TECHNICAL NOTE

Performing fluorescence polarization assays on the VICTOR Nivo.

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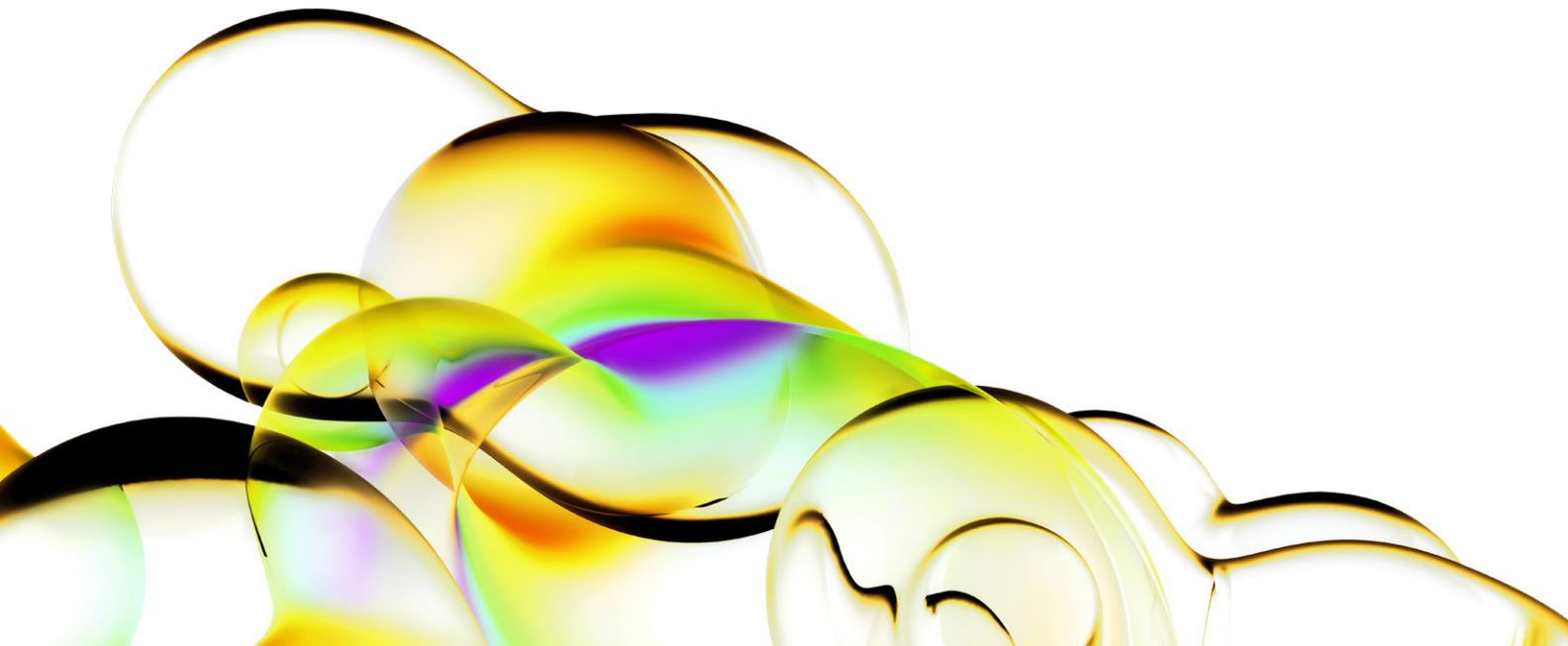
Introduction

Fluorescence Polarization (FP) is a homogeneous assay format that is highly suitable for many applications from occasional usage to high throughput screening, due to rather inexpensive reagents and its signal stability[1]. In FP assays, polarized light is used to determine the rotation capabilities of small fluorescently labelled molecules. With this assay principle, one can indirectly detect whether tracer molecules are bound to a much larger molecule or are freely rotating in solution. These are rather complex interrelationships on the assay as well as on the device side compared to other homogeneous, plate reader compatible assays. Hence, for users, it is often difficult to set up an FP assay correctly.

For this reason, we describe in this Technical Note how to set up a Fluorescence Polarization assay on the VICTOR® Nivo™ multimode plate reader and provide guidance for protocol optimization. The VICTOR Nivo is a compact multimode plate reader that provides all detection modes which are routinely used in drug discovery: Absorbance, Luminescence, Fluorescence Intensity, as well as options for Alpha, Time- Resolved Fluorescence and Fluorescence Polarization. Due to its intuitive control software and small footprint, the plate reader fits easily in any lab.



As an example assay, the Predictor™ hERG Fluorescence Polarization Assay[2] was used and its principle is shown in Figure 1, where the fluorescently labelled small molecules of the Predictor™ hERG Tracer Red can either bind to the hERG channel protein in Predictor™ membrane fraction or can rotate freely.

Blocking of the hERG potassium channel is known to be a potential off-target activity of drug candidates[2,3], that can lead to life-threatening arrhythmias. For this reason, effects on the hERG channel are investigated early in the drug discovery process using various methodologies, one of them being the Fluorescence Polarization assay.

VICTOR Nivo Multimode Plate Reader

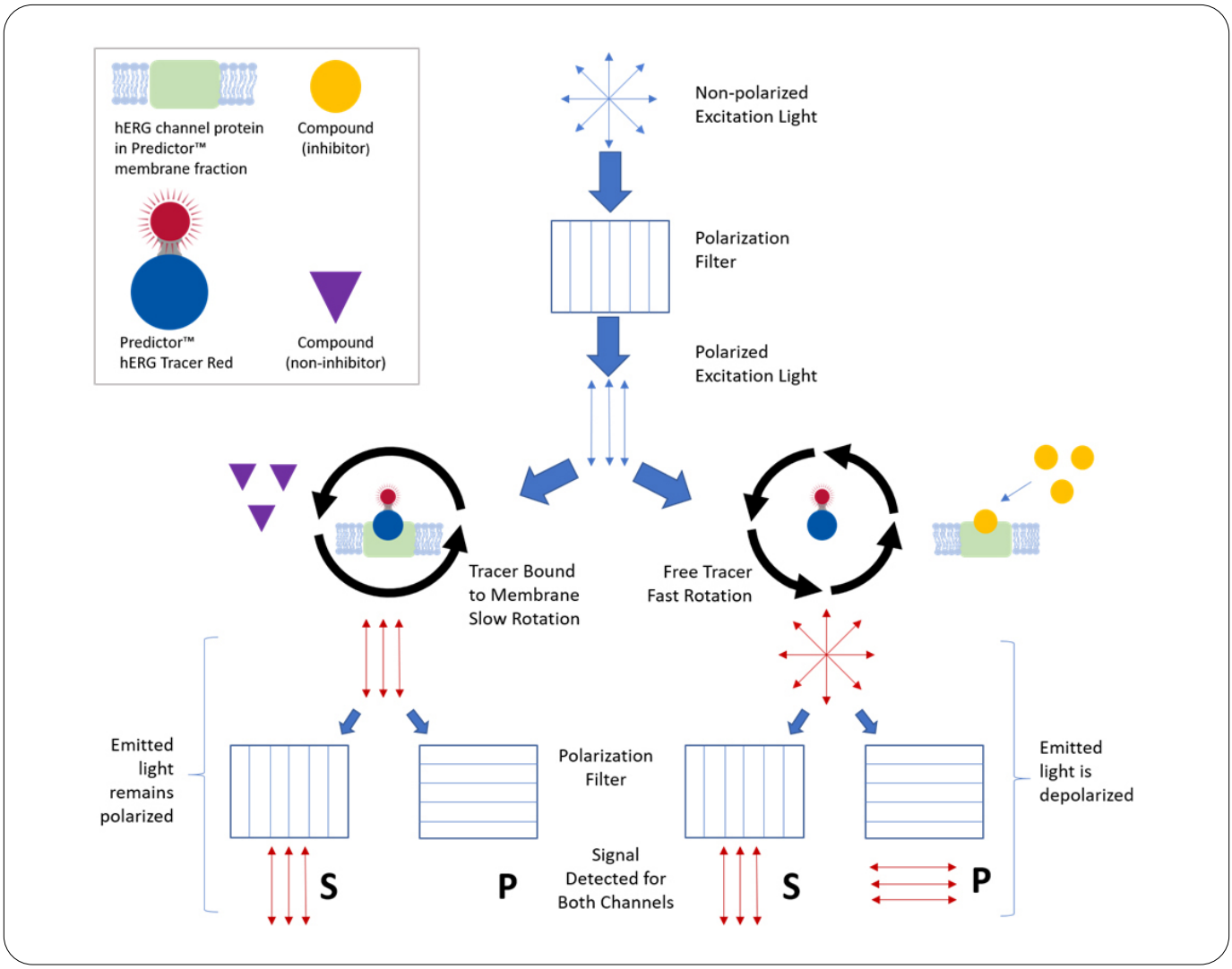


Figure 1: Assay Principle. If polarized light excites Tracer Red bound to the hERG channel protein, the emission light remains polarized, because the tracer-channel-complex rotates slowly during fluorescent lifetime. In contrast, inhibiting compounds in the ion channel block Tracer Red from binding. In case Tracer Red is replaced in the ion channel by a compound, it rotates quickly during fluorescent lifetime due to its small size. This is leading to highly depolarized emission light, which is detected by the instrument not only in S, but also in P orientation.

Materials and methods

Instrument setup run for Predictor™ hERG FP assay

The instrument setup run is a step used to optimize the FP measurement protocol specifically for the Predictor™ hERG Fluorescence Polarization Assay (Invitrogen, # PV5365) with regard to Z-height and G factor. For this experiment, a set of assay controls is needed: Buffer Blank, Assay Blank, Free tracer control, Negative control and Positive control. The controls were prepared according to the assay manual[5] and were transferred in triplicates to a black 384-well assay plate (Revvity, ProxiPlate # 6008260 or OptiPlate # 6007270) at a volume of 20 µL/well (Figure 2).

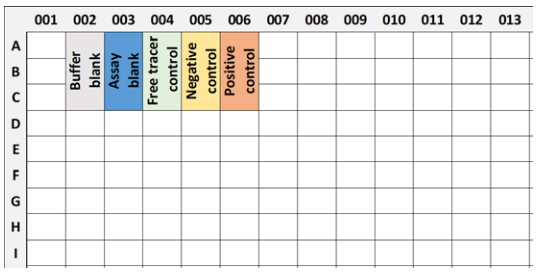


Figure 2: Plate layout for the instrument setup run on the VICTOR Nivo.

1. **Selection of filters**

In order to set up a FP measurement protocol on the VICTOR Nivo, three filters and a dichroic mirror are needed: a 530/30 nm excitation filter, two 580/20 nm emission filters and a 565 nm dichroic mirror. Alternatively, a 50/50 beam splitter can be used, but assay performance may be impaired. Dedicated polarization filters are not needed as the necessary polarizing components are already located inside the plate reader, if the instrument is equipped with FP technology.

2. **Z-focus height optimization**

Using a free tracer control well (reference polarization control), the Z-focus height optimization was demonstrated for a 384-well ProxiPlate and 384-well OptiPlate.
A FP Z-focus scan protocol was set up (excitation at 530 nm, emission at 580 nm) with 20 scan points between 0 and 20 mm (Figure 3).

The emission values (either S or P) were plotted in the VICTOR Nivo control software (Figure 4). The plate specific optimal Z-focus height was determined at the emission intensity maximum. For future FP measurements, this Z-focus height was transferred to the FP endpoint protocol of the control software.

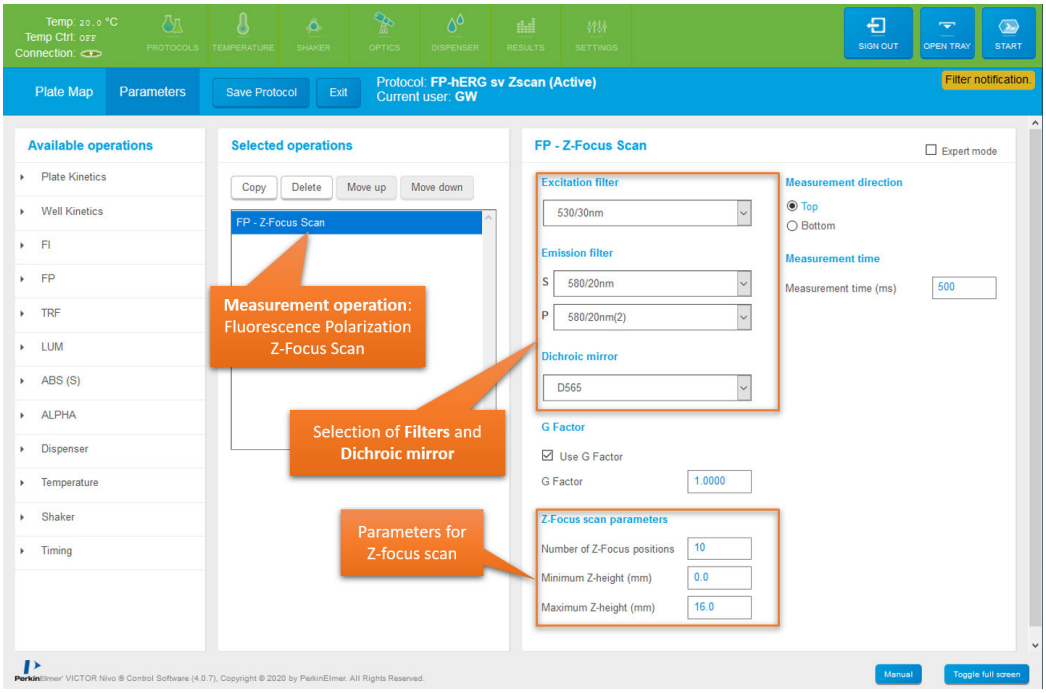


Figure 3: Z-focus scan protocol for the plate specific optimization of the Z-focus height.

3. **G Factor calculation**

The G factor is a correction factor used to compensate for differences in parallel and perpendicular optical components of the measurement device. Calculating the G factor is recommended, if the true polarization should be determined. Here, it was calculated using the free tracer control wells. In the Predictor™ hERG FP Assay, this reference control has a known value of 50 mP[5]. As a first step, the assay plate was measured once with the FP endpoint protocol including a G factor of 1. The S and P channel results were then used to calculate the G factor using Microsoft Excel according to the following formula:

$$G = \frac{S^*(1 - \frac{mP\,(Tracer)}{1000})}{P^*(1 + \frac{mP\,(Tracer)}{1000})}$$

As a rule of thumb, G is usually 0.8 < G < 1.2. The assay specific calculated G factor was inserted in the FP endpoint protocol of the control software and the measurement of the assay plate repeated. The G factor was determined correctly, if the known mP value of the reference control (here 50 mP, see above) is obtained as a result.

If the literature polarization value is not known for the used fluorophore, the relative change of polarization values (ΔmP) upon treatment can be plotted to create dose-response curves. For this, the G factor does not need to be adjusted and can be kept at 1. To calculate ΔmP, all resulting mP values of the curve are normalized to an assay relevant sample showing low polarization values such as the free tracer control, positive control or even the lowest compound concentration in this example.

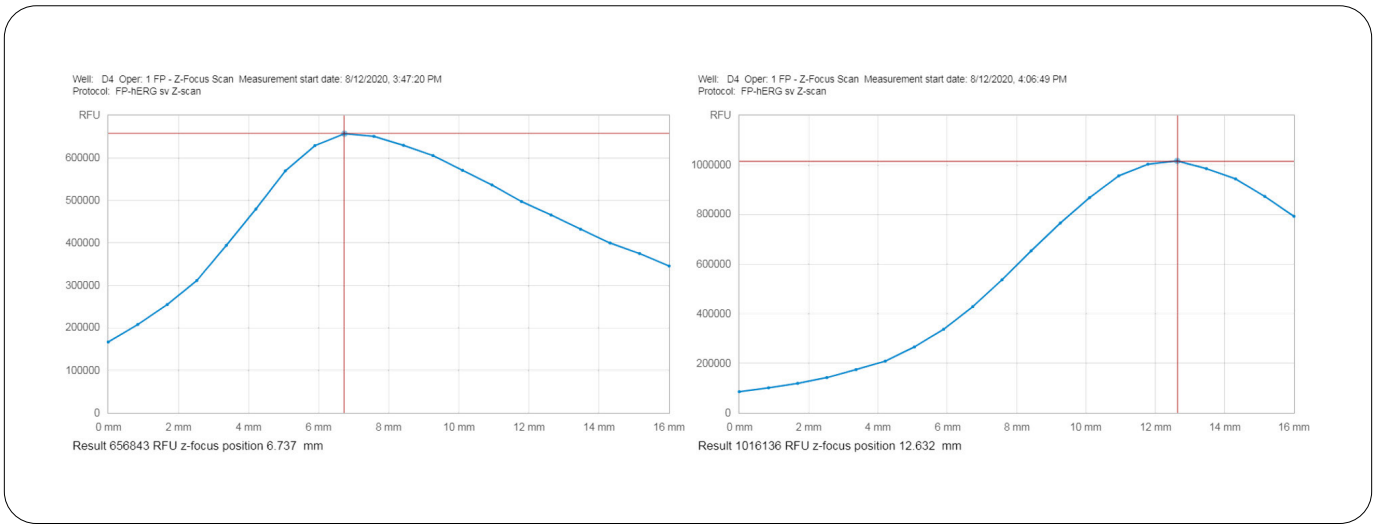


Figure 4: Z-focus height optimization was demonstrated in Revvity OptiPlate and ProxiPlate using a FP Z-focus scan protocol (excitation at 530 nm, emission at 580 nm) with 20 scan points between 0 and 20 mm. The emission intensity maximum (red intersecting lines) was determined directly in the VICTOR Nivo software.

Compound testing in the predictor™ hERG FP assay

The known hERG channel inhibitors Astemizole (Cayman chemical, #16967) and Terfenadine (Cayman chemical, #20305) were tested in 16-point dose response curves in a concentration range of 3.3 µM - 0.2 pM in the FP assay. To allow data correction in case of unspecific compound effects, both compounds were also tested in the presence of a saturating concentration of the inhibitor E-4031 (30 µM). The plate layout is shown in Figure 6.

First, the test compounds were dissolved in DMSO and a 3-fold dilution series was prepared. Afterwards, all samples were diluted 1:25 in assay buffer. Compounds were transferred to the assay plate at a volume of 5 µL/well. The tracer was diluted to 4 nM and 5 µL/well were transferred into the assay. Finally, 10 µL/well of the Predictor™ hERG Membrane were dispensed into a ProxiPlate (Revvity, # 6008260). After 2 hours of incubation at room temperature, the assay plate was placed in the VICTOR Nivo to run the FP protocol with the measurement settings shown in Figure 5.

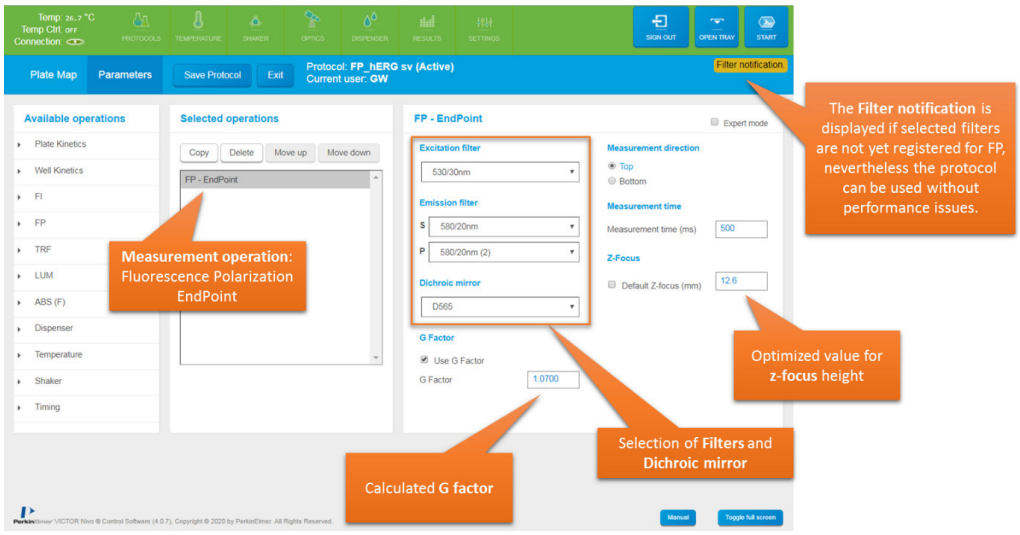


Figure 5: Final VICTOR Nivo measurement protocol for the Predictor™ hERG FP assay shown here for 384-well ProxiPlates.

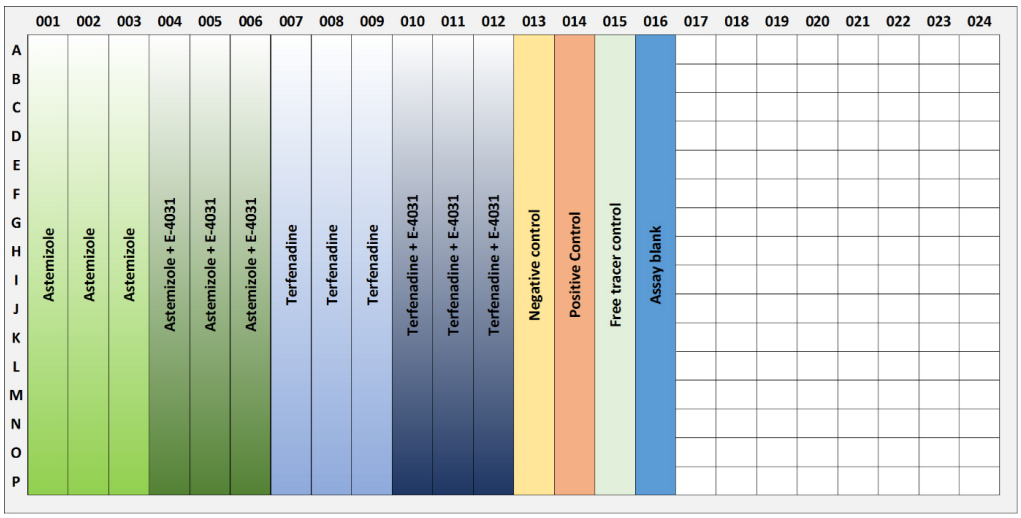


Figure 6: Plate layout for Compound Profiling in the Predictor™ hERG assay. The compounds Astemizole and Terfenadine were tested in 16-point dose response (3.3 µM - 0.2 pM, triplicates per concentration) in the presence and absence of the inhibitor E-4031.

Results

After optimizing the FP protocol on the VICTOR Nivo, it was used to measure the assay plate containing controls. As shown in Figure 7, the free tracer control results in 50 mP on average, showing that the G factor has been optimized correctly using the literature value[5]. Nevertheless, the actual assay window is the span between the negative (tracer and membrane) and positive control (tracer, membrane and 30 µM E-4031) in these experiments ~100 mP. Comparable results were obtained in the OptiPlate and ProxiPlate at a volume of 20 µL (data not shown).

In addition, it can be helpful to look not only at the mP results but also to calculate the total intensity with the formula 2*P+S. For example, background signal (assay blank and buffer blank) is often highly polarized, but the intensities are actually very low. Taking the total intensity into account during data analysis can therefore help avoid misinterpretation of results.

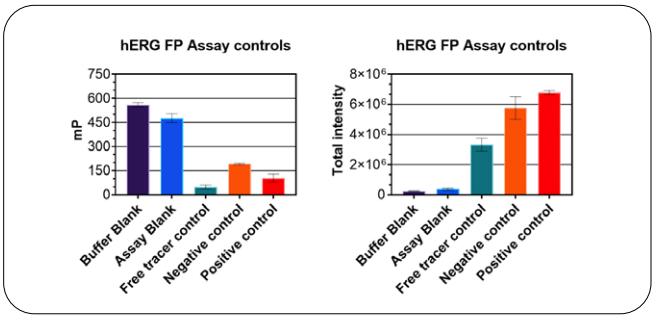


Figure 7: Resulting mP values (left) and total intensity (right) of assay controls in ProxiPlate after protocol optimization on the VICTOR Nivo. For each sample, the mean and standard deviation of three wells are shown.

In a subsequent experiment, the known inhibitors Terfenadine and Astemizole were tested in dose response in the FP assay, results are shown in Figure 8. The FP signal was detected 2 hours after incubation. Assay statistics for the two independent experiments are summarized in Table 1. For the calculations, 16 positive control wells and 16 negative control wells were used. The Z prime values of 0.73 and 0.87 indicate a robust assay performance for both experiments.

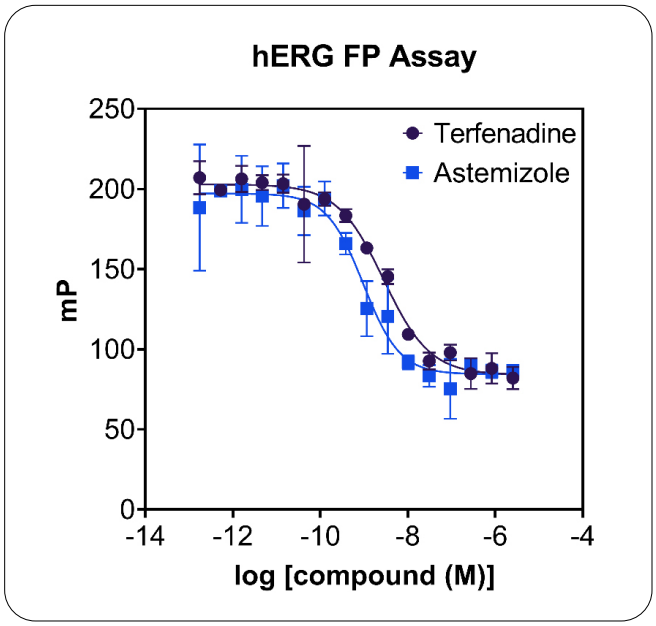


Figure 8: The compounds Astemizole and Terfenadine were tested in dose response experiments. The following IC_{50} values were determined: IC_{50} (Astemizole)= 0.97 nM and IC_{50} (Terfenadine)= 2.8 nM. For comparison, the assay manual5 reports an IC_{50} value of 1.9 nM for Astemizole. For each data point, the mean and standard deviation of three wells are shown.

Table 1: Assay statistics.

	Experiment 1	Experiment 2
Positive control, mP	82 ± 6	98 ± 9
Negative control, mP	195 ± 12	186 ± 17
Assay window, mP	113	88
Z prime	0.87	0.73
IC_{50} Astemizole	0.97 nM	0.51 nM
IC_{50} Terfenadine	2.8 nM	3.2 nM

Conclusion

We demonstrated the steps for FP protocol setup and optimization on the VICTOR Nivo and used the established measurement protocol for testing hERG inhibitors in dose response in two independent experiments. Using the protocol optimization steps described in this technical note, VICTOR Nivo's simple and flexible software enables users to quickly optimize FP assays. Software features such as the graph view for Z-focus scans and the applied G factor make it easy for users to determine the correct measurement height and to directly export the polarization values. Also, the innovative filter wheel with its built-in polarizing components makes it possible to use any Fluorescence Intensity filter combination for FP assays. No dedicated polarization filters are needed, only a second identical emission filter is required. In summary, this demonstrates that its ease of use of FP assays is a valuable addition to the VICTOR Nivo, along with its standard detection technologies.

References

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