APPLICATION NOTE

Avoiding biotin interference in AlphaLISA assays.

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

Introduction

AlphaLISA® technology allows the detection and quantitation of molecules of interest in buffer, cell culture media, serum, plasma, and other biological matrices in a homogenous, highly sensitive, easy-to-use, and reproducible manner[1,2]. In the presence of streptavidin-coated Donor beads, the biotinylated anti-analyte antibody binds to streptavidin-coated Donor beads and the beads come into close proximity. The excitation of the Donor beads at 680 nM induces the release of singlet oxygen molecules and triggers an energy transfer cascade in the Acceptor beads, which generates a sharp emission peak at 615 nM that can be detected using Revvity's EnSight™, EnSpire™, or EnVision® Multilabel Plate Reader. Figure 1 shows an example of AlpahLISA biomarker kits in which human albumin, the analyte of interest, is recognized by two antibodies, a biotinylated anti-analyte antibody and anti-analyte-conjugated AlphaLISA Acceptor beads.

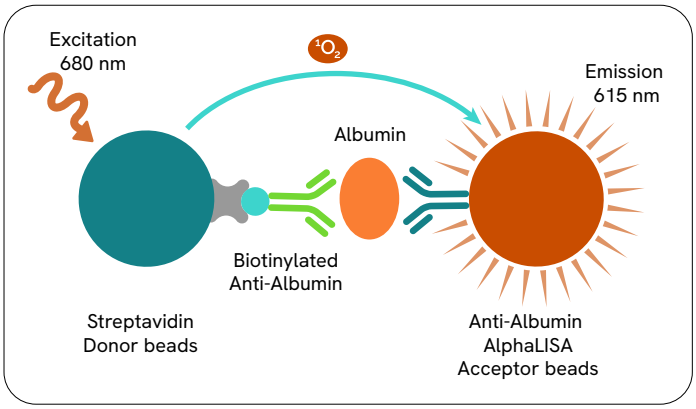
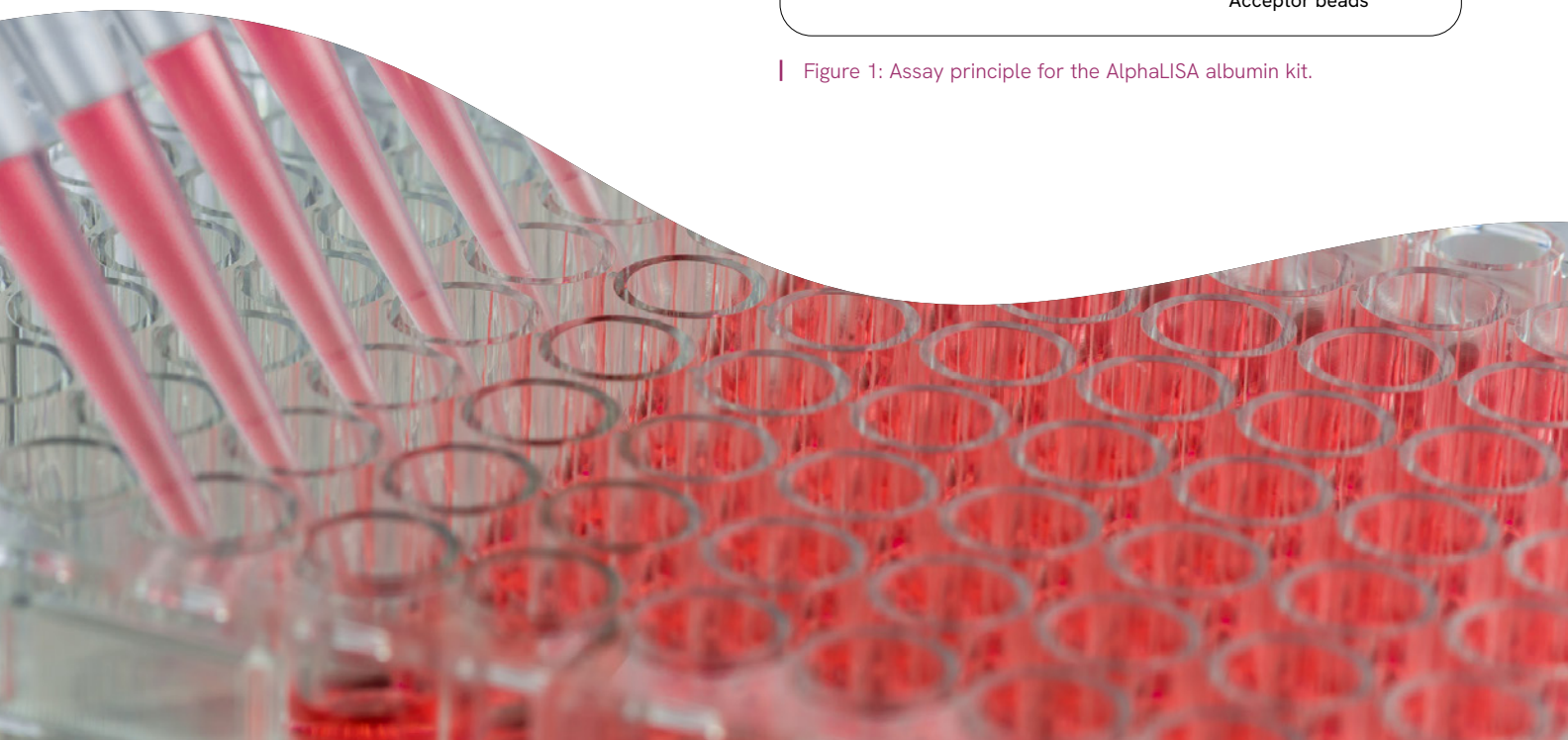


Figure 1: Assay principle for the AlphaLISA albumin kit.



Analyte quantitation can be performed by preparing a standard curve in buffers or diluents and interpolating the raw counts obtained from the analyte present in the sample to the standard curve. However, some cell culture media (e.g. RPMI and EX-CELL® 420) contain high levels of biotin (Table 1), which interferes with AlphaLISA and other assay technologies that rely on a streptavidin-biotin binding event for detection[3]. Biotin, also known as vitamin H or B7, is a water-soluble B-complex vitamin produced by a variety of cell types. Biotin plays a vital role in the metabolism of fatty acids and leucine, gluconeogenesis, the citric acid cycle, and the regulation of transcription and DNA repair. It is therefore present in many biological samples including serum. The presence of high levels of free biotin in the sample matrix can result in a decrease in total counts, lower signal to background ratios, and reduced AlphaLISA assay detection limits.

This application note demonstrates the value of using AlphaLISA biotin-free kits to reduce the effects of biotin interference in sample and standard preparations. In the biotin-free kits, the biotinylated antibody was replaced with digoxigenin (DIG)-labeled antibody and the streptavidin Donor bead was replaced with Anti-DIG Fab' Donor beads (Figure 2). To demonstrate reduced biotin interference using the AlphaLISA biotin-free detection kits, we selected two detection kits for comparison against their respective biotin-free versions: AlphaLISA Albumin biotin-free detection kit, AlphaLISA TruHits biotin-free kit, AlphaLISA Albumin kit, and AlphaLISA TruHits kit.

Materials and methods

Reagents, instruments, and data analysis

The kits evaluated were AlphaLISA Albumin biotin-free kit (Revvity AL363C), AlphaLISA TruHits biotin-free kit (Revvity AL901D), AlphaLISA Albumin kit (Revvity AL294C), and AlphaLISA TruHits kit (Revvity AL900D). RPMI 1640 (Cat # 30-2001) containing high levels of biotin was obtained from ATCC. RPMI 1640 without biotin (custom made) was purchased from Mediatech. EX-CELL® 420 (Cat # 14420C) and Biotin (Cat # B4591-1G) were purchased from Sigma-Aldrich. TopSeal™-A Plus Adhesive Sealing Film (Cat # 6050185) and OptiPlate-384, white opaque 384-well microplates (Cat # 6007290) were purchased from Revvity.

Table 1: Biotin content in commonly used cell culture media.

Medium	Biotin content (µg/mL)
FreeStyle CHO Expression Medium	1760
BME Eagle	1000
RPMI 1640	200
McCoy's	200
EX-CELL® 420	186
MEM a	100
FBS (Fetal Bovine Serum)	47
Iscove's MDM	13
Grace's Insect Medium	10
DMEM	0
DMEM/F12	0
HAT	0
EMEM	0

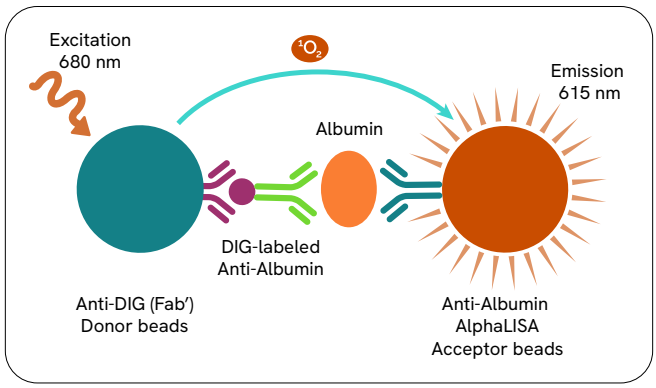


Figure 2: Assay principle for the AlphaLISA Albumin biotin-free detection kit.

AlphaLISA assay plates were read on a Revvity EnVision Multilabel Plate Reader (Figure 3) equipped with the ALPHA option using the AlphaScreen® standard settings (e.g. Total Measurement Time: 550 ms, Laser 680 nM Excitation Time: 180 ms, Mirror: D640as, Emission Filter: M570w, Center Wavelength 570 nM, Bandwidth 100 nM, Transmittance 75%). Data were analyzed and graphs were created using GraphPad Prism and Microsoft Excel.

Figure 3: EnVision multilabel plate reader.

Assay protocols

Unless otherwise stated, assays were performed following the provided technical data sheet (TDS) protocols for each kit. To test pure biotin interference, minor modifications to assay protocols were made to both AlphaLISA kits and AlphaLISA biotin-free detection kits, as described below.

Testing the interference of biotin on AlphaLISA biotin-free detection kits

Experiment 1

Biotin interference was tested using AlphaLISA Albumin and AlphaLISA Albumin biotin-free detection kits. Assays for both kits were performed on the same plate and repeated at least three times.

Albumin analyte was prepared at the EC_{80} concentration (100 ng/mL) determined by the standard curve. All other assay components were used as recommended on the kit technical data sheets. A biotin titration was prepared starting with a 20 mM biotin stock solution prepared in 1X PBS diluted down to 1 mM in the presence of NaCl. The 1 mM stock of biotin was then serial diluted in NaCl buffer. Assays were performed by adding the prepared reagents to 384-well plates in the following order: 2.5 µL of biotin standard dilutions, 2.5 µL analyte (100 ng/mL final), 2.5 µL anti-albumin Acceptor beads (10 µg/mL final), and 2.5 µL biotinylated anti-albumin antibody (1 nM final) or DIG-labeled anti-albumin antibody (1 nM final). Plates were sealed with TopSeal®-A, tapped gently to ensure that samples settled to their respective well, and incubated for 60 minutes at 23 °C. After the incubation, 40 µL of 1.25x streptavidin Donor beads or anti-DIG Donor beads was added and incubated an additional 30 minutes at 23 °C protected from light. Plates were read on an EnVision multilabel plate reader.

Experiment 2

Biotin interference was tested using AlphaLISA TruHits and AlphaLISA TruHits biotin-free kits. Reagents were prepared using each kit's recommended buffers. Biotin solution was prepared as in Experiment 1. Each concentration of biotin was tested in triplicate. Assays were performed by adding the prepared reagents to 384-well plates: 5 µL streptavidin Donor beads (20 µg/mL final) or anti-DIG Donor beads (20 µg/mL final) was added and followed by 10 µL of biotin preparation. Plates were sealed with TopSeal, tapped gently to settle liquids, and incubated 60 minutes at 23 °C protected from light. Afterwards 5 µL of Biotin-BSA Acceptor beads or DIG-IgG Acceptor beads (20 µg/mL, final) was added and incubated an additional 30 minutes at 23 °C protected from light. Plates were read on an EnVision multilabel plate reader.

Experiment 3

Biotin interference from high-biotin media (EX-CELL® 420) was also evaluated using the AlphaLISA TruHits and AlphaLISA TruHits biotin-free products. EX-CELL® 420 culture media was serially diluted with the assay buffer (1X Universal Buffer or 1X PBS as indicated in kit tech data sheet) and tested in triplicate. Assays were performed by adding the prepared reagents to 384-well plates: 5 µL streptavidin Donor beads (20 µg/mL final) or 5 µL anti-DIG Donor beads (20 µg/mL, final), followed by 10 µL EX-CELL® 420 culture media dilutions. The plate was then sealed with TopSeal-A, tapped gently to settle, and incubated 60 minutes at 23 °C protected from light. After the incubation, 5 µL of Biotin-BSA-Acceptor beads or 5 µL DIG-IgG Acceptor beads (20 µg/mL, final) were added. After an incubation of 30 minutes at 23 °C protected from light, the plate was then read on an EnVision multilabel Plate Reader.

AlphaLISA biotin-free detection kit evaluation

The performance of AlphaLISA Albumin biotin-free detection kit and the AlphaLISA Albumin kits were evaluated in different buffer conditions. All assays were performed following protocols found in the respective technical data sheets (TDS). Analyte standard curves were prepared either using the recommended assay buffer (NaCl buffer), in RPMI, or in RPMI without biotin. All other assay components were prepared in the NaCl buffer. Each assay was repeated (three or four times) and the mean results were used to establish assay parameters: LDL, LLOQ, EC_{50}, S/B ratios, maximum, and minimum counts.

Three known concentrations of albumin analyte (30, 10, and 3 ng/mL) were spiked into NaCl buffer, RPMI, and RPMI without biotin. The spiked samples were assayed along with the standards prepared in NaCl buffer, RPMI, and RPMI without biotin. Raw signals from spiked samples were interpolated to the standards to obtain the concentrations of analyte in the spiked samples and the percent recoveries were calculated. Briefly, the analyte standard dilutions and reagents were prepared. To a 384-well OptiPlate, 5 µL standards or spiked samples were added first, followed by 5 µL of a mixed solution of anti-albumin Acceptor beads and biotinylated anti-albumin antibody, or add 5 µL of a mixed solution of anti-albumin Acceptor beads and DIG-anti albumin antibody.

The plate was sealed using TopSeal-A, gently tapped, and incubated 60 minutes at 23 °C. After incubation, 40 µL of freshly prepared 1.25x streptavidin Donor beads or anti-DIG Donor beads (60 µg/mL) were added followed by an additional incubation for 30 minutes at 23 °C. The plate was then read on an EnVision multilabel plate reader.

Results

Biotin interference

The effects of biotin on AlphaLISA assays were evaluated in three separate experiments. Experiment 1 demonstrates that biotin at high concentrations (>30 nM) reduces AlphaLISA signal in the AlphaLISA Albumin kit, while the AlphaLISA Albumin biotin-free kit signal was not reduced by high concentrations of biotin (Figure 4). These results were further confirmed in Experiment 2 where biotin was tested in both an AlphaLISA TruHits kit and AlphaLISA biotin-free TruHits kit (Figure 5). Again, AlphaLISA signal did not change with increasing concentrations of biotin in the AlphaLISA biotin-free TruHits assay, whereas the signal in the AlphaLISA TruHits kit was blocked at biotin concentrations > 30 nM. This indicates that only the biotin/streptavidin binding is hindered by the presence of biotin, while the DIG/anti-DIG binding remains unaffected by even high concentrations of biotin.

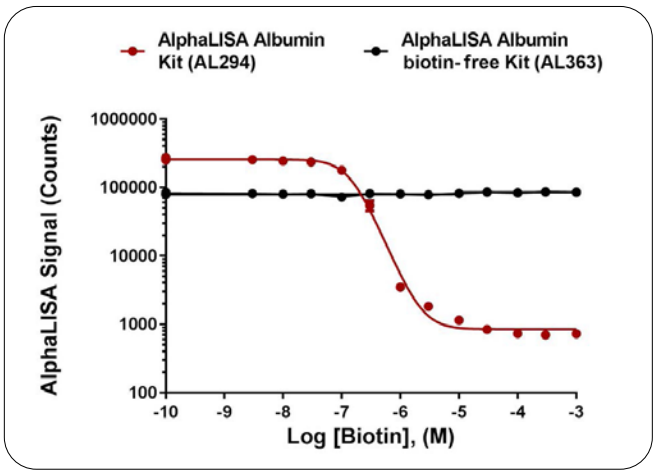


Figure 4: The interference of biotin on AlphaLISA Albumin and lack of interference on AlphaLISA Albumin biotin-free kits.

In Experiment 3, EX-CELL® 420 cell culture media containing high levels of biotin was used to evaluate the effect of biotin on TruHits assay performance. These results were consistent with those from Experiment 2. Even when used at 1x, media containing a high concentration of biotin did not affect the signal in AlphaLISA biotin-free TruHits assays. Conversely, in the assay performed using the AlphaLISA TruHits kit, the presence of biotin in the media reduced the assay signal. A dilution of ≥ 30 fold was necessary to remove biotin interference in the AlphaLISA TruHits assay (Figure 6).

AlphaLISA biotin-free detection kit assay performance

To determine the benefits of biotin-free detection kits, assays were performed to show the interference of biotin containing media (RPMI) in the AlphaLISA Albumin kit. In Figure 7, typical albumin standard curves were generated in NaCl buffer, RPMI, and RPMI without biotin. A dramatic signal reduction was seen when the standard was prepared in RPMI compared to the standards were prepared in NaCl buffer or in RPMI without biotin. These results suggest that biotin in RPMI interferes with the binding of the biotinylated anti-albumin antibody to streptavidin Donor beads resulting in reduced signals. The assay performances and specifications were summarized in the Table 2. It is clear that RPMI reduced the maximum counts and LDL/LLOQ (sensitivity) compared to the assays performed in NaCl buffer or in RPMI lacking biotin. When the assays were performed using AlphaLISA Albumin biotin-free detection kit (Figure 8), the biotin interference from RPMI was eliminated. In Figure 8, standard curves generated in all three diluents (NaCl buffer, RPMI, and RPMI without biotin) were almost identical showing that the biotin in RPMI did not affect the signal and alter the assay performance. As summarized in Table 2, all assay specifications (LDL, LLOQ, EC_{50}, S/B, and Maximum and Minimum signals) are similar among NaCl buffer, RPMI with biotin, and RPMI without biotin confirming that biotin had no effect on assay performance of AlphaLISA Albumin biotin-free detection kit.

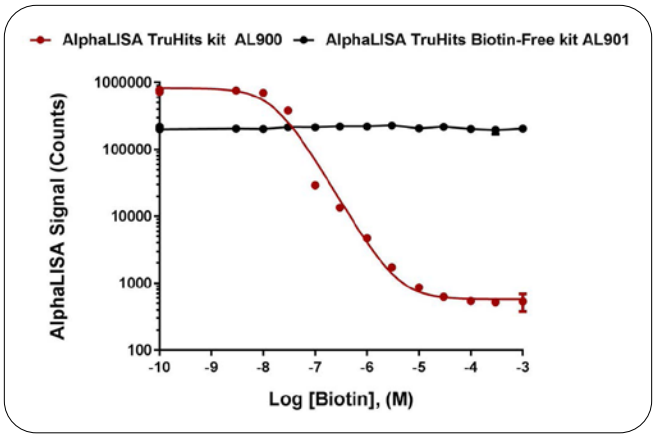


Figure 5: The interference of biotin on AlphaLISA TruHits and lack of interference in AlphaLISA biotin-free TruHits kits.

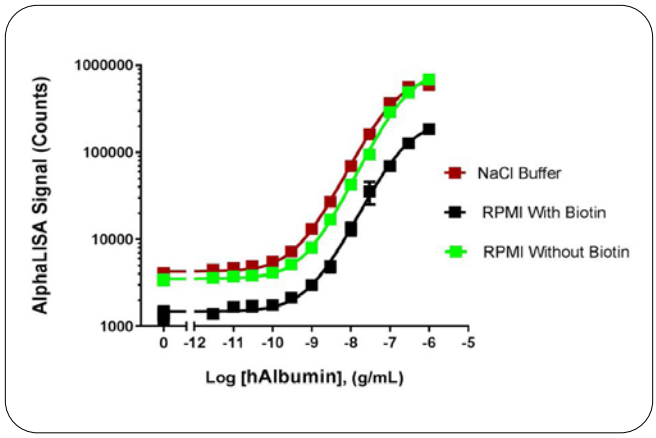


Figure 7: AlphaLISA Albumin kit (AL294) assays performed using NaCl buffer, RPMI, and RPMI without biotin as the diluent. The standards were prepared in these three diluents and all other assay reagents were prepared in NaCl buffer.

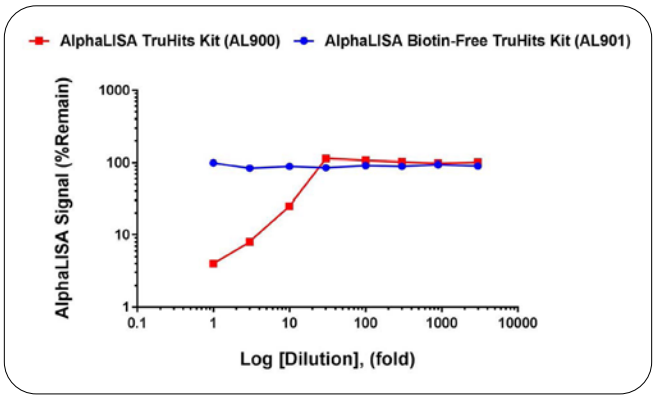


Figure 6: The interference of biotin in EX-CELL® 420 on AlphaLISA TruHits and lack of interference in AlphaLISA biotin-free TruHits kits.

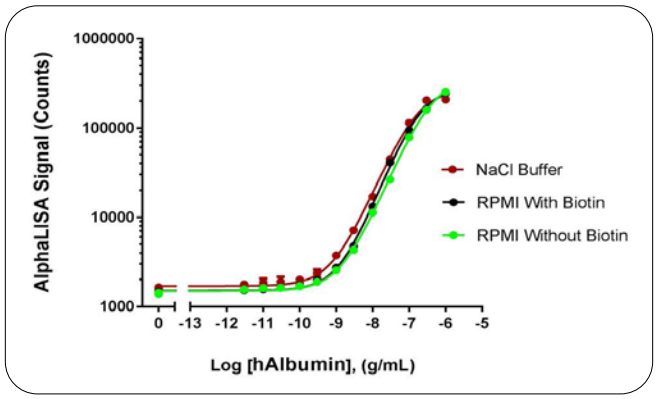


Figure 8: AlphaLISA Albumin biotin-free kit (AL363) assays were performed in NaCl buffer, RPMI, and RPMI without biotin. The standards were prepared in these three diluents and all other assay reagents were prepared in NaCl buffer.

Table 2: AlphaLISA Albumin kit and AlphaLISA Albumin biotin-free kit performance in NaCl buffer, RPMI, and RPMI media without biotin. The standards were prepared in these three diluents and all other assay reagents were prepared in NaCl buffer.

Assay specifications	AlphaLISA Albumin kit			AlphaLISA Albumin biotin-free kit		
	NaCl buffer	RPMI with biotin	RPMI without biotin	NaCl buffer	RPMI with biotin	RPMI without biotin
LDL (pg/mL)	48	333	45	78	121	137
LLOQ (pg/mL)	231	1401	212	413	531	570
EC_{50} (ng/mL)	120	235	263	159	187	256
S/B Ratio	157	136	204	143	177	179
Maximum Signal	596970	185719	681676	208422	244462	222622
Minimum Signal	4058	1394	3414	1577	1442	1280

Spike recovery

To further validate the AlphaLISA Albumin biotin-free detection kit, the spike recoveries of three known amounts of albumin analyte were analyzed and the results are summarized in Table 3. Overall, 80 to 94% of spiked analytes were recovered in all assays (Table 3). These results showed that the spike recoveries in the biotin-free kit are as good as in the AlphaLISA Albumin kit.

Table 3: AlphaLISA Albumin kit and AlphaLISA Albumin biotin-free kit performance: spike recoveries of three known concentrations of human albumin in NaCl buffer, RPMI, and RPMI without biotin.

Spiked hAlbumin (ng/mL)	% Recovery					
	AlphaLISA Albumin kit			AlphaLISA Albumin biotin-free kit		
	NaCl buffer	RPMI with biotin	RPMI without biotin	NaCl buffer	RPMI with biotin	RPMI without biotin
30	91	79	85	93	77	77
10	88	89	87	89	77	86
3	92	81	81	94	91	77

Summary

The presence of biotin can interfere with assays that are based on streptavidin/biotin capture. In certain AlphaLISA assays, this interference reduces the overall signal and can affect assay sensitivity (LDL and LLOQ). To overcome the effects caused by biotin, assay kits based on DIG/anti-DIG capture have been developed. In this application note we have demonstrated that the performance of the AlphaLISA biotin-free detection kits (Table 4) is not affected by high biotin concentration. These kits are recommended for the analysis of samples that contain high levels of biotin, such as cell culture media (RPMI, EX-CELL® 420, McCoy's) or other biological samples that contain high endogenous levels of biotin.

References

1. Immunogenicity Assessment using the AlphaLISA Technology. Application Note (2011), Revvity.
2. AlphaLISA Immunogenicity Assay Development Guide (2012), Revvity.
3. Performing AlphaLISA Assays with RPMI-1640 (2013), Revvity.

Table 4: AlphaLISA biotin-free detection kits

AlphaLISA biotin-free detection kits	100 assay points	500 assay points	5,000 assay points
Albumin (human serum)	AL363HV	AL363C	AL363F
Chloramphenicol	AL393HV	AL393C	AL393F
Factor VIII	AL362HV	AL362C	AL362F
HIV p24	AL332HV	AL332C	AL332F
IFN-γ (human)	AL327HV	AL327C	AL327F
IL-2 (human)	AL333HV	AL333C	AL333F
IL-6 (human)	AL3025HV	AL3025C	AL3025F
IL-8 (human)	AL328HV	AL328C	AL328F
IL-15 (human)	AL360HV	AL360C	AL360F
IL-17A (human)	AL346HV	AL346C	AL346F
IL-17A (mouse/rat)	AL544HV	AL544C	AL544F
TGF-1β (human)	AL361HV	AL361C	AL361F
TNFα (human)	AL325HV	AL325C	AL325F
TNFα (mouse)	AL541HV	AL541C	AL541F
	1,000 assay points		10,000 assay points
AlphaLISA TruHits biotin-free detection kit	AL901D		AL901M

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