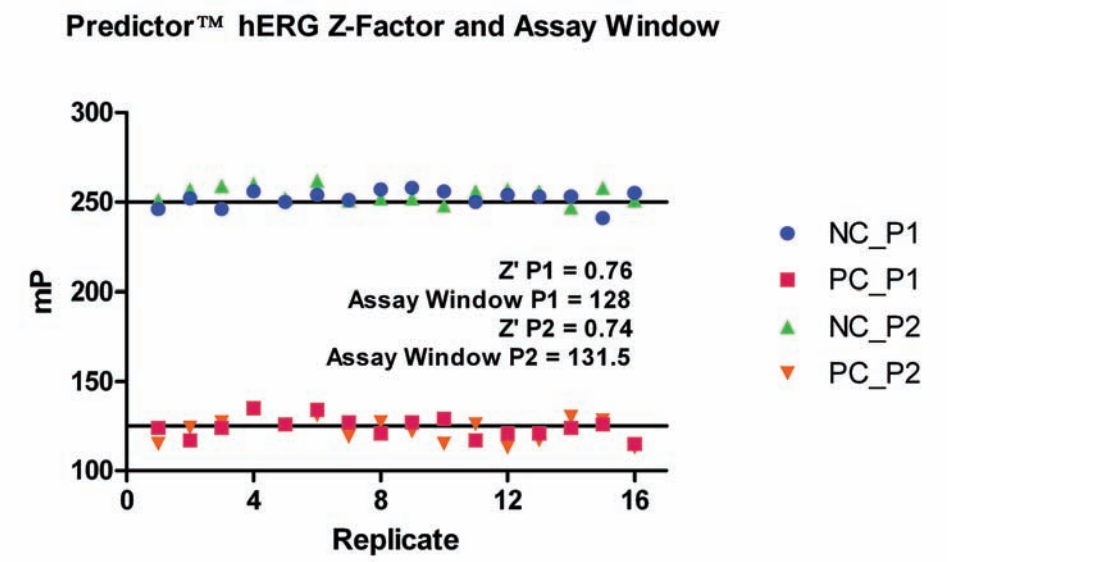


Figure 7 – Representative data showing Z' factor and assay window results at 2 hour incubation and 10 m/w with a read time of 1 minute 17 seconds. An assay window of 111-153 is typical for this assay at incubation times of 1-4 hours, with corresponding Z' of 0.56 – 0.77 respectively. Z' factors were calculated using the method of Zhang et al. (1999)<sup>3</sup>.

Compound	NEO_Predictor™	Patch Clamp	Assay Specific IC <sub>50</sub> (nM)	
			[ <sup>3</sup> H]-astemizole	[ <sup>3</sup> 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<sub>50</sub> values for Synergy NEO\_Predictor hERG validation data vs comparative methods<sup>4</sup>. Data shown for two hour assay incubation and 10 m/w with a read time of 1 minute 17 seconds.

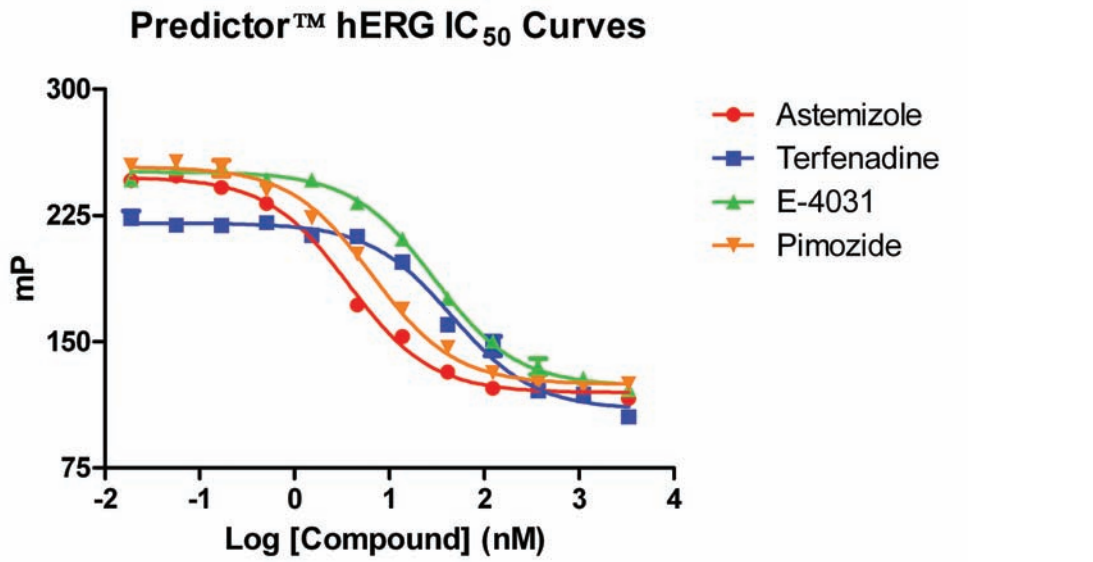


Figure 8 – Representative dose response curves. Assay performance was validated using four established hERG channel blockers to show rank order and potency of their IC<sub>50</sub> values. Comparative data shown in Table 2.

## Conclusions

- hERG HTS safety testing can be enhanced using the Predictor assay and the Synergy NEO
- The sensitivity of the Synergy NEO optical system reliably detects fluorescence polarization from a 20 µL reaction in low volume 384-well format in under 2 minutes
- Synergy NEO provides hERG inhibition data that agrees with established literature values for the Patch Clamp and Radioligand methods at 10 m/w with a read speed time of 1 min 17 sec
- Synergy NEO provides Z' and Assay Window data consistent with expected values for the assay, regardless of m/w or read speed, and independent of incubation time (data not shown above two hour incubation)
- Assay precision and performance data supports consistent pharmacology results at a variety of m/w and read speeds and independent of assay incubation time (data not shown above two hour incubation)

<sup>1</sup>Perrin, F. (1926) Polarization de la Lumiere de Fluorescence. Vie Moyenne de Molecules dans l'etat Excite. J. Phys Radium 7:390. | <sup>2</sup>Held, Paul, "Invitrogen's™ Predictor™ hERG Fluorescence Polarization Assay Using BioTek's Synergy 2 Multimode Reader" Application Note, BioTek Instruments, Inc. Winooski VT, 2008.

<sup>3</sup>Invitrogen™ Predictor™ hERG Fluorescence Polarization Assay Protocol. | <sup>4</sup><http://tools.invitrogen.com/content/sfs/manuals/Predictor-hERG-FAQ-General.pdf>