AlphaLISA for biomarkers in urine: Measuring the renal tubular injury indicator, β2-microglobulin.

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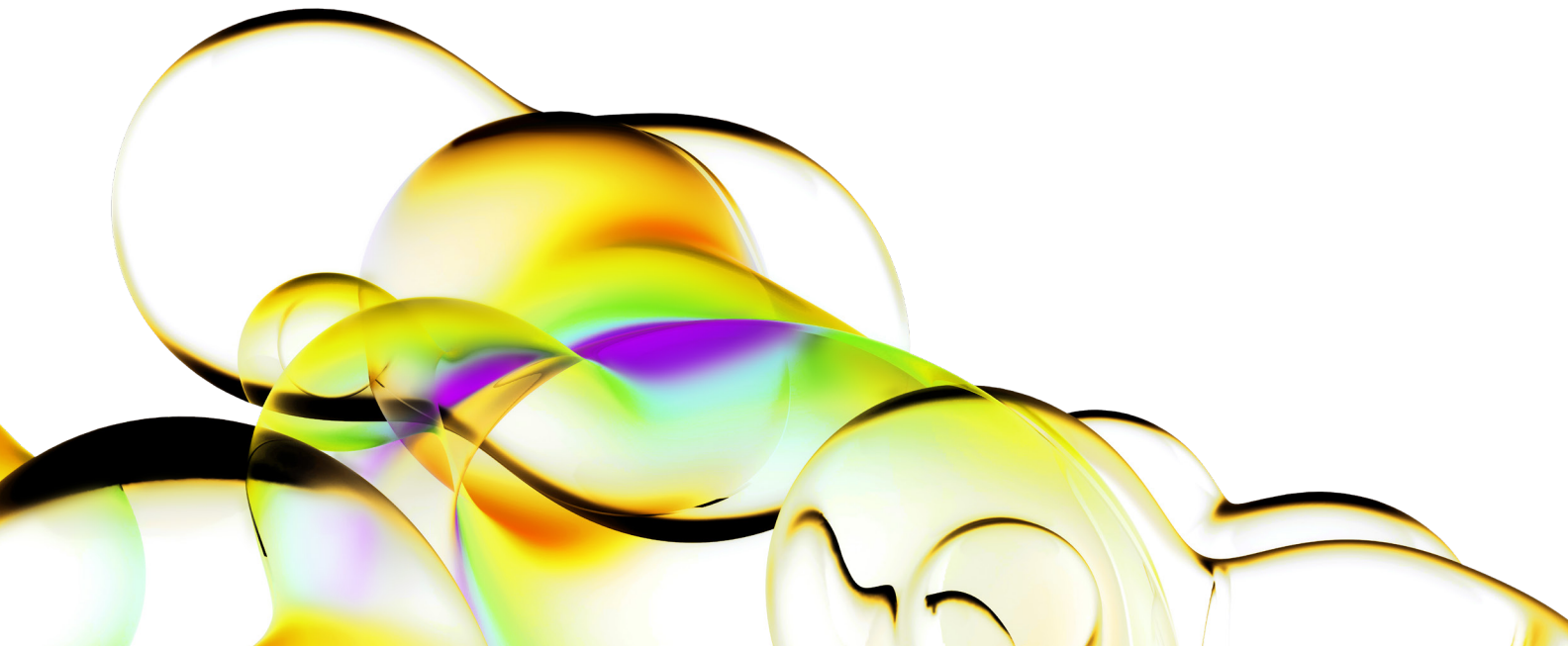
Introduction

Beta2-microglobulin is a single-chain, low molecular weight polypeptide that is found on the membrane of all nucleated cells.[1] If renal proximal tubular cells are functioning properly, after kidney filtration in the glomeri, the majority of β2-microglobulin is reabsorbed and catabolized. If the tubular function of the kidney is impaired, urinary levels of β2-microglobulin will be elevated over trace normal functioning levels. Thus, an abnormal level of β2-microglobulin in urine indicates renal filtration or reabsorption disorders.[2] Measurement of urinary β2-microtubulin is emerging as a method of assessing tubular function. However, the variability of urine matrix components such as organic compounds, pH, and electrolytes can affect antibody binding and assay performance.[3]

In this study, AlphaLISA™ technology is demonstrated for the first time in the complex matrix of urine, and is shown to detect the renal tubular injury marker Beta2-microglobulin (β2-microglobulin), in urine at a sensitivity of 77 pg/mL. AlphaLISA technology allows for the detection of molecules of interest in buffer, cell culture media, serum, plasma and now urine in a highly sensitive, quantitative, reproducible and user-friendly mode. In an AlphaLISA assay, a biotinylated anti-analyte antibody binds to the Streptavidin-coated Alpha Donor beads, while another anti-analyte antibody is conjugated to AlphaLISA Acceptor beads. In the presence of the analyte, the beads come into close proximity. The excitation of the Donor beads provokes the release of singlet oxygen molecules that



triggers a cascade of energy transfer within the Acceptor beads, resulting in a sharp peak of light emission at 615 nM (Figure 1). This light emission can then be detected on an Alpha-enabled reader such as the EnVision™ multilabel plate reader.

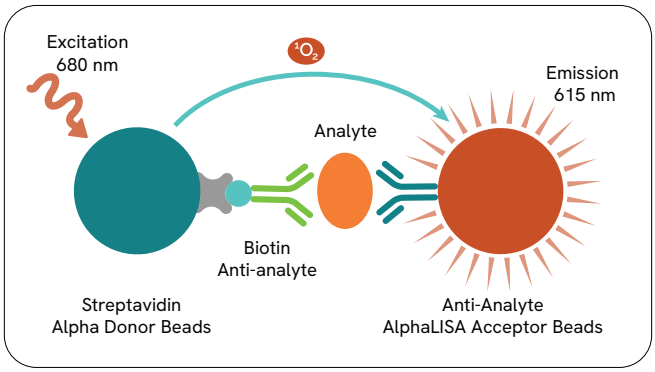


Figure 1: AlphaLISA assay schematic. In the presence of analyte, antibodies sandwich the analyte and bring the Donor beads and Acceptor beads within close proximity. Upon excitation, singlet oxygen from the Donor beads diffuses and activates nearby Acceptor beads to generate light that is proportional to the amount of analyte.

Here, we demonstrate how AlphaLISA technology can be used to quantify levels of β2-microglobulin in human urine samples. To ensure proper and accurate detection of biomarkers in urine, dilution linearity and spike-and-recovery experiments were performed to determine the proper dilution factors required for accurate recovery.

Materials and methods

Human urine samples

Pooled normal human urine, used for lower detection limit (LDL), dilution linearity, and spike-and-recovery experiments, was purchased from Innovative Research (Cat # IR100007P).

Detection of β2-microglobulin using AlphaLISA technology

The AlphaLISA immunoassay for human beta2-microglobulin (Revvity #AL3067C/F) was performed according to the recommended protocols provided with each kit (Figure 2).

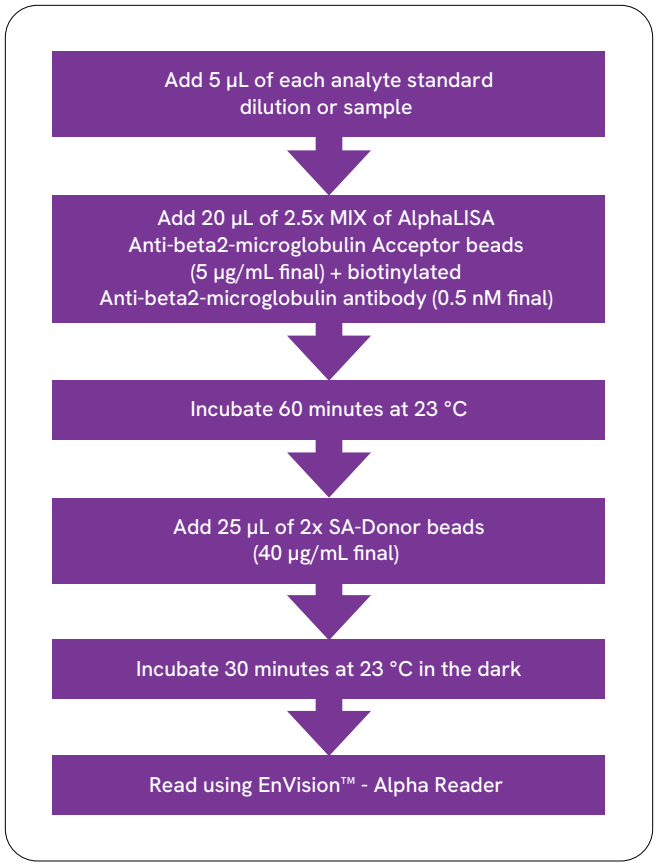


Figure 2: Workflow for human β2-microglobulin AlphaLISA detection assay.

Dilution linearity experiments

Neat normal human urine was diluted with immunoassay buffer (IAB). The assay was performed along with a standard curve using the analyte prepared in IAB. Concentrations of β2-microglobulin in diluted human urine were determined by interpolating to the standard curve generated in IAB.

Spike-and-recovery experiments

To ensure the chosen diluent, IAB, was optimal for recovering and quantifying analyte in urine samples, a spike-and-recovery experiment was performed. Three known amounts of β2-microglobulin were spiked into normal human urine (100, 30 and 10 ng/mL in spiked samples) and then the samples were diluted eight-fold into IAB. The standard was prepared in IAB. It is important to load analyte standards into assay plates within 60 minutes of preparation to avoid significant loss of analyte on vial walls at lower concentrations. The standard and all other reagents were prepared in IAB. The spike recoveries were determined. Recoveries were calculated after correction using a "no spike" β2-microglobulin sample.

Instrumentation

All AlphaLISA measurements were performed on the Revvity EnVision multilabel plate reader (Figure 3) which provides fast, sensitive Alpha technology detection, in addition to fluorescence intensity, luminescence, absorbance, fluorescence polarization, and time-resolved fluorescence detection technologies. The system is based on hybrid technology, combining filters and a monochromator for enhanced flexibility. It incorporates proprietary Direct Double Optics™ technology for high speed and sensitivity in simultaneous filter-based readouts, such as FRET assays, and for TRF, lamp-based excitation is standard, with the option of a high energy laser for higher speed and sensitivity.

Figure 3: EnVision multilabel plate reader.

Data analysis

Diluted samples were compared to standard curves prepared in IAB. Standard curves were prepared using the recombinant standards provided in the AlphaLISA kit. Standard curves were plotted in GraphPad Prism® version 6.0 using nonlinear regression and the four-parameter logistic equation (sigmoidal dose-response curve with variable slope) with $1/Y^2$ weighting method. For dilution linearity experiments R-squared (R^2) values were calculated to represent linearity and approximate percent recovery was calculated by dividing the interpolated value (from the standard curve) by the expected value then multiplied by 100 to convert to percent. For spike-and-recovery experiments, percent recovery was calculated by dividing interpolated serum spikes by the associated interpolated diluent spikes then multiplied by 100 to convert to percent. Spike values were determined by comparing to a standard curve prepared in the diluent used.

Additionally, all urine sample concentrations were interpolated onto a standard curve prepared in the same diluent as that used to dilute the sample. Further, the lower limit of detection (LDL) was calculated by averaging the blank wells (wells without analyte) and adding three times the standard deviation then interpolating the concentration from the standard curve. Below are the formulas:

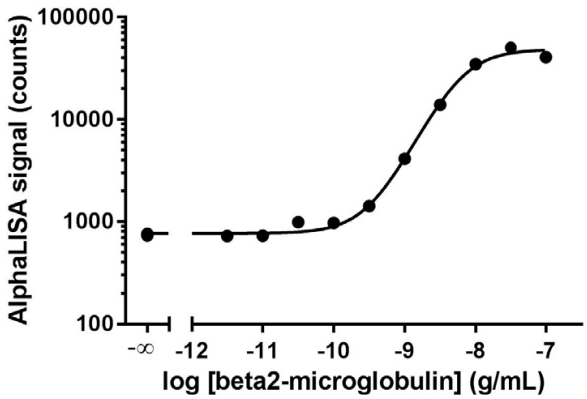
$$\% \text{ Recovery} = \frac{\text{(spiked sample value/expected sample value) X 100}}{}$$

$$\text{LDL} = \text{mean (blanks)} + 3 \text{ X SD}$$

Results

Standard curve

Figure 4 shows a representative AlphaLISA human β2-microglobulin calibration curve performed with 5 µL of β2-microglobulin standard ranging from 3-100,000 pg/mL under optimized assay conditions. The AlphaLISA signal obtained was plotted against the β2-microglobulin concentrations tested.

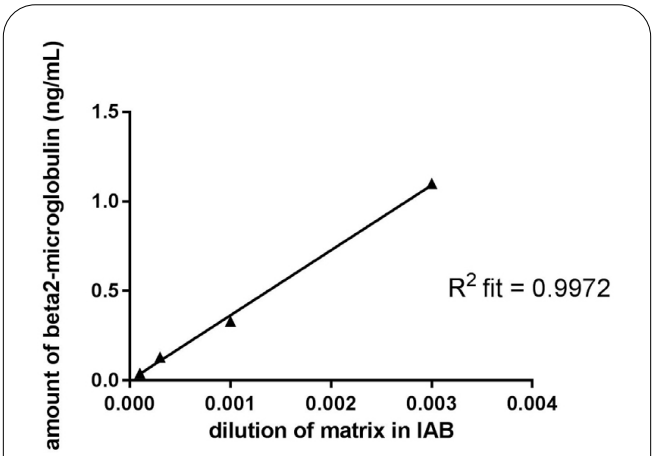


Kit	LDL (pg/mL)	Dynamic range (pg/mL)	S/B
β2-microglobulin	77	77-100,000	62

Figure 4: Performance of β2-microglobulin AlphaLISA Immunoassays. The standard curve shown was performed in AlphaLISA Immunoassay buffer. The table displays the performance characteristics for the assay.

Diluent linearity

Another benefit of Alpha technology is its compatibility with complex biological matrices like human urine. Excellent dilution linearity (R^2 =0.9972) was achieved in the β2-microglobulin-spiked human urine sample that was diluted ≥ eight fold. The results are shown in Figure 5.



Dilution factor (x)	Observed beta2-microglobulin (pg/mL)
100	2,700
300	1,100
1,000	330
3,000	130
10,000	40

Figure 5: Dilution linearity experiment performed using the β2-microglobulin AlphaLISA kit. The y-axis is the interpolated concentrations of β2-microglobulin at each dilution and the x-axis is the dilution. High points were excluded until the R-squared value was greater than 0.997.

Spike-and-recovery experiments

The spike recoveries of β2-microglobulin were determined and the results are shown in Table 1. Excellent recovery was seen for all three known amounts of β2-microglobulin spiked into normal human urine.

Table 1: Spike-and-recovery experiment performed using β2-microglobulin AlphaLISA kit showing the percent recovery of the urine spike samples compared to the diluent spike samples.

	Diluent: IAB	
	Spiked sample (human urine)	
Spike (µg/mL)	Concentration (µg/mL)*	Recovery (%)
No spike	0.033	NA
10	7.62	76.2
3	2.12	70.5
1	0.91	91

Conclusion

In this application note, we demonstrate the performance of the β2-microglobulin AlphaLISA detection kit for the first time in urine. AlphaLISA is a bead-based assay technology that doesn't require wash steps and provides excellent assay performance in terms of signal-to-background, dynamic range, and sensitivity. These data demonstrate how Alpha technology is an exceptional immunoassay technology that can be optimized to work with complex matrices such as urine. Assay dilution linearity and exceptional spike and recovery in urine were demonstrated for the clinically relevant biomarker, β2-microglobulin.

References

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