TECHNICAL NOTE

Optimized EnVision Nexus Alpha performance using apertures and crosstalk minimization mode.

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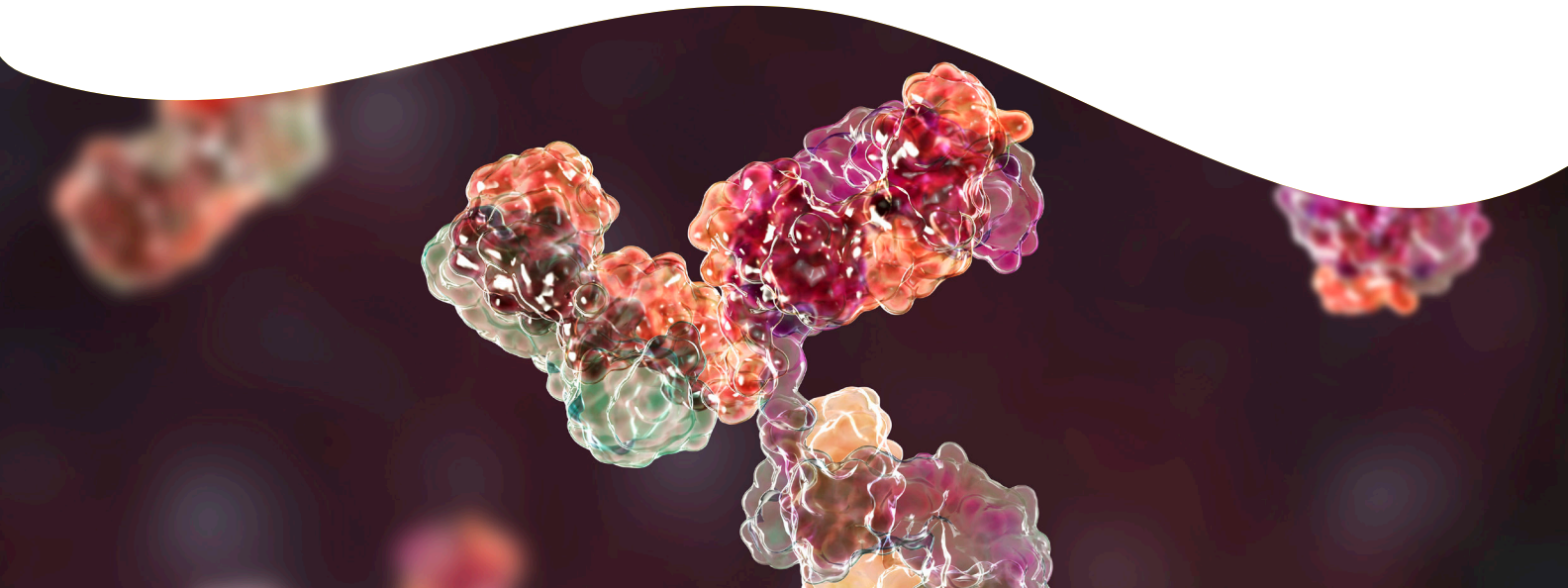
Introduction

In the field of life sciences and biomedical research, detecting and monitoring protein expression, activity and interaction are pivotal for understanding cellular processes and developing potential new treatments. AlphaLISA™ and AlphaScreen™ are bead-based assay technologies specifically designed to explore these intricate interactions and properties. Both employ a no-wash and no-separation approach, offering exceptional sensitivity and a wide dynamic range, making them compatible with various sample types, including serum, plasma, cell lysates and more.

Data quality for luminescence-based measurement approaches like Alpha can be limited by light emitted from adjacent wells of the currently measured sample. For utmost flexibility and best possible data quality the EnVision™ Nexus™ offers two Alpha detection options: Alpha Enhanced (ENH), which can be used with various filter modules, including AlphaScreen and different AlphaPlex™ filters, and Alpha high-throughput screening (HTS), designed for fast data acquisition. Both Alpha options utilize apertures that effectively block stray light from adjacent wells, minimizing crosstalk. Users can easily switch apertures using the new aperture transfer plate and a wizard in the EnVision Nexus software (Kaleido™ 4). Additionally, a crosstalk minimization reading pattern was developed, further reducing crosstalk to neglectable levels.

In this technical note we compare data quality of Alpha measurements on EnVision Nexus with its predecessor EnVision™ 2105 with a focus on the influence of crosstalk.



Materials and methods

AlphaLISA TruHits kit

The AlphaLISA™ TruHits™ kit (Product No.: AL900D) was used as a high signal Alpha sample. To achieve a high homogeneity of samples a mastermix was prepared in 50 ml reaction tubes. AlphaLISA™ BSA-biotin Acceptor beads and Streptavidin Alpha Donor beads were both diluted in dH_2O to a final concentration of 5 µg/mL. The solution was incubated overnight in the dark at room temperature.

The AlphaLISA TruHits bead dilution and water were pipetted into a StorPlate-384 deepwell, V-bottom (Part Number: 6008690) using the plate layout shown in Figure 1. To minimize pipetting errors, a JANUS™ G3 automated liquid handling workstation was used to pipette 20 µl from the StorPlate-384 into the wells of a 384-well AlphaPlate™ (Part Number: 6005359). Plates were sealed with TopSeal A, black (Part Number: 6050173) which was removed before the start of the measurement.

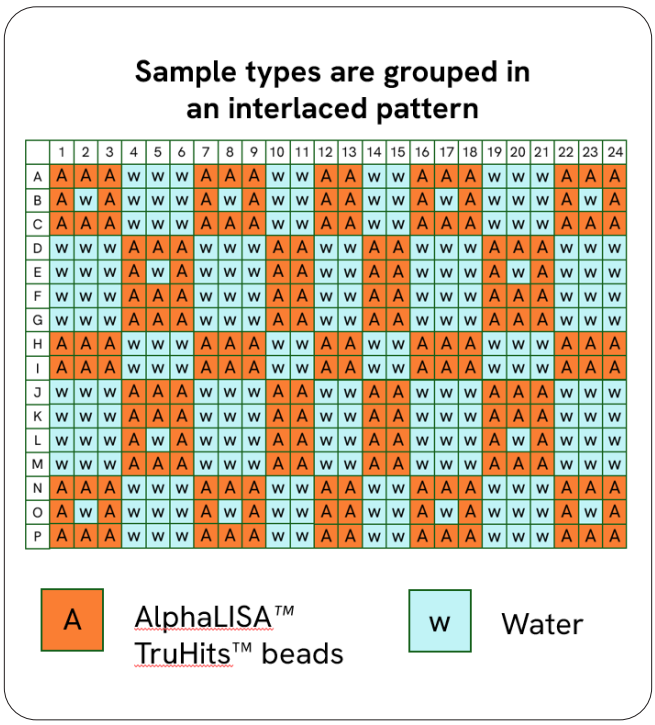


Figure 1: Plate layout. Wells of a 384-well AlphaPlate were filled with 20 µL AlphaLISA TruHits donor and acceptor beads (5 µg/mL) or water. These were arranged in an alternating pattern across the entire plate to determine sensitivity and signal-to-background taking into account the crosstalk from adjacent wells. For the crosstalk analysis shown in Figure 2 wells B2, B8, B17, B23, E5, E20, L5, L20, O2, O8, O17 and O23 were used.

Measurements

Alpha measurements on the EnVision Nexus or EnVision 2105 were performed either with the Alpha (ENH/STD) or the Alpha (HTS) option, as specified in Table 1. For high-precision settings we used an excitation time of 180 ms and a detection time of 370 ms (550 ms in total). High-throughput settings utilized a 35 ms excitation time and a 65 ms detection time (100 ms in total).

Table 1: Settings used for data acquisition.

	EnVision Nexus		EnVision 2105	
Technology	Alpha (ENH)	Alpha (HTS)	Alpha (STD)	Alpha (HTS)
Aperture	ELD	ULD	-	A384
Filter module	AlphaScreen 5001	-	AlphaScreen emission 570	-
Excitation source	680 nm laser	680 nm laser	680 nm laser	680 nm laser
Emission filter	575/110 nm	650 nm shortpass	570/100 nm	650 nm shortpass
Crosstalk minimization reading mode	On	On	-	-
Measurement duration (start to end) 384-well plate	4min 31s	1min 36s	4min 33s	1min 41s
Ambient temperature	On	On	-	-

Calculations and data analysis

Revvity Signals Research Suite™ was used for data analysis.

Formulas:

$$\text{Sensitivity} = \frac{3^{*}\sigma(\text{Water control}) \cdot C(\text{Alpha sample})}{\mu(\text{Alpha sample}) - \mu(\text{Water control})}$$

$$\text{Signal-to-Background} = \frac{\mu(\text{Alpha sample})}{\mu(\text{Water control})}$$

C: Concentration; µ: Average; σ: Standard Deviation

Abbrevations

Alpha	Amplified Luminescent Proximity Homogeneous Assay
CTM	Crosstalk minimization
ENH	Enhanced
HTS	High-throughput screening
STD	Standard

Results

1. Achieve minimal crosstalk with the EnVision Nexus

- The apertures of Alpha (ENH) and Alpha (HTS) on the EnVision Nexus highly reduce crosstalk and cross excitation effects
- The crosstalk minimization reading mode reduces crosstalk to negligible levels

The optical design of both Alpha technologies available for the EnVision Nexus aims for minimal crosstalk. The key components are the apertures placed directly above the well to block the light emitted from adjacent wells and to prevent cross excitation that results in bleaching of neighboring Alpha samples.

The EnVision Nexus uses an additional level of crosstalk minimization by optimizing the order in which samples are measured. This crosstalk minimization reading pattern reads each row multiple times, measuring only every other well in each run. This novel reading pattern utilizes a certain characteristic of the Alpha signal: after the initial excitation with the Alpha laser the signal decreases fast (see Figure 2). By skipping adjacent wells, the surrounding wells either have not been excited yet or the signal already decayed to much lower levels so that their contribution to the measured crosstalk is minimal.

Important consideration for minimal crosstalk: plate type selection

One often overlooked factor in minimizing crosstalk is the choice of the appropriate plate type. Most white plates show light leakage through the walls between wells. This cannot be prevented by the optical design of a plate reader. Revvity AlphaPlates were specifically designed to minimize light leakage through the walls of the wells while still preserving a high Alpha signal and are the recommended plate type for Alpha and luminescence measurements. For detailed comparison of the crosstalk in white plates and AlphaPlates, see also References 1.

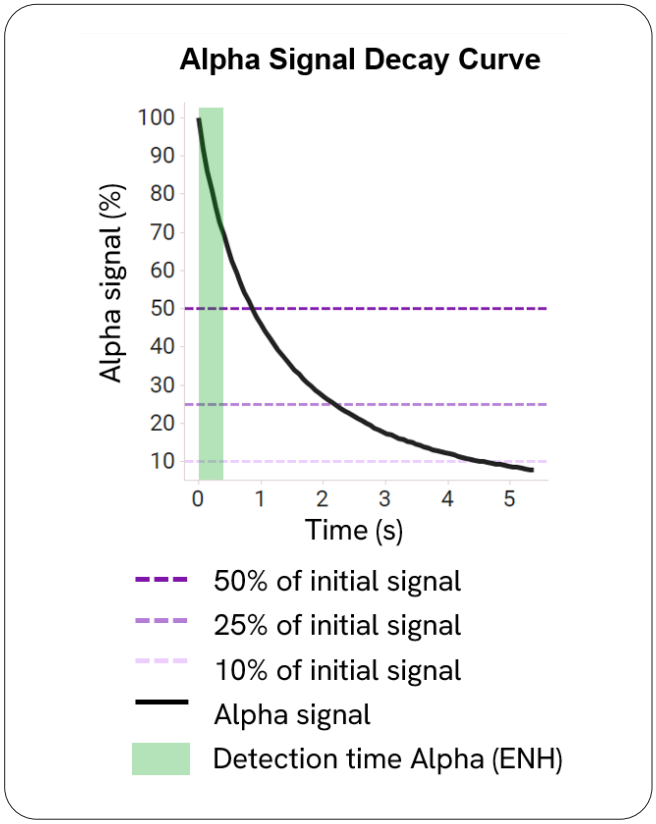


Figure 2: Alpha signal decay. AlphaLISA TruHits bead signal decay over five seconds. A default Alpha (ENH) measurement detects the Alpha counts during the high signal phase indicated in light green. If the crosstalk minimization reading mode of the EnVision Nexus is used, the signal of adjacent wells excited before already decayed to a low level and the resulting crosstalk is minimal.

To compare the crosstalk levels of the different instruments and measurement conditions, we analyzed the crosstalk into a well fully surrounded by high Alpha signal wells. The Alpha (STD) option on the EnVision 2105, which operates without an aperture, exhibited the highest crosstalk at approximately 0.3% (see Figure 3). In contrast, the EnVision Nexus Alpha (ENH) option with aperture, but crosstalk minimization reading mode (CTM mode) turned off, exhibited significantly lower crosstalk of 0.013% — a more than 20-fold reduction compared to Alpha (STD). Utilizing the crosstalk minimization reading mode further reduced crosstalk more than 4-fold to only 0.003%, which, even for very bright Alpha samples, is only slightly above background signal levels. Similar low crosstalk levels were achieved with the Alpha (HTS) mode of the EnVision.

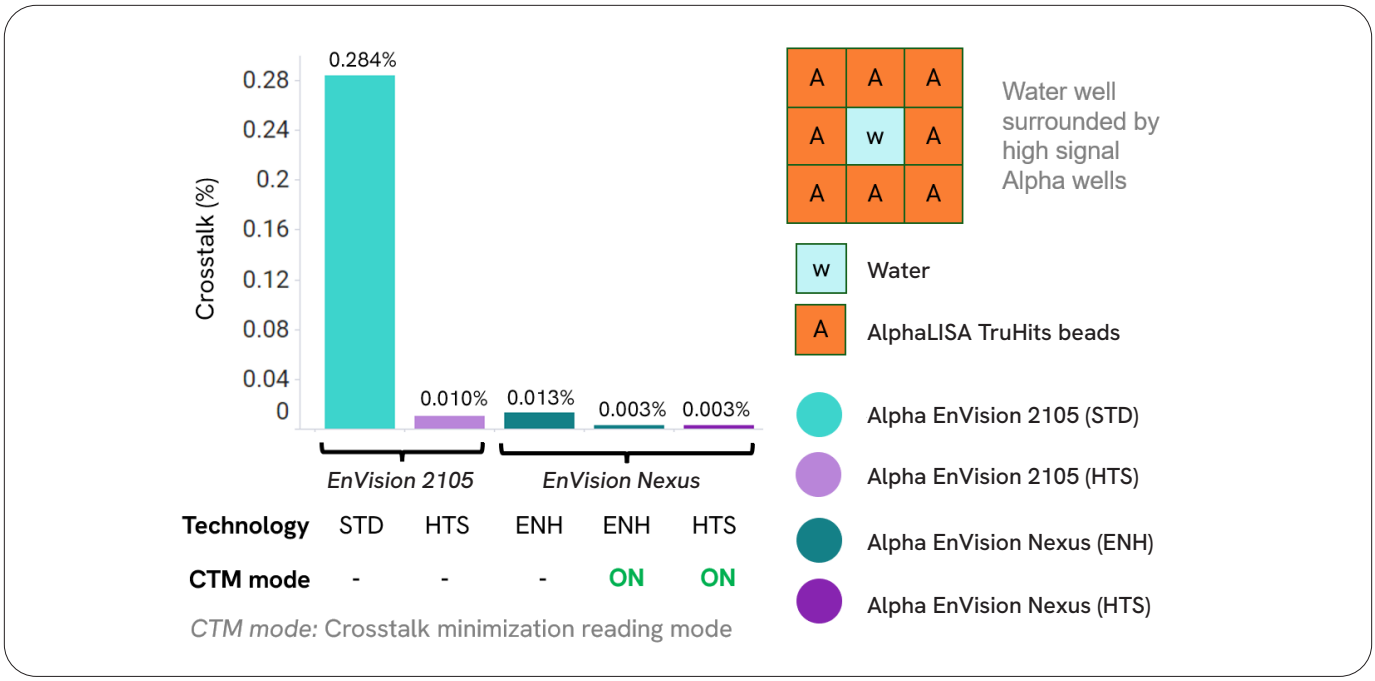


Figure 3: Crosstalk minimization. We analyzed the crosstalk measured in a well fully surrounded by wells filled with high signal AlphaLISA TruHits samples (5 µg/mL acceptor and donor beads), modelling an inhibition assay. Measurements on the EnVision 2105 with standard Alpha (no aperture) were compared to HTS and ENH Alpha on the EnVision 2105 and EnVision Nexus. Shown is the average of crosstalk measured in 12 different wells distributed over the plate (see Figure 1).

2. Excellent sensitivity and signal-to-background ratios

- The optical design of the EnVision Nexus ensures outstanding sensitivity and signal-to-background ratios for Alpha (ENH) and Alpha (HTS) even in high crosstalk scenarios
- The crosstalk minimization reading mode further improves the Alpha performance

Assay windows are often limited by the underlying biology and other factors, and positive hits in a high-throughput screen can already be indicated by minimal changes in the signal. Combining the optimized EnVision Nexus Alpha detectors with the high signal levels of the laser excitation allows for a large assay window, high signal-to-background ratios, and excellent sensitivity.

To illustrate the importance of minimal crosstalk for the sensitivity and signal-to-background ratio we chose a plate layout where instead of separating Alpha samples and controls, they were grouped in an interlaced pattern, a scenario much closer to real assay conditions (Figure 1). The Alpha (STD) option of the EnVision 2105 uses no aperture and crosstalk from adjacent wells limits the sensitivity and signal-to- background ratio (Figure 4A and B). The Alpha (ENH) option of the EnVision Nexus already shows greatly improved sensitivity and signal-to-background ratios compared to Alpha (STD) with a more than 15-times lower sensitivity and 20-times higher signal-to-background ratio. Optimal results are achieved by utilizing the crosstalk minimization reading mode, which is the recommended default setting for Alpha measurement. The Alpha (HTS) mode of the EnVision Nexus was improved, and furthermore uses the crosstalk minimization reading mode, resulting in excellent sensitivity and high signal-to-background ratios compared to the EnVision 2105. In contrast to the Alpha (ENH) option, the Alpha (HTS) mode on the EnVision Nexus exhibits a higher signal-to-background ratio, attributed to its shorter measurement window for the decaying Alpha signal (see Figure 2) and lower noise levels of the detector. Although the Alpha (HTS) mode nearly matches the sensitivity of Alpha (ENH), the measurement time for an entire plate is nearly three times faster. The rapid measurement time is the primary advantage of the Alpha (HTS) option.

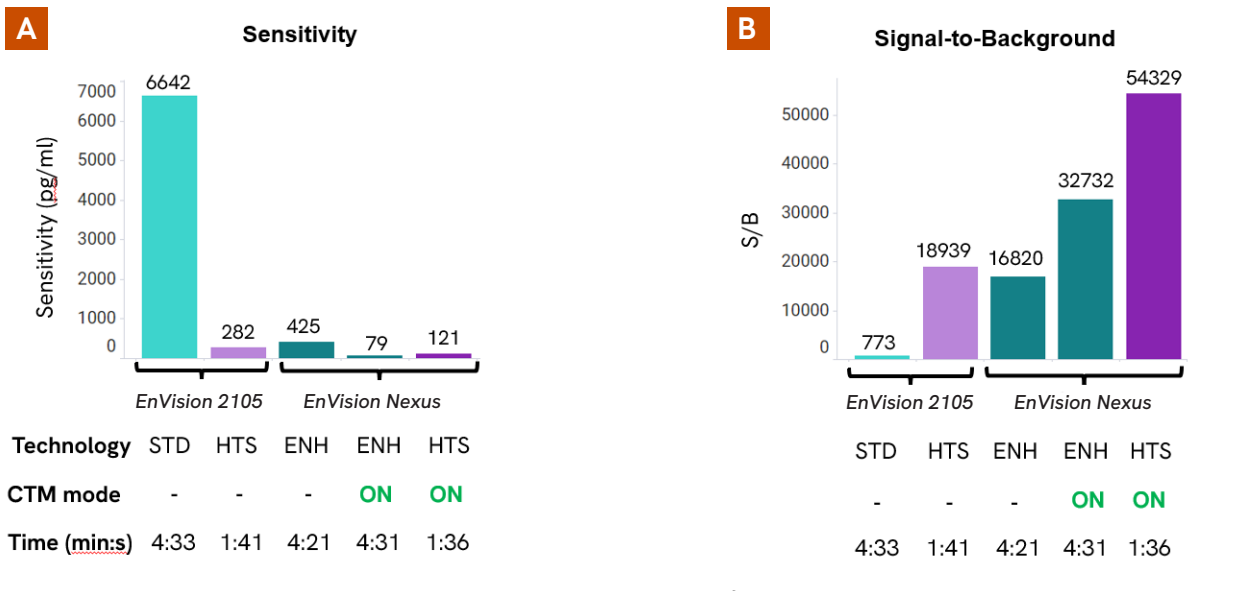


Figure 4: Sensitivity and signal-to-background ratio of AlphaLISA TruHits beads. Sensitivity (A) and signal-to-background ratio (B) were calculated for samples distributed across a 384-well plate as shown in the layout in Figure 1.

Conclusion

Here we present the advanced Alpha crosstalk minimization strategies employed in the EnVision Nexus. In contrast to the Alpha (STD) mode on other devices, the Alpha (ENH) mode on the EnVision Nexus exhibits only minimal inter-well crosstalk levels. This is a result of several improvements in the optical design, including an aperture located above the well and the crosstalk minimization reading mode. The Alpha (HTS) mode is specifically designed for highest speeds, combining the fastest measurement times with minimal crosstalk.

By leveraging various technologies such as the ambient temperature feature (see References 2), apertures, optical design, and crosstalk minimization, the EnVision Nexus ensures optimal measurement conditions. These advancements contribute to high reproducibility, exceptional sensitivity, and high signal-to-background ratios.

References

1. Advanced luminescence performance on the EnVision Nexus Tech Note (Revvity.com)

2. EnVision Nexus Ambient Temperature Feature for Exceptional Alpha Performance Tech Note (Revvity.com)