APPLICATION NOTE

Demonstration of the capability of the EnVision Nexus plate reader in a fluorescence polarization assay.

Authors

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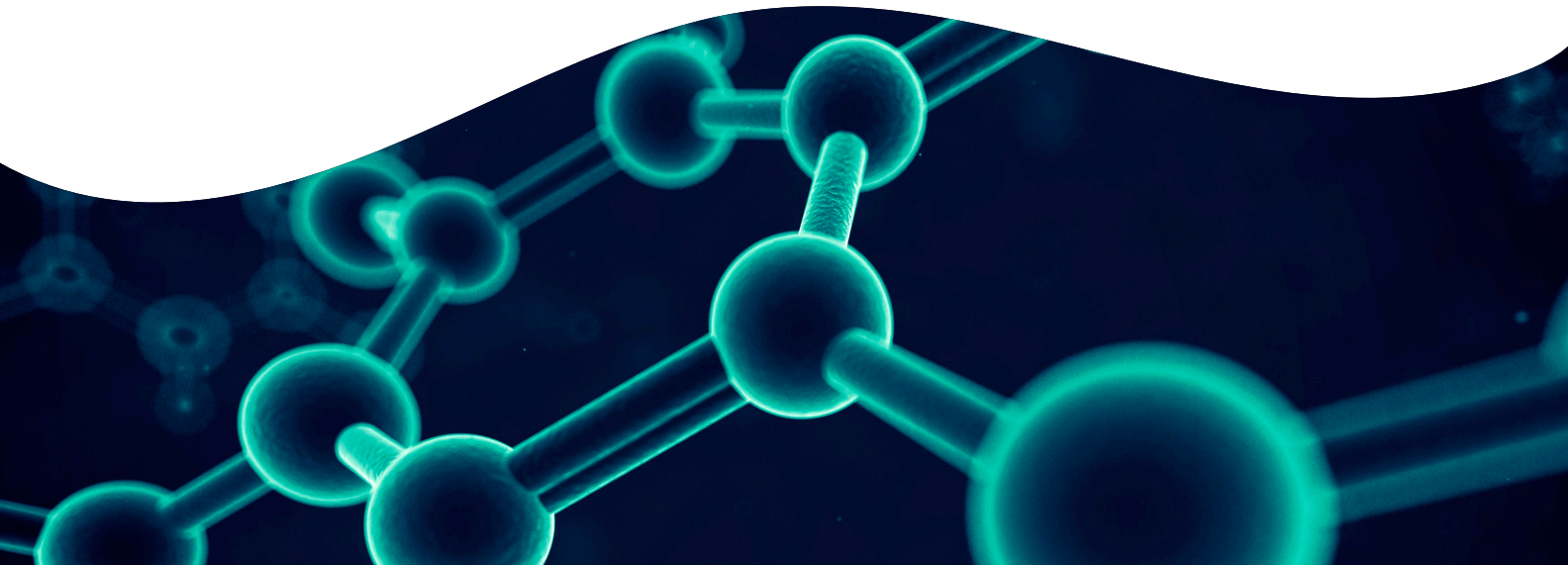
Introduction

The EnVision Nexus™ is a high-throughput multimode plate reader purpose built for speed and sensitivity. As the next generation of Revvity's EnVision™ plate reader technology, it continues to deliver the high sensitivity and optimal results expected from EnVision instruments. Standard filter-based technologies enable luminescence, absorbance, fluorescence intensity, fluorescence polarization, and time-resolved fluorescence (TRF) measurements. Optional add-on technologies are also available for Ultrasensitive Luminescence, Alpha (amplified luminescent proximity homogeneous assay), and a dedicated TRF Laser, making the EnVision Nexus a high-performance instrument for all assay applications. Fluorescence polarization technology measures changes in light polarization emitted by a fluorescent tracer in a sample and is quite different from fluorescence intensity, which measures the intensity of emitted light at a specific wavelength. Fluorescence polarization is a powerful tool used to study molecular interactions by monitoring the change in rotational mobility of fluorescently labeled molecules upon binding to a partner molecule. This application note demonstrates the capability of the EnVision Nexus to measure fluorescence polarization and compares it to the EnVision 2105.



Materials and methods

Instrumentation

The EnVision Nexus multimode plate reader was evaluated for its ability to measure fluorescence polarization using the Keap1:Nrf2 inhibitor screening assay kit (BPS Bioscience, #72020). The resulting fluorescence polarization values were compared to values obtained from an EnVision 2105 multimode plate reader.

Figure 1: Revvity's multimode plate readers: EnVision Nexus and EnVision 2105.

Fluorescence polarization assay information

In a fluorescence polarization assay, the excitation of a fluorophore bound to a small molecule with polarized light leads to the emission of depolarized light due to the rapid rotation rate of the small molecule complex. However, when the fluorophore is bound to a larger molecule, the rotation rate is slower, resulting in the emission of primarily polarized light. Fluorescence polarization is the measure of the molecular rotation that occurs in the time between excitation and emission. The measure of fluorescence polarization (mP) is calculated by the equation shown in Figure 2. Fluorescence polarization assays require polarized excitation, one mirror and two polarizing emission filters in the instruments, typically labelled in the software as S (parallel or straight) and P (perpendicular). The G-factor (g) is used to correct for the effects of optical components like filters, polarizers, and monochromators, which can affect polarization values. The G-factor setting can be used to match the polarization to literature values. This does not affect the change of measured polarization over the assay, characterized by delta mP.
In the experiments shown here, the G-factor was set to 1 for EnVision Nexus, where it is optional, but does not affect data quality. On EnVision, the G-factor is set during a gain-optimization, which is important for good data quality.

$$mP = \left(\frac{I_{//} - (g \times I_{\perp})}{I_{//} + (g \times I_{\perp})} \right) \times 1000$$

Figure 2: mP calculation where I// is the intensity with polarizers parallel to the excitation, I ⊥ is the intensity with polarizers perpendicular to the excitation, and g is the g-factor.

The Keap1:Nrf2 Inhibitor Screening Assay Kit (BPS Bioscience, #72020) is designed for the identification of inhibitors of Keap1:Nrf2 binding using fluorescence polarization. The manufacturer provided protocol was followed using a final assay volume of 50 µL in a black, 96-well ½-area OptiPlate (Revvity, #6002270). To determine the effect of the inhibitor on the formation of Keap1:Nrf2 complex, the Keap1 protein and the fluorescently labeled Nrf2 peptide are incubated with or without the test inhibitor for 30 minutes. The control small molecule inhibitor for this assay was KI696 (Fisher Scientific, #50-202-8648) which disrupts the interaction of Keap1 and Nrf2 and has a reported IC_{50} of 0.093 µM in the Keap1:Nrf2 inhibitor screening assay. Changes in the rotational mobility of the fluorescently labeled Nrf2 peptide are quantified using a plate reader capable of measuring fluorescence polarization. When the labeled Nrf2 peptide is bound to Keap1 protein in the absence of any inhibitor, the calculated mP value is high. In the presence of an inhibitor, the Nrf2 peptide is no longer bound to Keap1 and the calculated mP value is low.

Results and discussion

Fluorescence polarization results

Prior to running the Keap1:Nrf2 inhibitor screening assay, the z height (measurement height) was optimized for both instruments using 50 µL of a 1 nM fluorescein standard prepared from a 100 nM stock solution (Revvity, #C557-100) in a black, 96-well ½-area OptiPlate using the optimization option available in the software for each plate reader. After obtaining the optimized measurement height on each instrument, one assay plate was set up for manual verification by loading twelve replicate wells with 50 µL of 1 nM fluorescein per well. Measurement height settings above and below the optimized height were evaluated and the average mP value for fluorescence polarization was calculated as described in the methods section. The measurement height for each plate reader that yielded the tightest standard deviation for the twelve replicates was selected. In the case of the EnVision Nexus results seen in Figure 3, the original optimized height of 9 mm was selected as it performed the best.

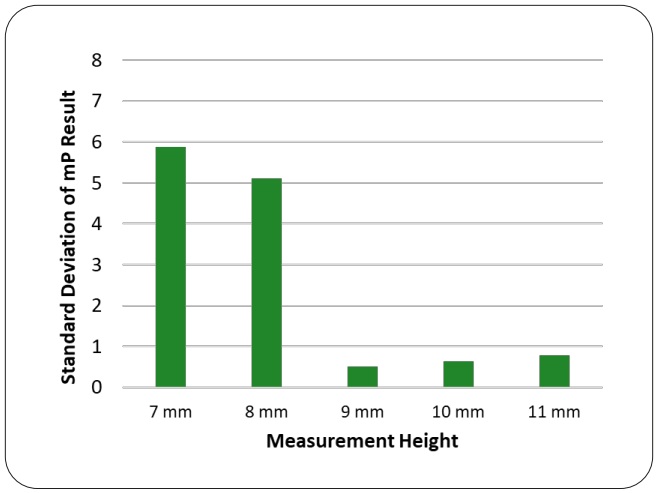


Figure 3: Measurement height verification on the EnVision Nexus using 1 nM fluorescein. Initial measurement height was determined by the software after multiple reads of a single well. Measurement heights above and below the initial optimal height in 1 mm increments were set up to test on twelve replicate wells. Standard deviation of the mP results at each manually tested measurement height are shown.

Figure 4 shows the average mP values on the EnVision Nexus are higher than the EnVision 2105, however the calculated IC_{50} values from the KI696 dose-response curves for each instrument were similar: EnVision Nexus 0.077 µM and EnVision 2105 0.074 µM. The calculated IC_{50} values were within an acceptable range of the manufacturer's example data (0.093 µM) for KI696 in the Keap1-Nrf2 inhibitor screening assay. The overall shift to lower mP values on the EnVision 2105 is caused by the different G-factor settings, this difference does not affect the information on the assay.

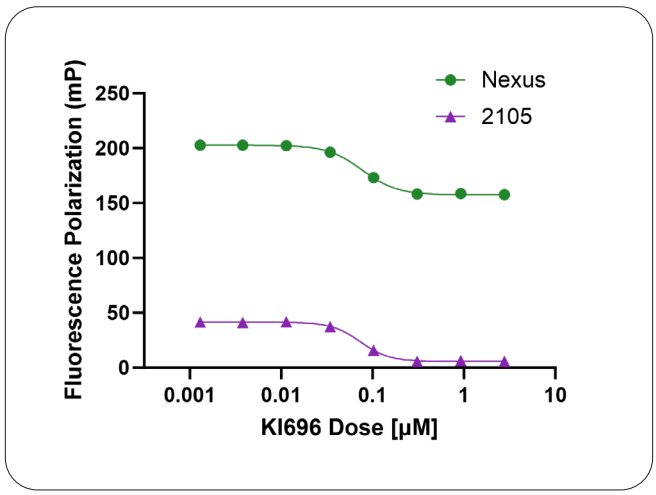


Figure 4: Fluorescence polarization results from the Keap1:Nrf2 inhibitor screening assay. IC_{50} values of the resulting curves were similar for both instruments: EnVision Nexus 0.077 µM and EnVision 2105 0.074 µM.

In addition to calculating the mP values in the Keap1:Nrf2 inhibitor screening assay, the delta mP value showing the overall change in fluorescence polarization was plotted to align the data more closely between instruments, also eliminating differences due to G-factor settings. Figure 5 shows similar curve fits for both instruments after calculation of the delta mP value where the negative control wells (no Keap1 protein) were subtracted from KI696 mP results. The EnVision Nexus showed a larger delta mP assay window, however the IC_{50} values remained similar: EnVision Nexus 0.077 µM and EnVision 2105 0.074 µM.

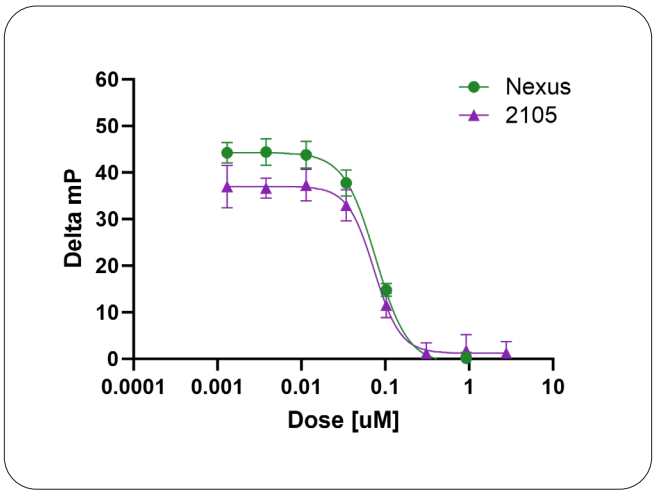


Figure 5: Delta mP results from the Keap1:Nrf2 inhibitor screening assay. The mP values for the negative control wells were subtracted from all values and the resulting delta mP value was plotted. IC_{50} values were similar for both instruments: EnVision Nexus 0.077 µM and EnVision 2105 0.074 µM.

Conclusions

The EnVision Nexus displayed superior fluorescence polarization performance which was achieved by utilizing the simple-to-follow default protocol and easy-to-install FITC fluorescence polarization filter module required by the assay. After optimizing the measurement height for each instrument, the Keap1:Nrf2 inhibitor screening assay was performed using the small molecule inhibitor KI696 as a control. When comparing the mP results of the KI696 dose-response curve (Figure 4), the absolute mP signal of the EnVision Nexus was higher than the EnVision 2105, however the curves were parallel resulting in similar IC_{50} values on par with the manufacturer's sample data. Normalizing the fluorescence values to delta mP (Figure 5) highlighted a benefit of the EnVision Nexus with a larger assay window and tighter standard deviation. Despite the differences in curve shape in the delta mP graph, the IC_{50} results for KI696 remained similar for both instruments. Overall, given the performance seen with the EnVision Nexus and considerations such as the Kaleido™ software ease-of-use and simple protocol set up, the EnVision Nexus delivers the speed and accuracy needed for fluorescence polarization assays.

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