AlphaLISA in urine: Detecting kidney injury marker KIM-1.

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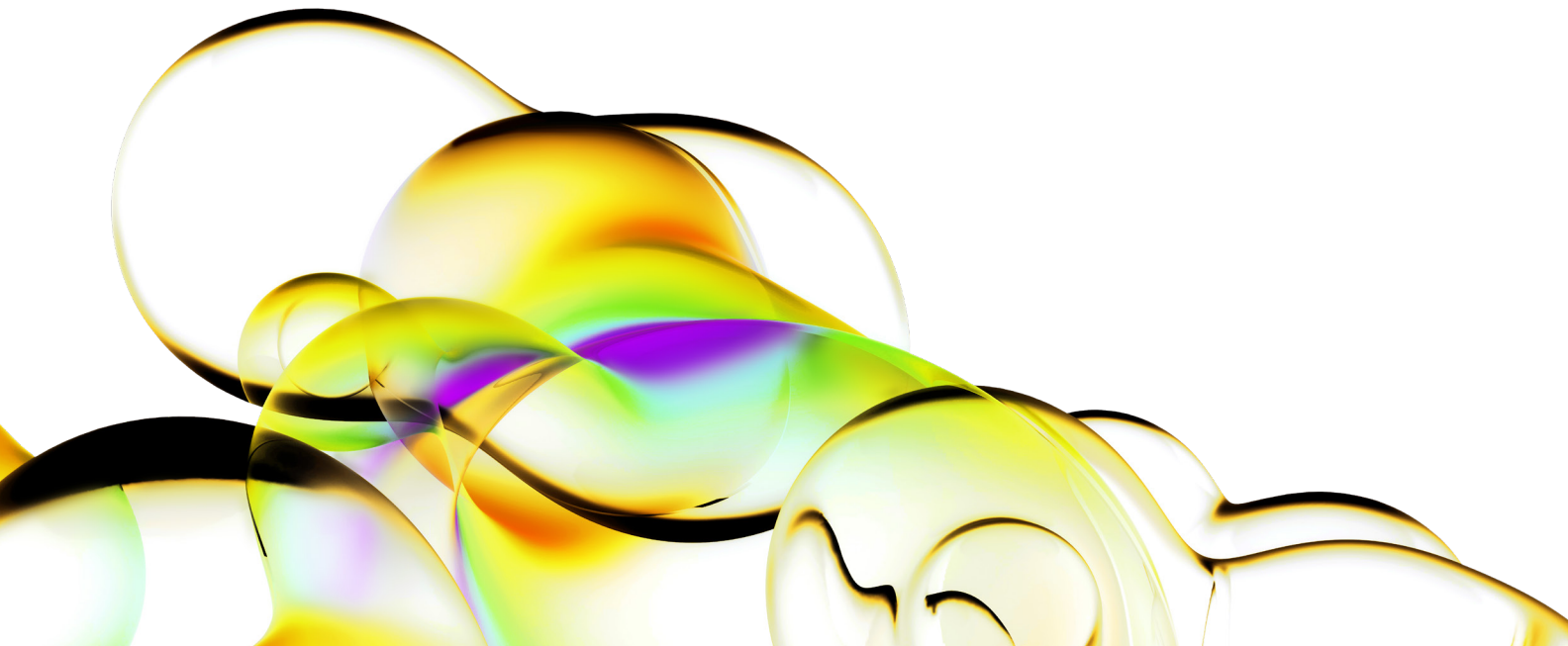
Introduction

AlphaLISA™ technology is demonstrated for the first time in the complex matrix, urine. The variability of urine matrix components such as organic compounds, pH, and electrolytes can affect antibody binding and assay performance.[1] In this note, AlphaLISA technology was shown to detect the kidney injury marker, KIM-1, in urine down to 14 pg/mL. Acute kidney injuries (AKIs) are increasingly common and often are a complication that accompanies other severe illnesses. Acute kidney injury is a frequent and serious complication, occurring in 1 – 5% of all patients treated at a hospital. This figure jumps to 50% for patients being treated in the intensive care unit and often results from general infection or sepsis.[2] Acute kidney injury causes a build-up of waste products in the blood and makes it hard for the kidneys to balance fluid in the body. Morbidity and mortality occurring as a result of AKIs remain high despite improvements in general healthcare. Current diagnosis of AKIs may be made by urine tests, blood tests, urine output, imaging or biopsy. One approach of particular interest is to detect specific injury biomarkers in urine that will help diagnose AKI early and predict treatment outcome. Kidney injury molecule-1 (KIM-1), also known as Hepatitis A virus cellular receptor 1 (HAVcr-1) and T-cell immunoglobulin and mucin domain 1 (TIM-1), is a promising biomarker that is undetectable in urine when kidneys are functioning normally but becomes elevated as ischemia occurs.[3] Kidney injury molecule-1 is a trans-membrane structural glycoprotein that is expressed on renal proximal tube endothelial cells. These cells are particularly sensitive to injury and in response KIM-1 is shed into the urine.[4]



AlphaLISA technology allows for the detection of molecules of interest in buffer, cell culture media, serum, plasma, urine, and other complex matrices 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 (Fig. 1). This light emission can then be detected on an Alpha-enabled reader.

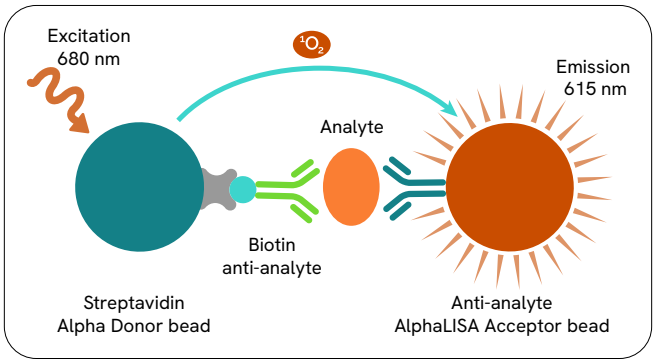


Figure 1: AlphaLISA assay schematic. In the presence of analyte, antibodies sandwich the analyte and bring the Donor beads and Acceptor beads within close proximity. Upon excitation, singlet oxygen from the Donor beads diffuses and activates nearby Acceptor beads to generate light that is proportional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

Here, we demonstrate how AlphaLISA technology can be used to quantify levels of KIM-1 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 dilution factors required for accurate recovery.

Materials and methods

Human urine samples

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

Detection of KIM-1 using AlphaLISA technology

The AlphaLISA immunoassay for human KIM-1 (Revvity #AL3075C/F) was performed according to the recommended protocols provided with each kit (Fig. 2).

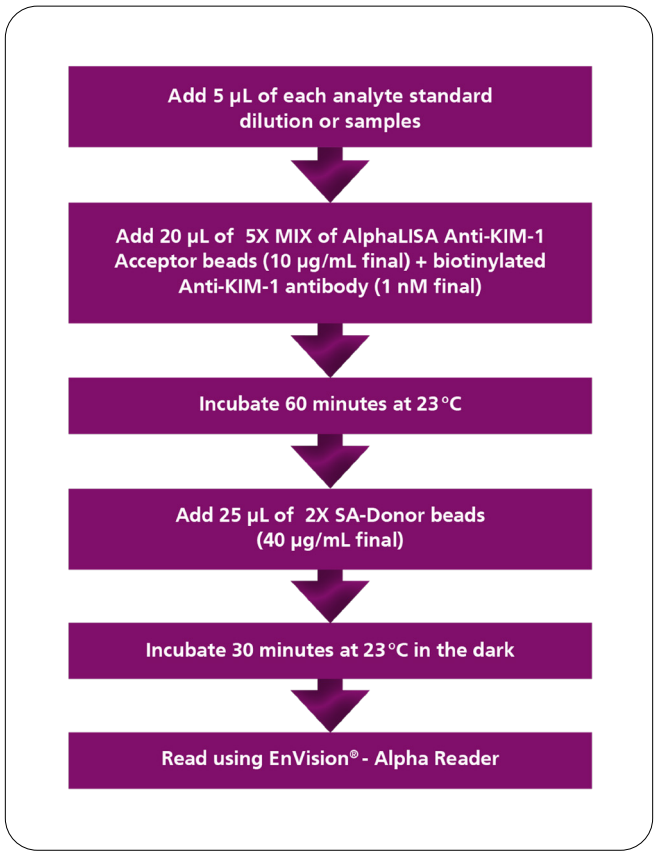


Figure 2: Workflow for human KIM-1 AlphaLISA detection assay.

Dilution linearity experiments

When optimizing an Alpha assay for detection of an analyte in serum or any other matrices, a linearity of dilution experiment should be performed. The purpose of this experiment is to identify the extent of sample dilution required to ensure accurate quantification of the analyte. The optimal dilution level will be one that achieves assay linearity (R-squared value ≥ 0.995). Linearity of dilution experiments were performed for the KIM-1 AlphaLISA kits. Neat normal human urine and KIM-1-spiked (100 ng/mL) normal human urine samples were diluted with immunoassay buffer (IAB) and the assay was performed along with a standard curve using an analyte prepared in IAB. Concentrations of KIM-1 in diluted human urine were determined by interpolating to the standard curve.

Spike-and-recovery experiments

Three known amounts of KIM-1 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 and all other reagents were prepared in IAB. The spike recoveries were determined. Recoveries were calculated after correction using a "no spike" KIM-1 sample.

Instrumentation

All AlphaLISA measurements were performed on the Revvity EnVision™ multimode plate reader (Fig. 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 the same diluent. 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 using the four-parameter logistic equation (sigmoidal dose-response curve with variable slope) with $1/Y^2$ weighting method. For dilution linearity experiments, 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 multiplying 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 multiplying by 100 to convert to percent. Spike values were determined by comparing to a standard curve prepared in the diluent used. Additionally, all real sample concentrations were interpolated onto a standard curve prepared in the same diluent as that used to dilute the sample. Further, 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.

$$\text{\% Recovery} = \frac{\text{(spiked sample value / expected sample value) X 100}}{\text{LDL = mean (blanks) + 3 X SD}}$$

Results

Standard curve

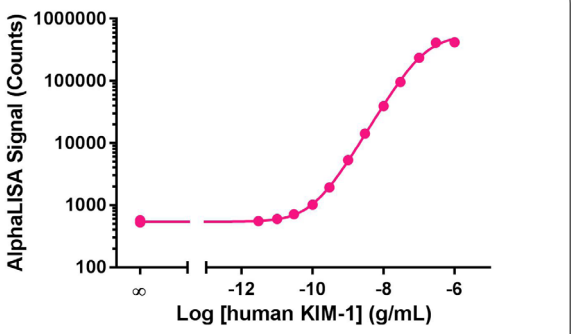
Figure 4 shows a representative AlphaLISA human KIM-1 calibration curve performed with 5 µL of KIM-1 standard ranging from 3 – 1,000,000 pg/mL under optimized assay conditions. The AlphaLISA signal obtained was plotted against the KIM-1 concentrations tested.

Dilution linearity

Another benefit of Alpha technology is its compatibility with complex biological matrices such as human urine. Excellent dilution linearity ($R^2 \geq 0.999$) was achieved in the KIM-1-spiked human urine sample that was diluted ≥ eight-fold. The results are shown in Figure 5.

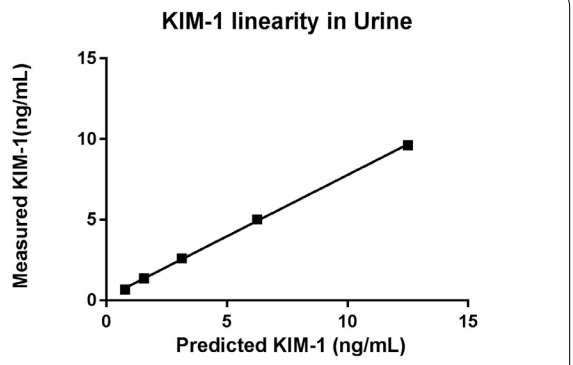
Spike-and-recovery experiments

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



Kit	LDL (pg/mL)	Dynamic range (pg/mL)	S/B
KIM-1	14	14 - 300,000	848

Figure 4: Performance of KIM-1 AlphaLISA Immunoassays. The standard curve shown was performed in AlphaLISA Immunoassay buffer. The table below the graph shows the performance characteristics for the assay.



Dilution factor (x)	Expected KIM-1 (ng/mL)	Observed KIM-1 (ng/mL)
8	12.5	9.62
16	6.25	5.02
32	3.13	2.60
64	1.56	1.35
128	0.78	0.67

Figure 5: Dilution linearity experiments performed using the KIM-1 AlphaLISA kit in IAB. The y-axis is the interpolated concentrations of KIM-1 at each dilution and the x-axis is the observed concentrations of KIM-1. Sample was diluted to yield R^2 value = 0.999.

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

	Diluent: IAB	
	Spiked sample (Pooled normal human urine)	
Spike (ng/mL)	Concentration (ng/mL)	Recovery (%)
No spike	0.2	N/A
100	72.7	73
30	29.3	98
10	8.5	85

Conclusion

In this technical note, we demonstrated the performance of the human KIM-1 AlphaLISA detection kit in human urine. AlphaLISA is a bead-based assay technology that requires no washing and provides excellent assay performance with regard to 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, KIM-1.

References

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