APPLICATION NOTE

Excellent performance from the EnVision Nexus multimode plate reader in two wash-based assays: absorbance-based ELISA and DELFIA TRF.

Authors

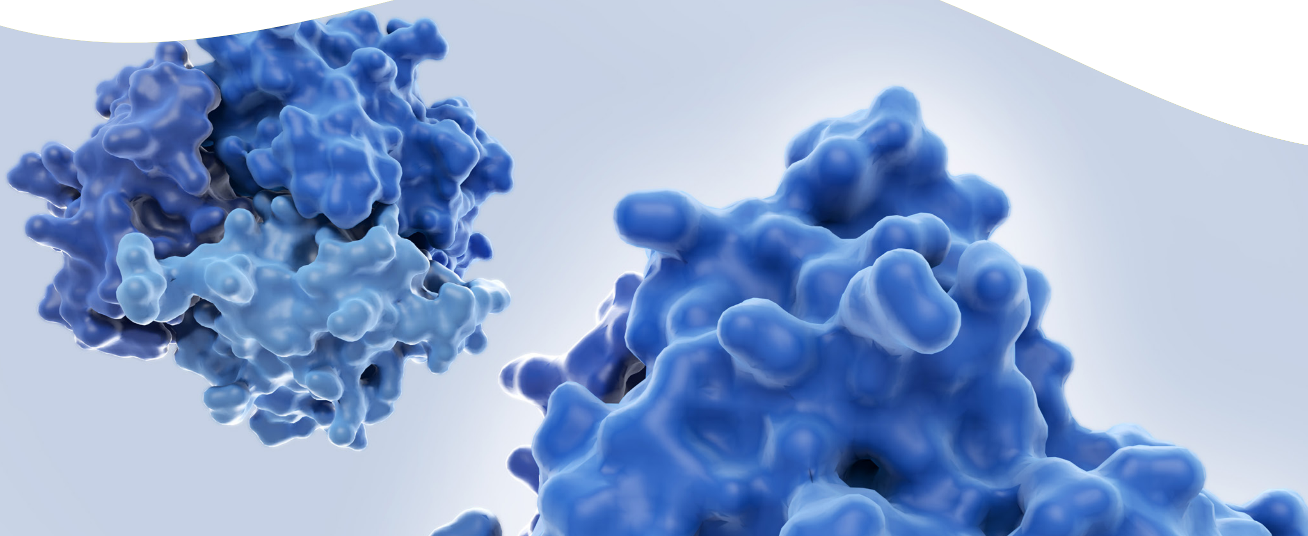
Adam Carlson
Katie Graham
Revvity, Hopkinton MA

Introduction

The EnVision® Nexus™ is a high-throughput multimode plate reader 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 modules enable luminescence, absorbance, fluorescence intensity, fluorescence polarization, and time-resolved fluorescence (TRF) measurements. Optional add-on modules 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.

This application note demonstrates the capability of the EnVision Nexus to measure standard absorbance from an ELISA (enzyme-linked immunosorbent assay) and TRF in the form of DELFIA® (dissociation-enhanced lanthanide fluorescence immunoassay). Both assay formats are wash-based sandwich assays with similar protocols and utilize tumor necrosis factor alpha (TNFα) as the target. The same pair of anti-TNFα antibodies was used in both the ELISA and DELFIA for the capture and detection steps (see materials and methods for information on DELFIA conversion from an existing ELISA). Results from both assay formats include a TNFα standard curve and interpolation of unknown TNFα concentrations from cell supernatants.

For research purposes only. Not for use in diagnostic procedures.



Materials and methods

Instrumentation

The newly launched EnVision Nexus multimode microplate reader (#HH36000002) was evaluated for its ability to read absorbance using filter-based modules typical of an ELISA: 450 nm and 570 nm. A separate filter module was required to measure the absorbance at each wavelength (see Table 1). The EnVision Nexus was also tested for its TRF capabilities by measuring a DELFIA assay. A dedicated filter module for TRF was used along with the factory-installed flash lamp for the excitation of the samples (excitation at 330 nm and emission at 615 nm).

Table 1: EnVision Nexus filter modules required for Absorbance and TRF (flash lamp)

Assay	Filter Module	Description	Catalog Number
ELISA	ELISA TMB	Abs 450 nm	HH36796002
ELISA	MTT Assay	Abs 570 nm	HH36796010
DELFIA	DELFIA	TRF 330 nm	HH36794001

Figure 1: Revvity's new multimode plate reader: EnVision Nexus.

ELISA information

ELISA MAX™ Deluxe Set Human TNFα (BioLegend, #430204) was performed following the manufacturer's protocol (see Figure 2A for assay schematic). The ELISA MAX Deluxe Set includes the anti-TNFα capture antibody, a biotinylated anti-TNFα detection antibody, and avidin-HRP as well as the assay sample diluent, coating buffer and horseradish peroxidase (HRP) detection reagents. Additional products required that are not provided with the kit include 96-well clear microwell assay plates (BioLegend, #423501), ELISA Stop Solution (BioLegend, #423001), and wash buffer made from PBS (Thermo Fisher, #14190-250) with Tween-20 (Fisher Scientific, #PI28320) at 0.05% final. The TNFα standard curve and samples were tested in 100 µl volume in triplicate. Cell supernatant samples were tested at a range of dilutions (neat, 1:10, 1:25, and 1:100) to find one dilution series that falls within the linear optical density (OD) range of the standard curve as is typical with absorbance-based assays. The results presented below are from the 1:10 dilution, prepared in Assay Diluent A. All wash steps were performed manually in a laboratory sink using the dump and blot method. Once the assay was complete, the microwell plate was read within 10 minutes of adding the stop solution. Soft plate moving was enabled on the instrument due to the large final volume in the assay plate (200 µL) to avoid sloshing of the samples and potential fluctuations in the absorbance reads. The absorbance of each sample was measured sequentially, first at a wavelength of 450 nm, followed by 570 nm (reference). The absorbance at 570 nm was subtracted from the absorbance at 450 nm to obtain a reference corrected value. This is a common practice to handle potential plate artifacts that can interfere with absorbance-based reads, such as scratches on the assay plate, and was recommended in the kit description. The average zero (background) was then subtracted from the reference corrected value to calculate the final OD, used to construct the TNFα standard curve. The TNFα standard curve was plotted in GraphPad Prism on a log-log axis and a 4-parameter logistic curve fit was used to interpolate concentrations of the unknown samples.

DELFIA information

DELFIA is a wash-based assay technology that was setup using the same anti-TNFα capture and biotinylated anti-TNFα detection antibodies from the ELISA MAX Deluxe Set Human TNFα (BioLegend, #430204). The assay followed a simple conversion protocol described in the Revvity Application Note[1]. Figure 2B shows the DELFIA schematic. In brief, after incubating the assay plate with a biotinylated detection antibody, the avidin-HRP reagent provided in the ELISA kit was replaced with a europium-labelled streptavidin (Eu-SA, Revvity, #1244-360). After a final wash step, DELFIA Enhancement Solution (Revvity, #1244-105) is added to release and activate the europium into solution in the assay wells. This change from the ELISA assay protocol converts the assay readout from absorbance (based on a colorimetric HRP reaction) to a fluorescent readout (based on the europium lanthanide). Europium is excited by a flash lamp or laser at 340 nm with emission detected at 615 nm using TRF settings. Additional materials required for DELFIA that are not included in the ELISA kit are DELFIA assay plates (Revvity, #AAAND-0001), PBS (Thermo Fisher, #14190-250) used to dilute the capture antibody and coat the assay plates, DTPA purified BSA (Revvity, #CR84-100) diluted in PBS to 1% final to block non-specific binding, DELFIA Wash Buffer (Revvity, #1244-114), and DELFIA Assay Buffer (Revvity, #1244-111) used to dilute the standard curve and biological samples. The results presented below are from a 1:10 sample dilution. All wash steps were performed using a BioTek 405TS plate washer set to dispense 300 µL per cycle (with three cycles per wash step) and aspirate the plate fully. Once the assay was complete, 200 µL of DELFIA Enhancement Solution was added. The DELFIA assay plate was covered with a lid and placed on a DELFIA orbital plate shaker (Revvity, #1296-003), on the slow setting (~600 rpm) and shaken for 10-15 minutes. Soft plate moving was set on the instrument due to the large final volume in the assay plate (200 µL) to avoid sloshing of the samples and potential fluctuations in the TRF read. TRF was measured using the EnVision Nexus default instrument settings for flash lamp excitation (60 µsec delay, 400 µsec counting window). The delay between sample excitation and capture of the resulting fluorescence intensity signal reduces the background autofluorescence of the assay plates and yields excellent signal to background results. Final TRF values for the TNFα standard curve were generated by subtracting the average zero (background) from individual TRF values. The TNFα standard curve was plotted in GraphPad Prism on a log-log axis and a linear regression curve-fit was used to interpolate concentrations of the unknown cell supernatant samples.

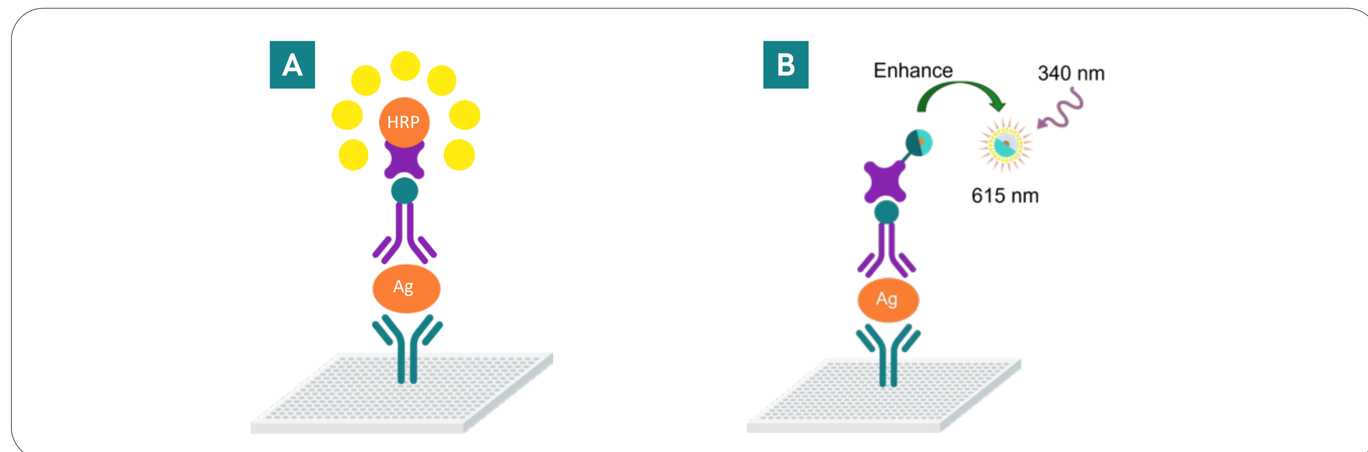


Figure 2: Assay schematics for (A) ELISA and (B) DELFIA. Both assays utilize a capture antibody adsorbed to a high bind assay plate. After binding the antigen of interest, biotinylated detection antibody is added to complete the sandwich assay. In an ELISA, avidin-HRP is used to generate the colorimetric signal in combination with a TMB substrate. In DELFIA, streptavidin-Europium is used to generate the fluorescent signal after addition of DELFIA enhancement solution to release Europium into solution in the assay plate.

Preparation of biological samples

THP-1 monocyte cells (ATCC, #TIB-202) were grown in suspension in T75 flasks (VWR, #BD353136) in RPMI Media (ATCC, #30-2001) supplemented with 10% heat-inactivated FBS (Thermo Fisher, #A3840002). Cell differentiation and subsequent conversion to an adherent cell morphology was achieved by treatment with phorbol 12-myristate 13-acetate (PMA, Fisher Scientific, #MP218388201) at 5 ng/mL in 6 well dishes (VWR, #82050-842) at a density of 1 x 10^6 cells/well for 48 hours following guidelines for differentiation from Park et al.[2] TNFα production was stimulated with a lipopolysaccharide solution (LPS, Fisher Scientific, #50-112-2025) of 100 ng/mL final in assay wells for 6 or 24 hours. After stimulation, cell supernatants were carefully collected, centrifuged briefly to remove any remaining cells and frozen in 0.25 mL aliquots at -20 °C until assayed. Samples included untreated wells at both the 6-hour and 24-hour time points to measure baseline TNFα levels produced by the differentiated THP-1 cells.

Results and discussion

ELISA results

The EnVision Nexus successfully measured absorbance data from both the 450 and 570 nm wavelengths. The standard curve for TNFα is shown in Figure 3 plotting concentration in pg/mL versus absorbance OD (reference read corrected). Curve fitting performed in GraphPad Prism showed an R^2 value of 0.9979 indicating an excellent fit.

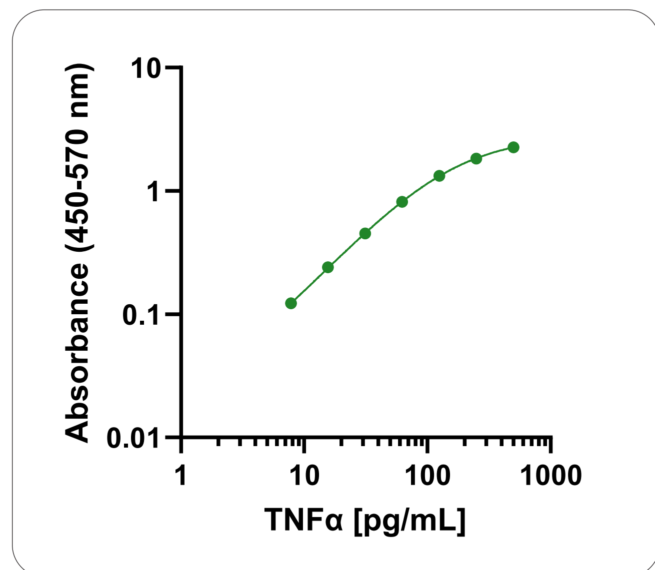


Figure 3: ELISA TNFα standard curve. Blank corrected OD results.

THP-1 supernatant samples were run at four dilutions and the results of the 1:10 dilution are shown in Figure 4. This dilution was chosen because all sample OD values fell within the linear range of the ELISA standard curve. Low levels of TNFα are present in the samples after differentiation without any stimulation. The 24-hour untreated values are higher than the 6-hour untreated values suggesting TNFα was slowly released into the supernatant. LPS induction strongly increased the TNFα levels in the samples and this effect was transient with 6-hour levels higher than those at 24 hours.

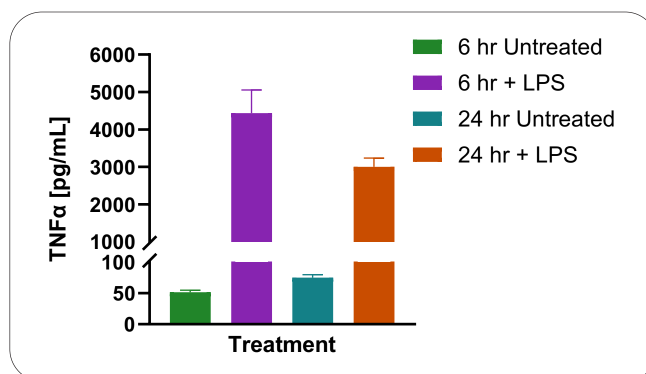


Figure 4: ELISA results from THP-1 cell supernatants. The supernatant samples were diluted 1:10 in Assay Diluent A and values interpolated from the TNFα standard curve. LPS induced TNFα production above the baseline at 6 and 24 hours.

DELFIA results

The EnVision Nexus effectively measured DELFIA TRF signal in the assay plate with default settings for TRF assays using the flash lamp. The standard curve for TNFα is shown in Figure 5 plotting concentration in pg/mL versus blank corrected TRF signal. Curve fitting in GraphPad Prism showed an R^2 value of 0.9977 indicating an excellent fit. The DELFIA assay format has a much larger dynamic range than a typical absorbance-based ELISA often allowing to extend the standard curve range at both the top and bottom of the curve. In these experiments a simple conversion from the ELISA was performed and the same concentrations of TNFα analyte were used so no extension of the dynamic range was displayed.

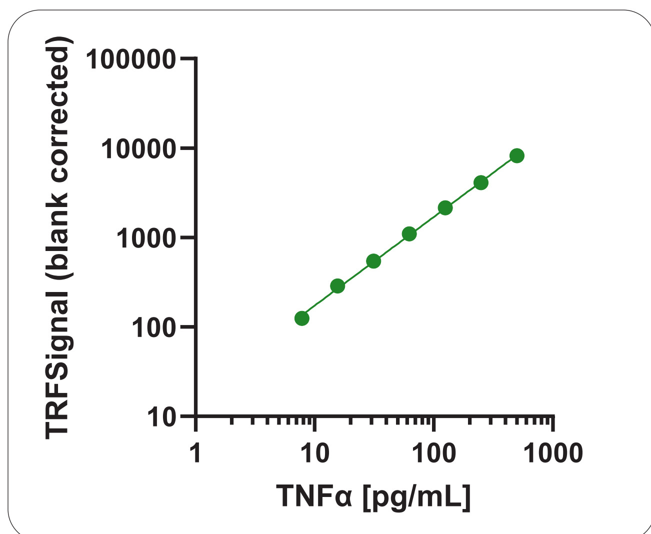


Figure 5: DELFIA TNFα standard curve. Blank corrected TRF results.

The THP-1 cell supernatant results from the 1:10 dilution are shown in Figure 6. The interpolated TNFα concentrations were similar between the ELISA and DELFIA. Consistent with the ELISA data, DELFIA measured a low-level baseline of TNFα present in the untreated controls, with higher levels at 24 hours than at 6 hours. LPS treatment induced a strong increase in TNFα in the supernatants with 6-hour treatment higher than the 24-hour treatment suggesting a transient effect on TNFα levels.

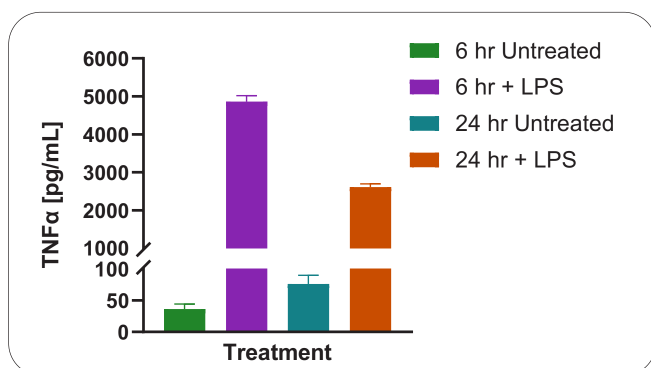


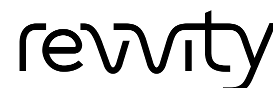
Figure 6: DELFIA results from THP-1 cell supernatants. The supernatant samples were diluted 1:10 in DELFIA Assay Buffer and values interpolated from the TNFα standard curve. LPS induced TNFα production above baseline at 6 and 24 hours.

Conclusions

The EnVision Nexus displayed strong performance with two wash-based immunoassays, one absorbance and one time-resolved fluorescence readout. Both the ELISA and DELFIA TNFα standard curves showed an excellent R^2 fit in GraphPad Prism. In general, DELFIA has the advantage of improved dynamic range relative to an absorbance-based ELISA (often up to a two-log increase) which can extend the standard curve range at both the top and bottom of the curve before seeing analyte saturation. This was not showcased here as a simple conversion was performed using the same concentrations for the TNFα standard curve as in the ELISA. In addition, each assay included the same THP-1 cell supernatant samples with unknown concentrations for testing. The ELISA and DELFIA assays correlated well resulting in similar interpolated values for all four samples tested (untreated versus LPS induction at 6 hours and 24 hours). To maximize the plate reading performance of the EnVision Nexus, the optional soft moving of the plate carrier arm was turned on to avoid sloshing of the high final assay volume for both assays (200 µL in a 96-well plate), which can improve accuracy. The EnVision Nexus assay protocols were easy to set up and run for both assay types when utilizing the correct filter module and the default instrument settings for each technology. Overall, the EnVision Nexus carries on the excellent performance expected from Revvity's line of multimode plate readers.

References

1. Revvity Application Note: Simple Conversion of ELISA to Revvity's High Sensitivity DELFIA Technology
2. Park E.K. et al. (2007) Optimized THP-1 differentiation is required for the detection of responses to weak stimuli. *Inflammation Research*; 56.



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

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