

## The Predictor™ hERG Fluorescence Polarization Assay

### Implementation on Tecan's Infinite® M1000 Multimode Reader



### Introduction

Fluorescence Polarization (FP) measurements play an essential role in life science applications investigating molecule interactions. The homogeneous mix-and-read format provides enabling technology for drug discovery and high throughput screening.

FP is based on the principle that when a small fluorescent molecule (the tracer) is excited with plane-polarized light, the emitted light is largely depolarized because the molecule rotates rapidly in solution during its fluorescence lifetime. If the tracer is bound to a large molecule, thereby increasing its molecular volume, its rotation is slowed and the emitted light remains in the same plane as it was excited [1]. Invitrogen developed a FP based assay to perform hERG (human Ether-a-go-go Related Gene) channel biochemical binding studies displacing radiometric binding assays [2].

### Predictor™ hERG FP Assay

#### hERG (human Ether-a-go-go Related Gene)

The hERG gene encodes the pore-forming subunits of a potassium ion channel contributing to the final phase of the action potential that returns the ventricular muscle cell to its resting state. If a drug suppresses the activity of the channel it can lead to prolongation of the action potential, which may result in a cardiac arrhythmia, ventricular fibrillation and finally in patient death.

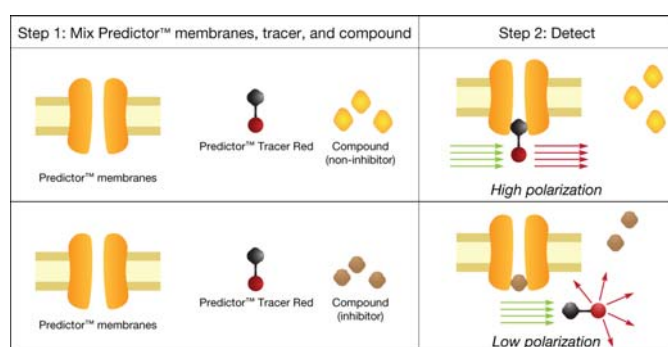
The Predictor™ hERG FP Assay allows prediction of potential hERG blockers early in a drug discovery process. The generation of a cell line expressing the protein in a sufficient amount is a precondition for investigations using membrane bound proteins [3].

#### Assay principle

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). When the tracer is bound by the hERG channel protein, the tracer produces a high fluorescence polarization.

Competitors (e.g. E-4031) that bind to the hERG channel protein displace the tracer. The released tracer tumbles in the solution emitting depolarized light relative to the excitation source (Fig. 1) [4].

In the current experiment E-4031 and astemizole were used as competitors. E-4031 is known to block hERG channels selectively. E-4031 is also used as positive control and is therefore provided by the assay kit [4].



**Figure 1:** Principle of the Predictor™ hERG FP Assay (Invitrogen)

## Materials and Methods

### Instruments

- Tecan Infinite M1000 premium Quad4 Monochromators™ multimode microplate reader equipped with a fluorescence polarization module

### Microplate

- 384 well micro plate, small volume, black, flat bottom, (Corning®, NY, USA)

### Assay

- Predictor™ hERG Fluorescence Polarization Assay (PV 5365, Invitrogen, CA)
- Astemizole (Sigma, MO)

Reagent preparation:

Assay compounds were prepared according to the protocol provided by the manufacturer (homogenization of the Membrane, E-4031 dilution series) [4].

Preparation of Astemizole dilutions: 100 mM Astemizole stock solution was initially diluted in water (or DMSO) to obtain different concentrations of Astemizole solutions (10 mM to 697 pM; threefold). These solutions were, further on, diluted in FP Assay Buffer (1/25) and then transferred to the assay plate. The final concentrations of the test compounds are given in Table 1.

Final conc. of E-4031 [nM]	Final conc. of Astemizole [nM]
30000	100000
10000	33333
3333	11111
1111	3703
370,4	1234,6
123,5	411,5
41,2	137,2
13,7	45,7
4,57	15,28
1,52	5,08
0,508	1,694
0,169	0,565
0,0565	0,1882
0,0198	0,0627
0,0063	0,0209
0,0021	0,0070

**Table 1:** Final concentrations of test compounds (E-4031 and Astemizole)

Assay protocol: 5 µl test compound (E-4031 and astemizole dilutions) were mixed with 10 µl Membrane and 5 µl 4 nM Tracer Red. The measurements were carried out in three replicates. After incubation for 4 hours at RT, the FP was measured. Assay blank (8 replicates): 5 µl water/DMSO (test compound solvent), 10 µl Membrane, and 5 µl FP Assay Buffer. Positive control (E-4031; 16 replicates): 5 µl 120 µM E-4031, 10 µl Membrane and 5 µl 4 nM Tracer Red. Negative control (16 replicates): 5 µl water/DMSO (test compound solvent), 10 µl Membrane and 5 µl 4 nM Tracer Red.

### Measurements

The measurement settings are given in Table 2.

G-Factor calculation: 5 µl water/DMSO (test compound solvent), 10 µl Assay Buffer, and 5 µl 4 nM Tracer Red. Reference polarization value: 40 mP, reference blank: the same as the assay blank.

Measurement Parameter	Instrument Settings
Plate	COS384fb
Mode	Fluorescence Polarization
Excitation wavelength	530 nm
Excitation bandwidth	5 nm
Emission wavelength	585 nm
Emission bandwidth	20 nm
Gain	optimal
Number of Flashes	10 (=100 ms integration time)
Settle Time	0 ms
Z-Position	Calculated from well
G-Factor	Calibrated
G-Factor: Reference Blank	Same as Measurement Blank
Measurement Blank	(A9-H9)

**Table 2:** Measurement parameter and instrument settings for the Predictor™ hERG Fluorescence Polarization on Tecan's Infinite M1000 multimode microplate reader.

### Calculations

Prior to calculating polarization values the assay blanks were used to subtract the background fluorescent intensity. This was done by the software by selecting the appropriate assay wells.

The blank subtracted fluorescence polarization values were plotted against compound concentration. IC<sub>50</sub> values were determined by nonlinear fits of a sigmoidal dose response curve to the data [4].

Z'-factors were determined using the blank subtracted polarization values from 16 wells of negative and positive controls using the method of Zhang et al. (1999) [5].

## Results

Predictor™ hERG Fluorescence Polarization Assay utilizes the principle of the fluorescence labeled tracer binding to the large hERG channel protein resulting in high polarization values. If the tracer is displaced by a competitor the polarization decreases, the lower the polarization value the greater the affinity of the competitor to the hERG channel protein.

In the present experiment both test compounds (E-4031, astemizole) show the capability to block the hERG channel (Fig. 2 and 3). The IC<sub>50</sub> values (inhibition constant) of 28.5 nM for E-4031 and 1.68 nM for Astemizole are in accordance with the specification criteria defined by Invitrogen [4]. The IC<sub>50</sub> refers to the test compound concentration which results in a half-maximum shift in polarization value.

The Z'-factor for the measurement is 0.79 (Fig. 4) and is an indication of the assay performance. Values of greater than 0.5 are generally considered good, while a value of 1 indicates a theoretically ideal assay with no variability [4].

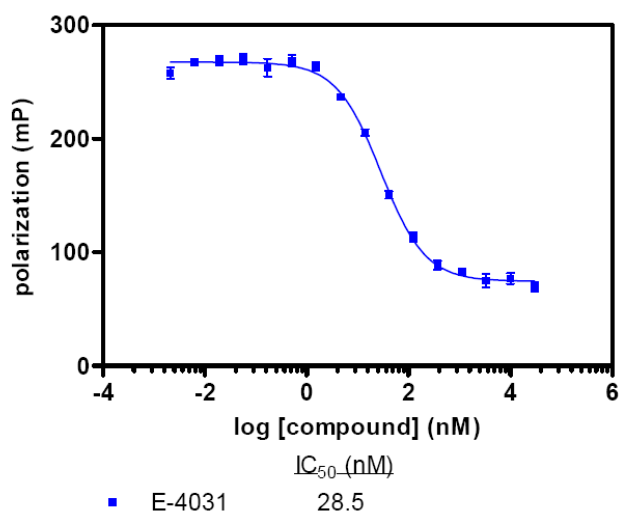


Figure 2: Concentration response curve for E-4031. Fluorescence polarization values were plotted against compound concentration.

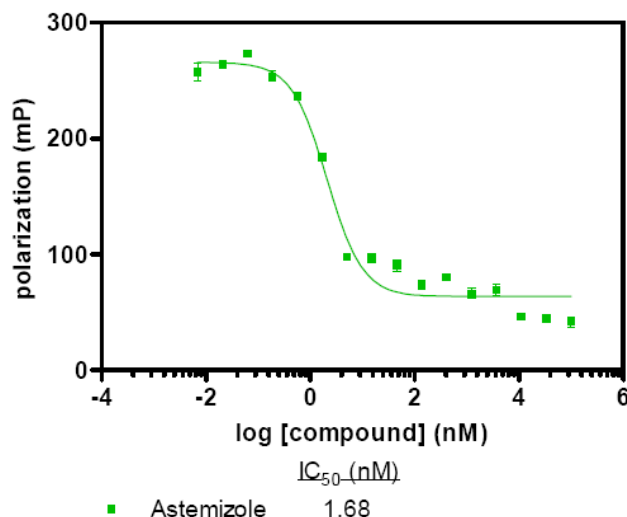


Figure 3: Concentration response curve for astemizole. Fluorescence polarization values were plotted against compound concentration.

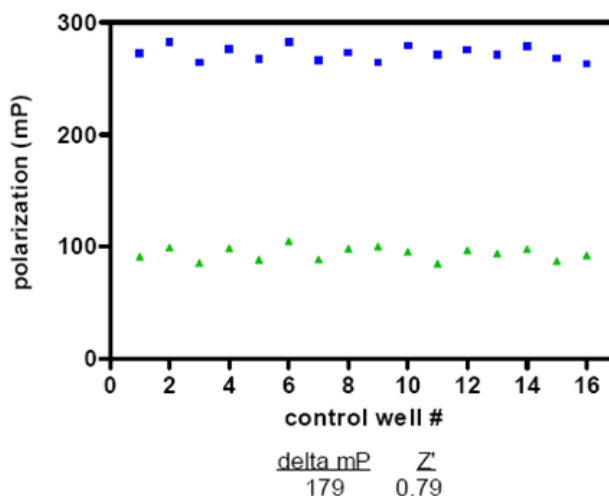


Figure 4: Blank corrected polarization values of positive and negative controls (16 replicates). Negative controls are given in blue, positive controls (30µM E-4031) in green.

## Conclusion

This application note describes the successful validation and implementation of the Predictor™ hERG Fluorescence Polarization Assay for research purposes within drug discovery on the Tecan Infinite M1000 premium Quad4 Monochromators™ based multimode detection system. Tecan's Infinite M1000 offers an easy-to-use and flexible way of accessing fluorescence polarization data.

## Acknowledgements

We express our acknowledgements to David Piper, PhD, Bryan Marks, and Kevin Lowitz from Invitrogen (Madison), who conducted the validation experiments on the Tecan Infinite M1000 microplate reader.

## List of abbreviations

FP	Fluorescence Polarization
hERG	human Ether-a-go-go Related Gene
DMSO	Dimethylsulfoxid
IC	Inhibition Constant

## Literature

- [1] J.R. Lakowicz, Principles of Fluorescence Spectroscopy, Springer Science & Business Media, 3rd edition, 2006
- [2] Invitrogen homepage, [www.invitrogen.com/predictor](http://www.invitrogen.com/predictor)
- [3] Piper D.R. et al., Development of the Predictor hERG Fluorescence Polarization Assay Using a Membrane Protein Enrichment Approach. Assay Drug Dev Technol. 2008, Vol. 6, No. 2:213-223
- [4] Predictor™ hERG FP Assay Manual, Product Insert
- [5] Zhang J.H. et al., A simple statistical parameter for use in evaluation and validation of high throughput screening assays. J Biomol Screen 1999, Vol. 4:67-73

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