revvity

APPLICATION NOTE

The EnVision Nexus multimode plate reader features sensitive HTRF detection technology.

Figure 1: EnVision Nexus multimode plate reader.

Introduction

In this application note, we use Revvity Homogenous Time-Resolved Fluorescence (HTRF®) immunoassay kits to demonstrate the performance of the new EnVision® Nexus™ multimode plate reader. Data from cell-based experiments and HTRF kit standard curves were collected and compared across the various time resolved fluorescence (TRF) modes (flash lamp, laser) available on the EnVision Nexus. Results demonstrate the EnVision Nexus continues to deliver the consistent and sensitive results expected from EnVision plate reader technology, with improved read speed for high throughput applications.

The EnVision Nexus, Revvity's newest multimode plate reader, builds on the 20+ year legacy of EnVision plate readers. Specifically designed for high-throughput screening, this next generation dual detector instrument excels in speed and sensitivity to reduce downtime and increase reliability. The EnVision Nexus comes standard with five filter-based technologies that enable absorbance, luminescence, fluorescence intensity, fluorescence polarization, and TRF measurements. Optional add-on technologies are available that enable ultrasensitive luminescence, Alpha, and a TRF laser, making the EnVision Nexus a high-performance plate reader compatible with all Revvity assay technologies (Alpha, HTRF®, LANCE®, and DELFIA®).

Materials and methods

The recently launched EnVision® Nexus™ multimode microplate reader (#HH36000002) was evaluated for its HTRF detection capabilities. Data were also collected from an established EnVision model, the EnVision multilabel plate reader, for reference. HTRF data was collected using flash lamp excitation which comes standard on the instruments, as well as using the TRF laser which is available as an optional add-on feature for fast and precise measurements. The EnVision Nexus features an UV Xenon flash lamp, and the EnVision includes a standard Xenon flash lamp. For laser excitation, the EnVision Nexus offers an optional dedicated low-maintenance, solid state laser and an "on-the-fly" detection mode for increased plate read speeds. The EnVision uses a traditional nitrogen-gas excitation laser for TRF measurements.

Instrument read time

To compare the speed and efficiency of the EnVision instruments, a reading time test was conducted in which a complete 384-well plate was measured using variable lamp and laser excitation settings on each instrument. One important difference to note between the plate readers is that the EnVision Nexus measures integration time in milliseconds whereas the EnVision measures integration time in flashes. 100 flashes on the EnVision is approximately equivalent to 433 msec on the EnVision Nexus. The lamp settings tested in this study included 433 msec integration time for the EnVision Nexus and 100 flashes for the EnVision. The laser settings tested for EnVision Nexus included integration times of 1100 msec, 1000 msec, 43 msec, or the "on-the-fly" mode (synchronized excitation and detection), while a setting of 50 flashes was used for the EnVision plate reader.

HTRF assays

HTRF® (Homogeneous Time-Resolved Fluorescence) is a simple, no-wash TR-FRET technology. It combines standard Fluorescence Resonance Energy Transfer (FRET) technology with time-resolved measurement of fluorescence, eliminating short-lived background fluorescence. In a sandwich HTRF assay, two antibodies that recognize the analyte of interest are used, with one antibody coupled to a donor (typically a Europium or Terbium cryptate) fluorophore and the other antibody tagged with an acceptor fluorophore (XL665 or d2 dye). If the two antibodies recognize the analyte, the donor will emit fluorescence upon excitation and the energy will be transferred to the nearby acceptor, resulting in specific acceptor fluorescence (Figure 2). The signal intensity is proportional to the number of antibody-analyte complexes formed.

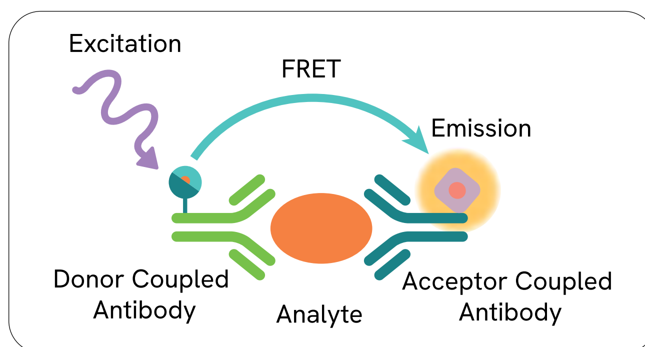

Figure 2: Schematic of HTRF assay technology (sandwich assay format) in which the analyte of interest is captured between two antibodies tagged with donor and acceptor fluorophores resulting in FRET which produces a measurable signal.

For this evaluation, HTRF assays performed represent several analyte targets within the Revvity HTRF portfolio (Table 1). All assays were sandwich format and used Europium donors to produce the fluorescence signal. Additional information related to the specific kits below can be found in their respective manual available on the Revvity website (www.revvity.com).

The EnVision Nexus multimode plate reader features sensitive HTRF detection technology.

Table 1: HTRF kits and sample types used for the EnVision Nexus evaluation.

Description	Analyte Family	Kit Name	Product Number	Samples Tested
IKKα is a key regulator of the NF-κB pathway downstream of several proinflammatory factors including interleukin-1, tumor necrosis factor alpha (TNFα) and toll-like receptor agonists.	Phosphorylation	HTRF Human Phospho-IKKα Ser176/180 Detection Kit	64KKAS6PEG	Cell lysate
TAR DNA binding protein 43 (TDP-43) is a nucleic acid binding protein involved in RNA-related metabolism. Aggregated TDP-43 has been identified as a hallmark of amyotrophic lateral sclerosis (ALS) and frontotemporal lobar dementia (FTLD), and more widely in several neurodegenerative diseases: TDP-43 proteinopathies.	Phosphorylation	HTRF Human Phospho-TDP43 Ser409/410 Detection Kit	64TDPS4PEG	Cell lysate
The MAPK/ERK signaling cascade is activated by a wide variety of receptors involved in growth and differentiation. ERK phosphorylates hundreds of cytoplasmic and nuclear substrates. The wide complexity and diversity of MAPK signaling makes ERK a key regulator and major signaling node in biology.	Phosphorylation	Advanced phospho-ERK (Thr202/Tyr204) cellular kit	64AERPEG	Cell lysate
IL1 beta, also known as leukocytic pyrogen or mononuclear cell factor, is a pro-inflammatory cytokine of the IL1 family. IL1 beta is considered to be a major endogenous pyrogen and induces prostaglandin synthesis, T cell activation, B cell activation, and subsequent antibody production. It is also a known promoter of T cell differentiation into Th17.	Cytokine biomarker	Human IL1 beta kit	62HIL1BPEG	Standard curve

Phosphorylation assays

Phosphorylated protein concentrations from cell lysates were measured using three HTRF kits: Phospho-IKKα (#64KKAS6PEG), Phospho-TDP43 (#64TDPS4PEG), and Advanced Phospho-ERK1/2 (#64AERPET). The Phospho-IKKα kit uses a sandwich HTRF assay format to enable the simple, rapid, and direct detection of endogenous cellular IKKα when phosphorylated at Ser176 and Ser180. Similarly, the Phospho-TDP43 kit is intended for the direct detection of endogenous levels of TDP-43 in cells, only when phosphorylated at Ser409/410. The Advanced Phospho-ERK1/2 kit is a sandwich assay used for the detection of endogenous levels of ERK1/2 in cells when phosphorylated at Thr202 and Tyr204.

To evaluate the plate reader measurements, serial dilutions of cell lysate samples were prepared for each assay from HeLa cells (Phospho-IKKα and Phospho-TDP43) or HEK293T cells (Phospho-ERK) cultured in a T175 flask at 37 °C, 5% CO2 to at least 80% confluency. For the Phospho-IKKα assay, HeLa cells were stimulated with a cocktail of 100 nM calyculin A (Tocris #1336) and 2 nM IL-1β (R&D Systems #201-LB) for 15 minutes in complete cellular medium. Cell medium was removed before addition of 3 mL of lysis buffer (LB #1). For the Phospho-TDP43 assay, HeLa cells were stimulated by calyculin A (100 nM) in complete cell culture medium for 30 minutes before removal of cell medium and addition of 3 mL of complete lysis buffer. Finally, for the Advanced Phospho-ERK1/2 assay, HEK293T cells were stimulated with 50 nM mouse EGF (mEGF; Sigma #SRP3196) in complete cell culture medium for 5 minutes. The cell culture medium was removed before adding 3 mL of complete lysis buffer. Following lysis, cell lysates were collected and serially diluted for all assays. Diluted lysates were then analyzed by adding 16 µL to a low-volume 384-well plate along with 4 µL of detection reagent mix and incubated overnight at room temperature before reading the plates on both EnVision instruments using lamp and laser excitation modes.

To gather additional experimental data to evaluate the EnVision Nexus plate reader measurements, dose-response curves were generated using compound stimulated cells for the Phospho-TDP43 and Advanced Phospho-ERK assays. For the Phospho-TDP43 assay, HeLa cells were plated to a density of 50,000 cells/well in a 96-well plate. Following an overnight incubation, cells were stimulated with doses of calyculin A (range: 0.1-200 nM) diluted in cell culture medium for 30 minutes at 37 °C, 5% CO2. The culture medium was then removed, and 50 µL of lysis buffer added. The lysate was collected and 16 µL was transferred to a 384-well plate and combined with 4 µL of detection reagent. The assay was incubated overnight at room temperature before measuring the HTRF signal with both EnVision plate readers using lamp excitation. For the Advanced Phospho-ERK assay, HEK293 cells were added to a 96-well plate at a density of 50,000 cells/well. After an overnight incubation, the cells were dosed with mEGF prepared in cell culture medium (doses: 0.03-50 nM) for 5 minutes at 37 °C, 5% CO2, after which media was removed and 50 µL of lysis buffer was added. A 16 µL volume of the collected lysates were transferred to a 384-well low-volume plate and combined with 4 µL of detection reagent mix. The plate was incubated for 4 hours at room temperature before reading on both EnVision instruments.

Cytokine biomarkers

The IL1β kit (#62HIL1BPEG) detects the cytokine IL1β in a sandwich assay format. For this assay, the measurement data consisted of the IL1β analyte standard curve, which was prepared as outlined in the kit product insert. Readings were then taken from a 384-well low-volume plate following lamp and laser excitation on both plate readers.

Assay reading parameters and data analysis

Filters and reading parameters used to generate the assay data are listed in Table 2 . Emission signals were collected at both 620 nm (donor) and 665 nm (acceptor) wavelengths. The HTRF Ratio was then calculated as: (665 nm/620 nm) x 104. The Delta Ratio (ΔR) was obtained by subtracting the background or negative control signal from the signal of each data point (Ratio signal - Ratio negative). Since these raw data signals are reader dependent, they have limited use for comparing the performance of separate instruments. Therefore, two reader independent parameters were calculated to compare the signal measurements between the EnVision instruments, including Delta F (%) and Signal to Background (S/B) ratio. Delta F (%) was calculated as (Delta Ratio (ΔR) / Ratio negative) x 100. The S/B was calculated as (Ratio signal / Ratio negative) and used to plot the assay data. For experimental cell-based assay data, the Fold of Change (FOC) and IC50 or EC50 measures were calculated to confirm similar values were generated from the data curves produced by each plate reader.

Table 2: Plate reader settings and filter modules part numbers used for generation of HTRF data.

Plate Reader	Excitation Source	Integration Time/ Flash Number	Filter Module Part Number
EnVision Nexus	Lamp	433 msec	HH36794002
	Laser	1100 msec	HH36794003
EnVision	Lamp	100 flashes (equivalent to 433 msec)	-
	Laser	50 flashes (equivalent to 1100 msec)	-

Results and discussion

Instrument read time

For both lamp and laser excitation, the EnVision Nexus demonstrated superior speed and performance, particularly using the “on the fly” laser mode, which completed an entire read of a 384-well plate in less than 30 seconds (Table 3). The “on the fly” mode works by synchronizing excitation and detection capabilities for increased plate reading speed. For the other laser settings, the EnVision Nexus exhibited read times that were approximately 1.5-12 times faster (depending on the integration time selected), compared to the EnVision, which required 12.5 minutes to read a full plate. The EnVision Nexus reading time was over 1 minute faster than the EnVision when using lamp excitation at comparable reading parameters (integration time to number of flashes).

Table 3: Comparison of the time required to read a full 384-well plate on the EnVision Nexus versus the EnVision using lamp and laser excitation.

Plate Reader	Excitation Source	Integration Time/ Flash Number	Reading Time (min:sec)
EnVision Nexus	Lamp	433 msec	3:25
	Laser	1100 msec	7:54
	Laser	1000 msec	7:15
	Laser	43 msec	0:47
	Laser	On the fly	0:08
EnVision	Lamp	100 flashes (equivalent to 433 msec)	4:39
	Laser	50 flashes (equivalent to 1100 msec)	12:19

Phosphorylation assays

For all phosphorylation assays, the EnVision Nexus displayed an improved S/B ratio relative to the EnVision when using lamp excitation (Figure 3). With lamp excitation, the S/B measurements for diluted cell lysates were on average 6.2% higher (range: 0.0 – 11.5%) for the Phospho-IKKα assay; 13.0% higher (range: 0.0 – 20.9%) for the Phospho-TDP43 assay; and 12.7% higher (range: 0.0 – 20.4%) for the Advanced Phospho-ERK assay on the EnVision Nexus compared to the EnVision.

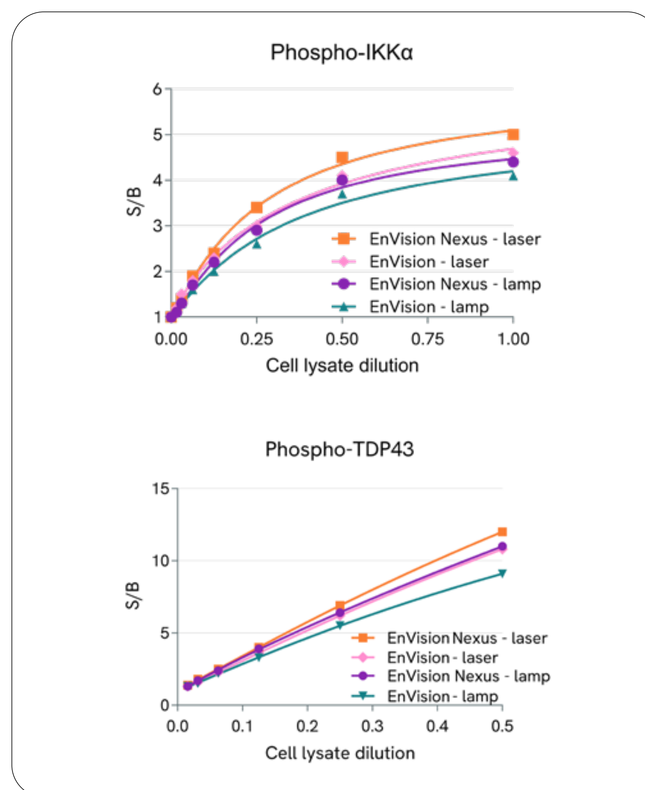

Figure 3: Comparison of lamp and laser excitation generated S/B measurements of Phospho-IKKα and Phospho-TDP43 from diluted HeLa cell lysates.

When comparing data generated by the EnVision Nexus using lamp excitation versus laser excitation, there was little difference (~1%) between the S/B values on the Advanced Phospho-ERK assay (Figure 4), suggesting that equivalent results were obtained using either excitation mode for this assay. However, for the Phospho-IKKα and Phospho-TDP43 assays, laser excitation improved the S/B by an average of 11.6% (range: 7.7-17.2%) and 6.2% (range: 2.6-9.1%), respectively, compared to lamp excitation (Figure 3), demonstrating that the use of EnVision Nexus laser excitation may improve sensitivity for some HTRF assays.

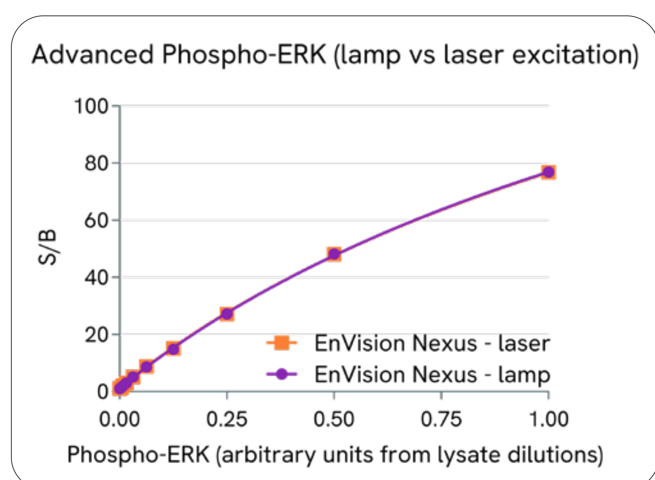

Figure 4: Comparison of EnVision Nexus lamp versus laser excitation results from the Advanced Phospho-ERK assay.

In general, measurements from the dose-response curves of stimulated cells were similar across readers and excitation modes for the phosphorylation assays. As expected, the calculated EC50 of the phosphatase inhibitor calyculin A was similar across instruments, with an EC50 = 47 nM measured with the EnVision Nexus and EC50 = 45 nM measured with the EnVision (using lamp excitation), indicating good agreement between the readers for the Phospho-TDP43 assay. In addition, a slight improvement to the calculated FOC was observed with the EnVision Nexus (FOC = 8) compared to the EnVision (FOC = 6.9), which aligns with the improvement in HTRF assay sensitivity observed with the diluted cell lysates for the Phospho-TDP43 assay. Likewise, for the Advanced Phospho-ERK assay, calculated compound efficacy for mEGF demonstrated good agreement across plate readers and excitation modes (Table 4). However, there was no difference in the FOC values between the plate readers (Table 4), despite the increased sensitivity of the EnVision Nexus measurements with diluted cell lysates. For this assay, the FOC was not impacted due to the presence of high basal levels of phosphorylation in non-stimulated HEK293 cells. Thus, the observed improvement to the FOC may be limited by biological factors (e.g., basal phosphorylation levels), regardless of reader sensitivity.

Table 4. Compound efficacy and FOC for mEGF stimulated HEK293 cells on the Advanced Phospho-ERK HTRF assay, as measured by lamp and laser excitation with both EnVision instruments.

Plate Reader	EC20 (nM)	EC50 (nM)	EC80 (nM)	Fold of Change
EnVision Nexus Lamp	0.33	4.5	8.4	4.1
EnVision Nexus Laser	0.35	4.6	8.7	4.1
EnVision Lamp	0.29	4.9	9.0	4.2
EnVision Laser	0.34	4.7	8.6	4.0

EnVision Nexus - lamp

EnVision Nexus - laser

EnVision - lamp

EnVision - laser

HTRF Ratio

log [mEGF] M

Figure 5: Comparison of EC50 values (dotted line) for mEGF stimulated HEK293 cells on the Advanced Phospho-ERK HTRF assay. EC50 values were consistent across plate readers and excitation sources.

Cytokine biomarkers

For the IL1β assay, with lamp excitation the EnVision Nexus produced an average increase in Delta F signal of 9.6% (range: 4.9-26.2%) relative to the EnVision, suggesting a mild improvement in sensitivity (Figure 6). The EnVision Nexus laser excitation mode yielded similar results to excitation using the lamp, indicating equivalent sensitivity can be achieved using either excitation mode of the EnVision Nexus for this assay type.

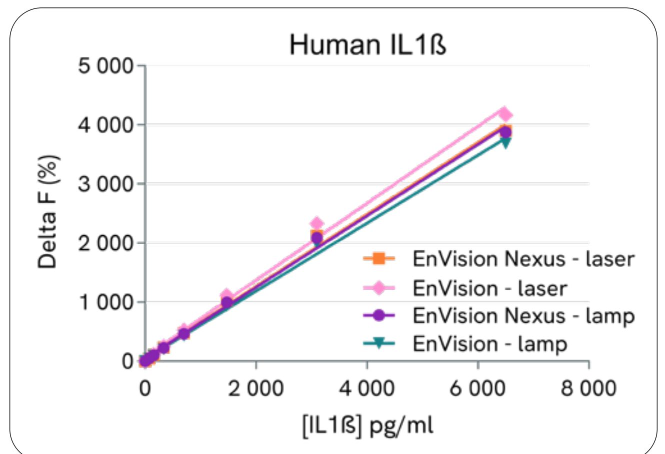

Figure 6: Comparison of Delta F (%) signal produced with lamp and laser excitation by the EnVision Nexus and EnVision plate readers.

The EnVision Nexus multimode plate reader features sensitive HTRF detection technology.

Conclusion

The EnVision Nexus multimode plate reader demonstrated equivalent or improved performance relative to earlier EnVision plate reader technology across multiple analytes (Table 5). In particular, the EnVision Nexus produced results at faster read speeds without negatively impacting sensitivity using both lamp and laser excitation sources. The 'on the fly' reading mode can produce a full 384-well plate dataset in less than 30 seconds, making this instrument well-suited for high throughput screening applications. For some assays, using the standard TRF flash lamp excitation mode resulted in data that was equivalent to laser excitation, however, for certain analytes and assay systems, the dedicated TRF laser may offer improved assay sensitivity. Overall, the EnVision Nexus builds on the strong legacy of robust and sensitive EnVision plate reader technology, while improving read speeds to meet the needs of high throughput labs.

Table 5: Assessment of EnVision Nexus versus EnVision measurements using lamp excitation, and laser versus lamp excitation data for the EnVision Nexus.

Analyte Family	Kit Name	EnVision Nexus vs EnVision lamp excitation	EnVision Nexus laser vs lamp excitation
Phosphorylation	HTRF Human Phospho-IKKα Ser176/180 Detection Kit	Improved	Improved
Phosphorylation	HTRF Human Phospho-TDP43 Ser409/410 Detection Kit	Improved	Slightly improved
Phosphorylation	Advanced phospho-ERK (Thr202/Tyr204) cellular kit	Improved	On par
Cytokine biomarker	Human IL1 beta kit	On Par	On par

revvity

Revvity, Inc.
940 Winter Street
Waltham, MA 02451 USA
www.revvity.com

For a complete listing of our global offices, visit www.revvity.com
Copyright ©2023, Revvity, Inc. All rights reserved.