APPLICATION NOTE

Excellent sensitivity from the EnVision Nexus multimode plate reader for AlphaLISA detection across a variety of assay formats.

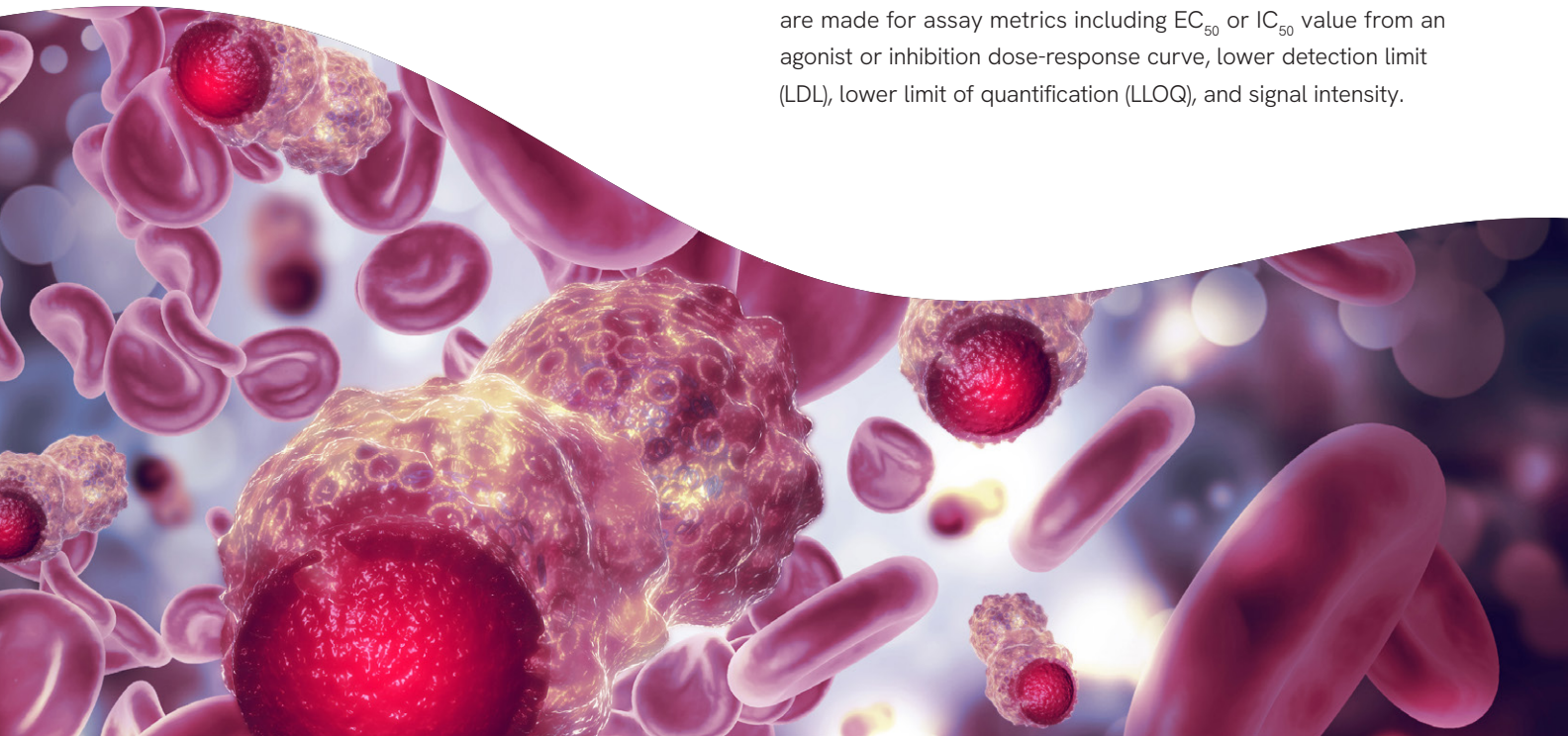
Authors

Adam Carlson
Dan Cardillo
Revvity, Hopkinton MA

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

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 delivers the high sensitivity and optimal results expected from EnVision instruments. The EnVision Nexus works with all standard microplates, including 24-, 48-, 96-, 384- and 1536-well formats. This state-of-the-art plate reading platform supports both standard and advanced detection technologies with excellent dynamic range, kinetic and scanning measurements, and bottom reading. 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 AlphaLISA® detection capabilities of the EnVision Nexus and compares it to the EnVision® 2105. Three different AlphaLISA formats were used to evaluate AlphaLISA detection and sensitivity measurements: High Performance Cytokine AlphaLISA kits, an AlphaLISA Binding Kit, and AlphaLISA® *SureFire® Ultra™* kits. The AlphaLISA assays were run in parallel on the two instruments using the "Enhanced Alpha" mode on the EnVision Nexus and "Standard Alpha" mode on the EnVision 2105. These two read modes are treated as equivalent as they are both the baseline Alpha read mode available on the reader. Comparisons are made for assay metrics including EC_{50} or IC_{50} value from an agonist or inhibition dose-response curve, lower detection limit (LDL), lower limit of quantification (LLOQ), and signal intensity.



Materials and methods

Instrumentation

The EnVision Nexus multimode microplate reader was evaluated for its ability to measure AlphaLISA using the Enhanced Alpha mode including the optional add-on Alpha laser and the Enhanced Alpha aperture. The aperture sits above the assay well and directs light to the PMT detector while blocking signal from neighboring wells. This helps reduce crosstalk issues that can be seen with other plate readers. Results from the EnVision Nexus in enhanced Alpha mode were compared to the EnVision 2105 multimode plate reader, which also contains an Alpha laser for Standard Alpha mode. Both instruments use filter and mirror-based detection of the AlphaLISA signal. Data was collected using an AlphaLISA filter (emission 615 nm +/- 8 nm) or the broadband signal capturing AlphaScreen® filter (emission 570 nm +/- 100 nm) for comparison of signal intensity.

Figure 1: Revvity's EnVision line of multimode plate readers (left to right): EnVision 2105 and EnVision Nexus.

AlphaLISA assay setup

AlphaLISA immunoassays offer a homogenous (no-wash) alternative to ELISAs, with a simple, streamlined workflow that can be used to detect and quantitate biomolecules in both simple and complex sample types. AlphaLISA is a bead-based luminescent amplification assay, offering greater sensitivity, a wider dynamic range, and smaller sample sizes over a traditional absorbance-based ELISA as well as other immunoassay techniques. Common applications for AlphaLISA are the detection of biomarkers, characterization of protein kinases, cytokines, and GPCRs, as well as epigenetics and protein-protein interactions.

Due to the reduced final volume of each AlphaLISA assay format (20 µL) from the standard protocol (50 µL), white ProxiPlate-384 Plus plates (#6008280) and light gray, AlphaPlate-384 shallow well plates (#6008250) were used. Both plate types are manufactured with shallow wells to bring the sample closer to the plate reader's detector and yield excellent results when low assay volumes are implemented. Gray AlphaPlates were specifically designed for AlphaLISA assays to reduce well-to-well cross talk and background fluorescence compared to white assay plates. One assay plate was prepared per instrument type, read type (AlphaLISA vs AlphaScreen*), and plate color resulting in twelve assay plates tested in total. The three High Performance Cytokine assays were run on the same plate as the Cereblon binding assay on Day 1. A separate set of plates was prepared for AlphaLISA *SureFire Ultra* testing on Day 2.

*AlphaScreen settings are acceptable to capture AlphaLISA results due to the broad range of detection on the EnVision reader settings (570 nm, bandwidth 100 nm) however AlphaLISA settings (615 nm, bandwidth 8 nm) allow the user to multiplex if so desired.

AlphaLISA High Performance Cytokine assay format

AlphaLISA High Performance Cytokine assays use two unique monoclonal antibodies specific for the cytokine of interest. One monoclonal antibody is biotinylated and binds to the streptavidin coated Alpha Donor bead whereas the AlphaLISA Acceptor bead is conjugated directly with an additional monoclonal antibody. In the presence of a cytokine the monoclonal antibodies in the assay bind to the cytokine, bringing both the Donor and Acceptor beads into proximity. Excitation of the Donor beads at 680 nm provokes the release of singlet oxygen that triggers a cascade of energy transfer reactions in the nearby Acceptor beads, resulting in a sharp peak of light emission at 615 nm (Figure 2). AlphaLISA assay signal generated is proportional to the amount of cytokine present in the sample. The following kits were evaluated on the EnVision Nexus and the EnVision 2105: AlphaLISA High Performance Human IL-1β Detection Kit (#AL3160), AlphaLISA High Performance Human TNFα Detection Kit (#AL3157), and AlphaLISA High Performance Human IFNγ Detection Kit (#AL3153). The recommended assay protocol was followed according to the Revvity instructions provided for each assay adapted to run with a final assay volume of 20 µL. Standard analyte curves were prepared from each kit in bulk in a 1.1 mL deep well StorPlate-96 (#6008390) and plated in duplicate into 384-well assay plates.

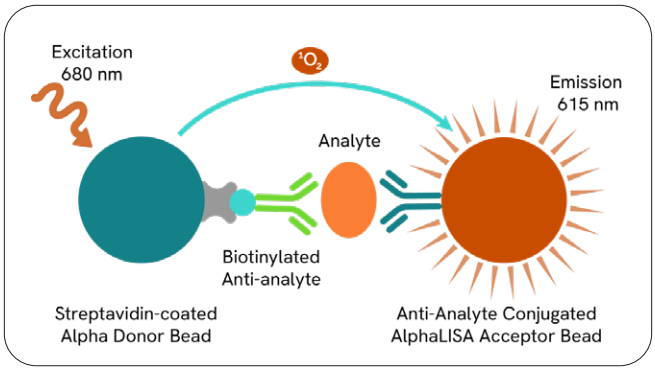


Figure 2: Assay schematic for AlphaLISA with biotinylated anti-analyte antibody and anti-analyte conjugated AlphaLISA Acceptor beads.

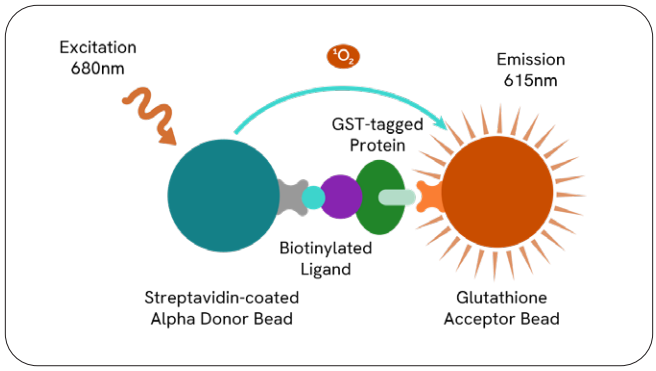


Figure 3: Assay schematic for AlphaLISA binding assay with biotinylated ligand and GST-tagged protein of interest.

AlphaLISA binding assay format

The AlphaLISA detection of protein binding showcased in these comparison studies uses Glutathione AlphaLISA Acceptor beads to capture a glutathione S-transferase (GST) tagged protein of interest and streptavidin coated Alpha Donor beads to capture the biotinylated ligand. Donor beads and Acceptor beads come into proximity through ligand binding to the protein of interest. Excitation of the Donor beads provokes the release of singlet oxygen that triggers a cascade of energy transfer reactions in the nearby Acceptor beads, resulting in a sharp peak of light emission at 615 nm (Figure 3). Typically, an AlphaLISA binding assay is utilized to screen for inhibitors of the ligand and protein interaction, wherein the inhibitor competes for binding of the protein of interest with the labelled ligand and results in a loss of AlphaLISA signal. The following kit was evaluated on the EnVision Nexus and the EnVision 2105: AlphaLISA Human Cereblon Binding Kit (#AL3147). The recommended assay protocol was followed according to the Revvity instructions provided adapted to run with a final assay volume of 20 µL. A dilution curve of the Cereblon targeting small molecule lenalidomide provided in the kit was prepared in bulk in a 1.1 mL deep well StorPlate-96 (#6008390) covering a broad range of dilutions (1000 µM to 10 nM). The lenalidomide dose-response curve was plated in duplicate into 384-well assay plates to generate a competition curve with the biotinylated ligand and GST-tagged Cereblon.

AlphaLISA *SureFire Ultra* assay format

AlphaLISA *SureFire Ultra* assay kits allow the rapid, sensitive, and quantitative detection of target-specific total and phosphorylated proteins from cells to track and characterize changes to cell signaling and cellular homeostasis. The Alpha Donor bead is coated with streptavidin to capture the biotinylated antibody while the AlphaLISA Acceptor bead is coated with a proprietary Capt*Sure*™ agent that immobilizes the other antibody which is labeled with a Capt*Sure* tag. In the presence of the target protein, the two antibodies bring the Donor and Acceptor beads into proximity. Excitation of the Donor beads provokes the release of singlet oxygen that triggers a cascade of energy transfer reactions in the nearby Acceptor beads, resulting in a sharp peak of light emission at 615 nm (Figure 4). The amount of light emission is proportional to the quantity of target protein present in the sample. The following kits were evaluated on the EnVision Nexus and the EnVision 2105: AlphaLISA *SureFire Ultra* p-ERK1/2 (Thr202/Tyr204) (#ALSU-PERK-A), and AlphaLISA *SureFire Ultra* p-SYK (Tyr525/526) (#ALSU-PSYK-A). The recommended protocol for AlphaLISA *SureFire Ultra* was followed according to the Revvity instructions provided for each assay adapted to run with a final assay volume of 20 µL. Positive control lysates for each kit were reconstituted as directed to create a 100% control lysate value and then serially diluted 2-fold for 8 points generating a standard dilution curve. The appropriate assay buffer was used as a no lysate data point to gauge background assay signal. Lysates were prepared in bulk in a 1.1 mL deep well StorPlate-96 (#6008390) and plated in duplicate into 384-well assay plates.

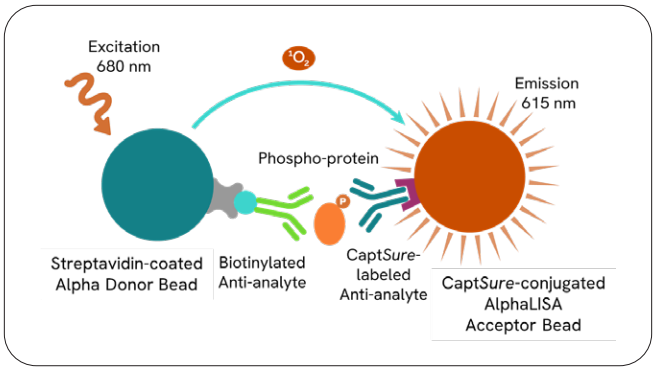


Figure 4: Assay schematic for AlphaLISA *SureFire Ultra* showcasing a phosphorylated target protein.

Data capture and analysis

Each assay plate was read individually on one instrument following the Revvity recommended protocol using default settings for either AlphaLISA (emission 615 nm, bandwidth 8 nm) or AlphaScreen (emission 570 nm, bandwidth 100 nm). Once all data was collected, it was entered into GraphPad® Prism™ to generate analyte standard curves, compound dose-response curves, or lysate dilution curves using nonlinear regression fitting (four parameter logistic with $1/y^2$ weighting). GraphPad Prism calculates the EC_{50} or IC_{50} values from agonist and antagonist dose-response curves after plotting the data. Additionally, assay blanks (assay buffer only) were run for each standard curve to provide sample data to calculate the LDL or LLOQ values, where appropriate. LDL is obtained using the following equation: Average of the blanks + 3x Standard Deviation of the blanks. This value was generated in Microsoft Excel and interpolated back onto the analyte standard curve in GraphPad Prism. Similarly, LLOQ is obtained using the following equation: Average of the blanks + 10x Standard Deviation of the blanks. LLOQ is thought to be the more biologically relevant measure as it often is used to gauge the lowest level of analyte or protein that can be detected accurately in an assay.

Results and discussion

High Performance Cytokines assays

Assays were performed as described in the Materials and methods section. Figure 5 shows the raw instrument counts for Human IL-1β in the white ProxiPlate-384 Plus assay plates to highlight the large assay window seen with an AlphaLISA assay between the baseline values and the top of the standard curve (~500-fold in the case of the EnVision Nexus AlphaScreen values). AlphaLISA (AL) raw values were consistently higher on the EnVision Nexus relative to the EnVision 2105, however the AlphaScreen (AS) raw values were comparable between instruments.

[Analyte] g/mL	Nexus (AS)		Nexus (AL)		2105 (AS)		2105 (AL)	
3.00E-08	806956	816091	526977	547895	874210	881264	337643	332573
1.00E-08	759691	772000	486654	477952	777552	812737	279335	285517
3.00E-09	534893	553121	347930	326301	572531	585012	204312	193740
1.00E-09	288890	297233	180874	174183	297429	306430	111407	104573
3.00E-10	107923	111942	69794	64045	114636	111896	38458	41131
1.00E-10	40248	43460	27318	25205	46018	43943	14440	15209
3.00E-11	14309	14957	9270	9293	14395	14370	5003	5246
1.00E-11	5808	6493	4330	4314	5772	5844	2280	2303
3.00E-12	2929	3005	2474	2317	3049	2508	1103	1182
1.00E-12	2004	2187	1799	1715	2029	1636	772	797
3.00E-13	1705	1731	1618	1494	1513	1466	857	712
1.00E-13	1683	1523	1550	1532	1379	1208	800	841

Figure 5: Analyte standard curve raw counts for Human IL-1β with AlphaScreen (AS) and AlphaLISA (AL) settings in a white ProxiPlate-384 Plus assay plate.

Figure 6 shows representative standard curves in GraphPad Prism for the High Performance Cytokine kits measuring Human IL-1β as all three assay kits tested showed similar trends in the data. Alpha signal intensity was comparable between the EnVision Nexus and the EnVision 2105 for AlphaScreen (AS) settings on both instruments regardless of plate color with the resulting curve fits nearly overlapping. As expected, the Alpha signal intensity for gray assay plates is lower than the results from white assay plates but did not affect the assay performance characteristics such as the LDL, signal-to-noise window, EC_{50}, or dynamic range demonstrating that the power of an AlphaLISA assay performance is unaffected by plate color. The Alpha signal intensity for the EnVision 2105 using AlphaLISA (AL) settings was consistently lower than the EnVision Nexus across both assay plate colors.

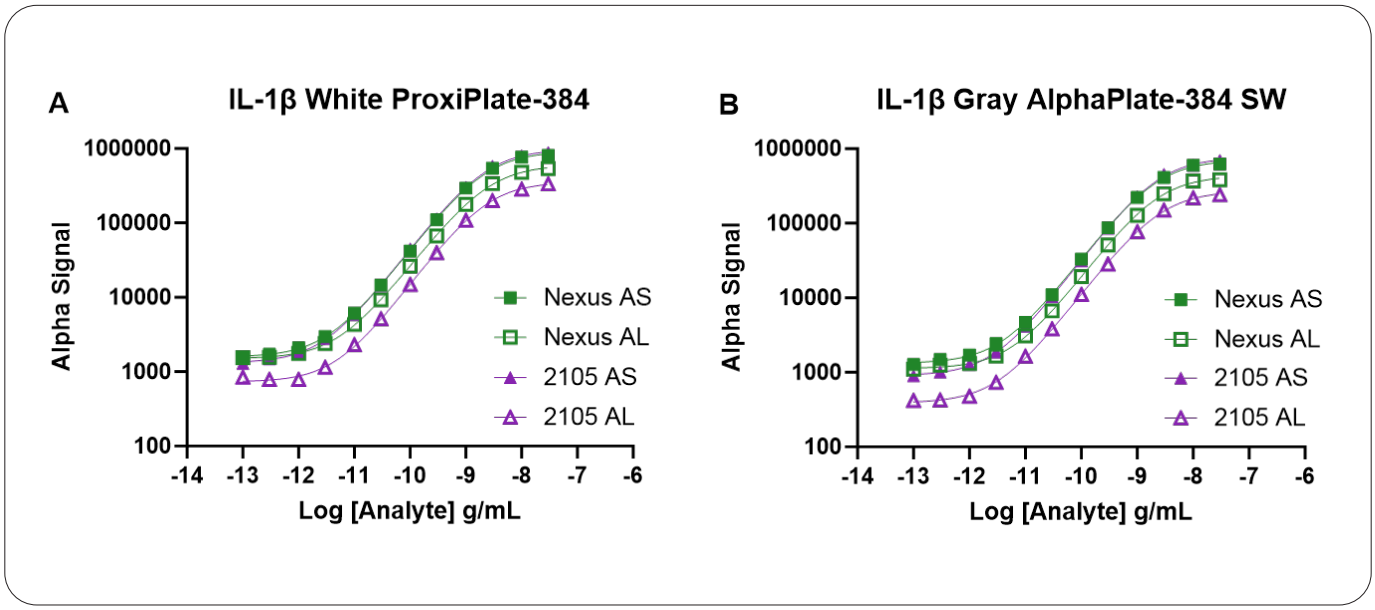


Figure 6: Analyte standard curve for Human IL-1β with AlphaScreen (AS) and AlphaLISA (AL) settings in white (A) or gray (B) 384-well assay plates.

When comparing the assay metrics measured across all three targets tested, the tables below show no significant difference in the trends for LDL, LLOQ or EC_{50} values between the two instruments indicating the EnVision Nexus is performing as well as the EnVision 2105 in these assays. The assay parameters and analytical results should stay consistent from reader to reader with improved performance and sensitivity.

Table 1: LDL Results [pg/mL] for AlphaScreen (AS) and AlphaLISA (AL)

	IL-1β AS	TNFα AS	IFNγ AS	IL-1β AL	TNFα AL	IFNγ AL
Nexus (White)	1.33	12.29	1.46	2.87	29.88	2.76
2105 (White)	0.24	14.44	3.55	1.95	26.53	4.32
Nexus (Gray)	0.79	8.30	0.70	3.89	19.50	11.15
2105 (Gray)	0.24	9.56	0.46	2.65	28.60	6.78

Table 2: LLOQ Results [pg/mL] for AlphaScreen (AS) and AlphaLISA (AL)

	IL-1β AS	TNFα AS	IFNγ AS	IL-1β AL	TNFα AL	IFNγ AL
Nexus (White)	4.46	37.17	4.15	8.43	87.14	9.15
2105 (White)	1.37	39.52	12.13	6.58	76.78	12.70
Nexus (Gray)	2.48	20.58	1.94	12.69	59.70	32.18
2105 (Gray)	1.12	33.26	1.99	7.37	77.95	18.43

Table 3: EC_{50} Results [g/mL] for AlphaScreen (AS) and AlphaLISA (AL)

	IL-1β AS	TNFα AS	IFNγ AS	IL-1β AL	TNFα AL	IFNγ AL
Nexus (White)	2.11E-09	2.10E-08	3.85E-09	2.49E-09	2.60E-08	4.08E-09
2105 (White)	2.27E-09	1.86E-08	4.26E-09	2.21E-09	3.07E-08	3.97E-09
Nexus (Gray)	2.21E-09	2.06E-08	3.61E-09	2.32E-09	2.20E-08	3.59E-09
2105 (Gray)	2.35E-09	2.61E-08	3.47E-09	2.57E-09	2.79E-08	4.16E-09

Cereblon binding assay

Assays were performed as described in the Materials and methods section. Figure 7 shows the dose-response of the inhibitor lenalidomide in the Cereblon binding assay. Alpha signal intensity was similar between the EnVision Nexus and the EnVision 2105 for AlphaScreen (AS) settings on both instruments regardless of plate color with the resulting curve fits nearly overlapping. As expected, the Alpha signal intensity for gray assay plates is lower than the results from white assay plates (approximately 30%) but did not affect the assay performance characteristics (as mentioned above). The Alpha signal intensity for the EnVision 2105 using AlphaLISA (AL) settings was consistently lower than the EnVision Nexus across both assay plate colors.

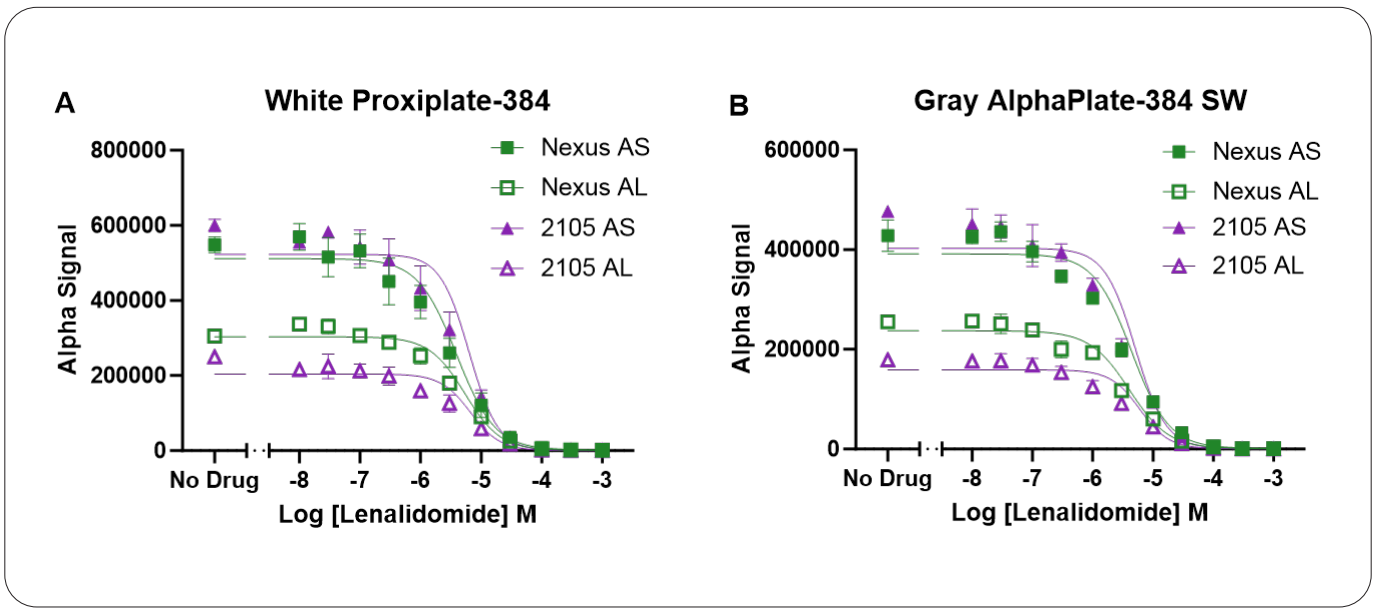


Figure 7: Lenalidomide dose-response curve with AlphaScreen (AS) and AlphaLISA (AL) settings in white (A) or gray (B) 384-well assay plates.

When comparing the IC_{50} values between the two instruments within one condition (e.g. AlphaScreen, gray plates) there is no significant difference in the overall results indicating the EnVision Nexus is performing as well as the EnVision 2105 in this Alpha assay format.

Table 4: IC_{50} Results [g/mL] for AlphaScreen (AS) and AlphaLISA (AL)

	Nexus AS	2105 AS	Nexus AL	2105 AL
White	3.91E-06	6.33E-06	5.39E-06	6.65E-06
Gray	4.39E-06	5.08E-06	4.05E-06	6.06E-06

AlphaLISA *SureFire Ultra* assays

Assays were performed as described in the Materials and methods section. Figure 8 shows representative data from the p-SYK assay with results in the gray AlphaPlate-384. In a similar fashion to the other AlphaLISA assays tested, the gray plates reduced the overall signal intensity but did not affect the assay performance characteristics. For these assays, signal intensity in the p-SYK AlphaLISA *SureFire Ultra* assay was higher with the EnVision Nexus relative to the EnVision 2105 plate reader for both the AlphaScreen (AS) data and the AlphaLISA (AL) data.

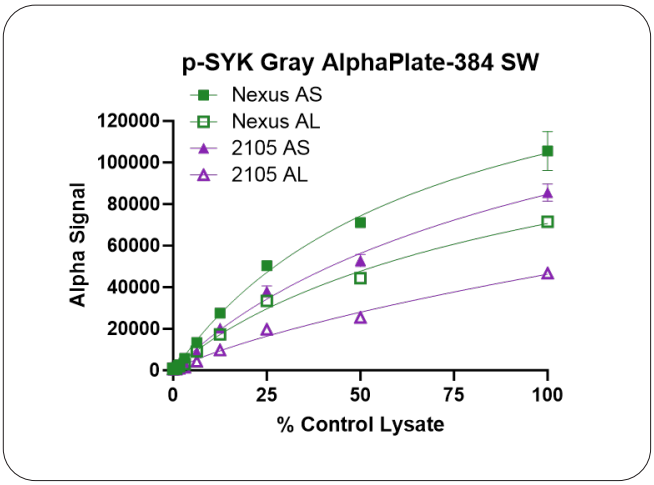


Figure 8: Control lysate dilution curve for p-SYK (Tyr525/526) AlphaLISA *SureFire Ultra* Assay with AlphaScreen (AS) and AlphaLISA (AL) settings in a gray 384-well assay plate.

Similar trends in the results were seen with the p-ERK AlphaLISA *SureFire Ultra* assay (Figure 9). The signal intensity was higher with the EnVision Nexus relative to the EnVision 2105 plate reader for the AlphaScreen (AS) data and consistently higher for the AlphaLISA (AL) data. For the AlphaLISA *SureFire Ultra* assay format there is no standard analyte curve to generate LDL, LLOQ or EC_{50} values. The control lysate dilution series was therefore generated to compare the two plate readers across several data points to determine differences in Alpha signal intensity.

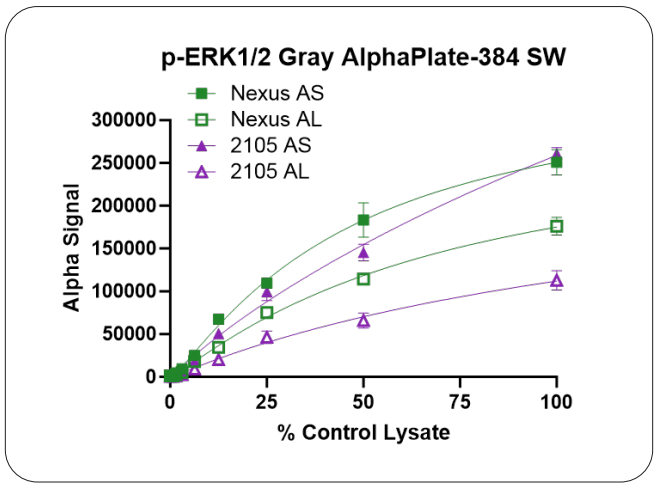


Figure 9: Control lysate dilution curve for p-ERK1/2 (Thr202/Tyr204) AlphaLISA *SureFire Ultra* Assay with AlphaScreen (AS) and AlphaLISA (AL) settings in a gray 384-well assay plate.

Conclusions

Some benefits of the EnVision Nexus include automated Alpha aperture loading, assay ready quick install filter modules containing pre-aligned mirror and filters, a maintain ambient temperature setting and a cross talk minimization option. Overall, the easy-to-use Kaleido™ software requires less hands-on optimization time from the end user. The EnVision Nexus displayed strong performance in all three AlphaLISA formats tested. The measured signal intensity was consistently higher with the focused AlphaLISA settings on the EnVision Nexus than the EnVision 2105. There was no significant difference in the AlphaScreen signal intensity across the assays tested between the EnVision Nexus and the EnVision 2105. As expected, the LDL, LLOQ, EC_{50} and IC_{50} values calculated for each assay format were similar between the two EnVision plate readers and Alpha detection modes demonstrating that AlphaLISA results are consistent regardless of the plate reader used as these measures are a function of the assay and should be independent of the instrument. AlphaLISA therefore demonstrated excellent performance on the EnVision Nexus as a superior no-wash immunoassay format compared to other immunoassay techniques. Combined with the simple, robust, and reproducible assay protocol for AlphaLISA, this work showcases the ease-of-use of AlphaLISA and the exceptional assay characteristics such as LDL, LLOQ, signal-to-noise window, and the remarkable dynamic range (4-5 logs) offered by this technology.

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