APPLICATION NOTE

Applicability of AlphaLISA technology to a wide spectrum of complex biological samples.

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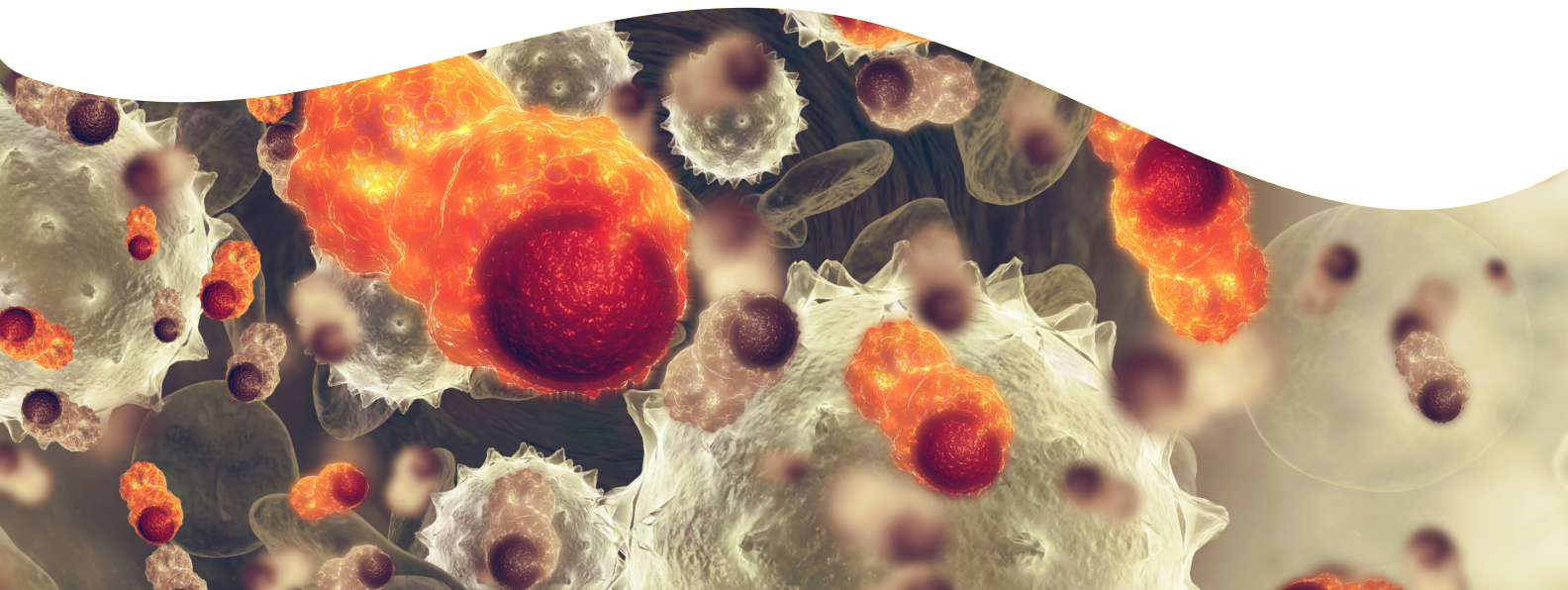
Introduction

ELISA assays have commonly been used for quantitation of biomarkers and other analytes in a variety of sample types. In an ELISA, separation of the target analyte-of-interest from other proteins and biomolecules in the sample occurs through repeated, stringent wash steps. Though these wash steps provide isolation of the target biomolecule for quantitation, they also render the assay tedious and time-consuming.

In contrast, AlphaLISA™ provides a homogeneous (no wash steps) assay with a simple, streamlined workflow that can be used to detect and quantitate biomolecules in both simple and complex sample types, including cell lysates, cell culture supernatants, tissue, urine, serum, plasma, cerebrospinal fluid, and other biological samples. In an AlphaLISA assay, streptavidin Alpha Donor beads bind to a biotinylated antibody specific for the target analyte. A second anti-analyte antibody is directly-conjugated to AlphaLISA Acceptor beads. In the presence of the target analyte, the beads come into proximity. Excitation of the Donor beads at 680 nm triggers the release of singlet oxygen molecules that causes a cascade of energy transfer in the nearby Acceptor beads, resulting in a sharp peak of light emission at 615 nm (Figure 1). The amount of analyte in the sample is proportional to the emission signal which can be quantitated by interpolation from a standard curve.

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



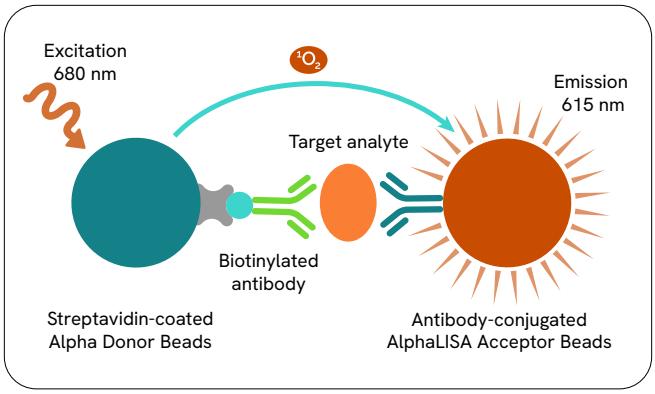


Figure 1: Assay principle for AlphaLISA biomarker detection assay (sandwich immunoassay format).

For this application note, we developed an AlphaLISA assay for the detection of mouse MMP12 and used this as a model to assess compatibility of AlphaLISA technology with various sample types using spike-and-recovery and linearity experiments. Though the target analyte for the model assay was a mouse protein, porcine tissue (pork chop), human urine, human serum, and human plasma were used to simulate actual samples due to commercial availability and to mimic matrix effects without concern for interference from endogenous MMP12. AlphaLISA provided a robust assay with high sensitivity and wide dynamic range that was suitable for a variety of complex sample types.

Materials and methods

Reagents

- Anti-mouse MMP12 antibody, polyclonal (R&D Systems, #AF3467)
- Mouse MMP12 recombinant analyte (R&D Systems, #3467-MPB-020)
- ChromaLink® biotinylation reagent (TriLink, #B1001-105)
- Zeba desalting columns (ThermoFisher, #89889)
- Streptavidin Alpha Donor beads (Revvity, #6760002)
- Unconjugated AlphaLISA Acceptor beads (Revvity, #6772001)
- Carboxymethylamine hemihydrochloride (Sigma, #C13408)
- Sodium cyanoborohydride (Sigma, #296945)
- AlphaLISA immunoassay buffer (Revvity, #AL000C)
- DPBS, no calcium, no magnesium (ThermoFisher, #14190-114)
- BSA (Revvity, #CR84-100)
- 96-well V-bottom StorPlates™ (Revvity, #6008290)
- 384-well AlphaPlates™ (Revvity, #6005350)

Samples

- Buffer: 1X AlphaLISA immunoassay buffer (Revvity, #AL000)
- Cell Lysates: HCT116-Red-Fluc Bioware® Brite cell line (Revvity, #BW124318), lysed with 1X AlphaLISA immunoassay buffer
- Supernatant: DMEM (ATCC, #30-2002) + 10% FBS (ThermoFisher, #26140079)
- Supernatant: RPMI-1640 (ATCC, #30-2001) + 10% FBS
- Tissue: Pig (porcine – pork chop) tissue homogenized in RIPA buffer with protease inhibitors, 20 mg/mL (local grocery store)
- Urine: Normal Human Pooled Urine, no filtration (Innovative Research)
- Serum: Normal Human Pooled Serum, sterile filtered (Sigma, #H4522)
- Plasma: K3EDTA Pooled Human Plasma, no filtration (BioIVT custom)

Diluents for standard curves

- AlphaLISA immunoassay buffer
- DMEM + 10% FBS
- RPMI + 10% FBS
- 0.5% BSA in PBS
- RIPA buffer (ThermoFisher, #89900)
- FBS

AlphaLISA assay development

The AlphaLISA detection assay was developed using the same polyclonal anti-mouse MMP12 antibody as both the biotinylated and AlphaLISA Acceptor bead-conjugated antibody. Biotinylated anti-mouse MMP12 antibody was prepared using the ChromaLink® biotinylation reagent as described in the ChromaLink® manual. Anti-mouse MMP12-conjugated AlphaLISA Acceptor beads were prepared using the recommended standard protocol. The concentration of biotinylated antibody, bead concentrations, order-of-addition, and optimal incubation times were determined in AlphaLISA Immunoassay Buffer (IAB) and 384-well AlphaPlates. The optimal bead concentrations that produced the best sensitivity and signal to background for this assay were 40 µg/mL Streptavidin Alpha Donor beads and 10 µg/mL AlphaLISA Acceptor beads in a standard, 50 µL final volume reaction. The final protocol developed is shown in Figure 2.

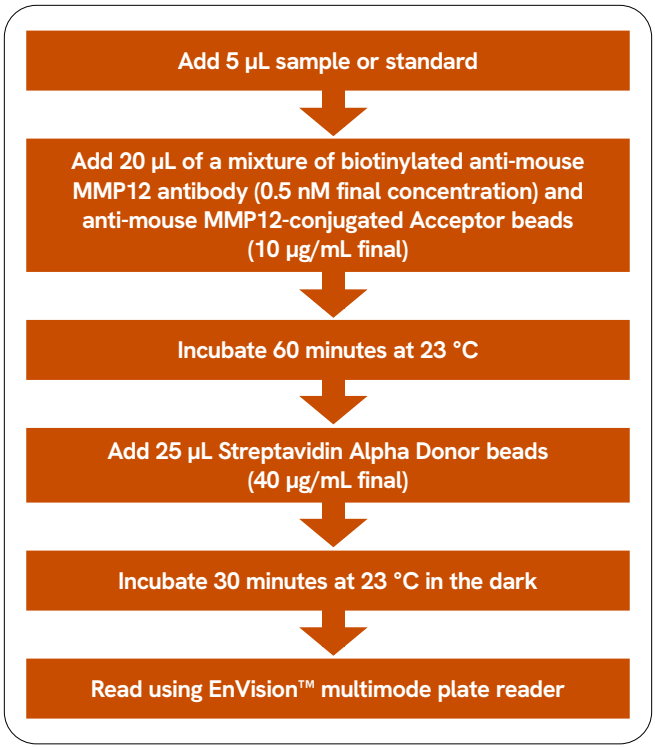


Figure 2: Optimized protocol for AlphaLISA mouse MMP12 detection assay.

Instrumentation and data analysis

The AlphaLISA assay was measured using a Revvity EnVision™ 2105 multimode plate reader using default values for standard Alpha detection. Standard curves for the AlphaLISA assay were performed in indicated sample diluents using recombinant mouse MMP12 protein as the standard. Curves were plotted in GraphPad Prism® according to a nonlinear regression using the four-parameter logistic equation (sigmoidal dose-response curve with variable slope) and $1/Y^2$ data weighting. The lower detection limit (LDL) of the assay was calculated by taking three times the standard deviation of the average background values and interpolating concentration from the standard curve.

Dilution linearity experiments

Linearity of dilution experiments were performed to preliminarily determine an appropriate diluent for each sample type. A known amount of standard analyte was spiked into each sample, then diluted in two-fold increments in the chosen diluents using a 96-well StorPlate (polypropylene microplate). Dilutions of the standard analyte in each proposed diluent were set up in a separate StorPlate. The optimized AlphaLISA assay protocol was run using spiked samples and standards in proposed diluent. To assess linearity, the concentrations of each spike and spike dilution were interpolated from the standard curve in the proposed diluent and plotted against the dilution factor. A linear regression was performed, and linearity was assessed by correlation coefficient.

Spike-and-recovery experiments

For spike-and-recovery experiments, each experimental sample was spiked with 1,500, 5,000, or 15,000 pg/mL mouse MMP12 analyte. In a second set of tubes, each proposed diluent was spiked with the same concentration of analyte. A standard curve was then run using each proposed diluent. The concentration of each spike-in was interpolated from the corresponding standard curve. Percent recovery was calculated using the following equation:

% Recovery = (spiked sample value / expected sample value) X 100

Results

Determining the appropriate diluent that matches the biological sample of interest is critical for accurate detection in any assay format. Substances within the sample such as organic compounds, proteins, and electrolytes can interfere with the chemistry or binding interactions involved in the detection technology. Sample matrix effects can shift the standard curve, making it essential to prepare the standard curve in a diluent that is as similar as possible to the biological sample for accurate quantification.

We used linearity and spike-and-recovery experiments to determine the optimal diluent for each sample type and assess overall sample compatibility. Criteria for sample compatibility included a linearity correlation coefficient >0.995 and spike-and-recovery values within the range of 70-130%. In some cases, several potential diluents were tested. Recovery results for optimal diluents are shown in Table 1. The serum sample required a 2-fold dilution for good linearity, indicating that serum samples would need to be diluted 2-fold in 100% FBS prior to running the assay. All other samples types did not require dilution for linearity (data not shown) and could therefore be used "neat" (i.e., without sample dilution). Suitable recoveries were obtained for all samples.

Table 1: Recovery of analyte spiked in each sample type with standard curves prepared in optimal diluents in an AlphaLISA mouse MMP12 assay.

Sample type	% Recovery
Buffer (Diluent: AlphaLISA Immunoassay Buffer)	116
Cell Lysate (Diluent: AlphaLISA Immunoassay Buffer)	78
DMEM + 10% FBS (Diluent: DMEM + 10% FBS)	129
RPMI + 10% FBS (Diluent: RPMI + 10% FBS)	103
Urine (Diluent: PBS + 0.5% BSA)	93
Tissue (Diluent: RIPA Buffer)	88
Serum (Diluent: 100% FBS)	125
Plasma (Diluent: 100% FBS)	81

From the standard curve in each diluent, the sensitivity, dynamic range, and signal-to-background were calculated. Standard curves are shown in Figure 3. Slight shifts in the standard curve can be observed as the diluent is changed.

These shifts can be attributed to differences in matrix effects, which can have a significant impact on the interpolated concentration of a given test sample and illustrate the importance of identifying an appropriate diluent for each sample type through linearity and recovery experiments. As seen in Table 2, the AlphaLISA assay maintained excellent sensitivity with broad dynamic range and large signal-to-background ratio for all sample types tested.

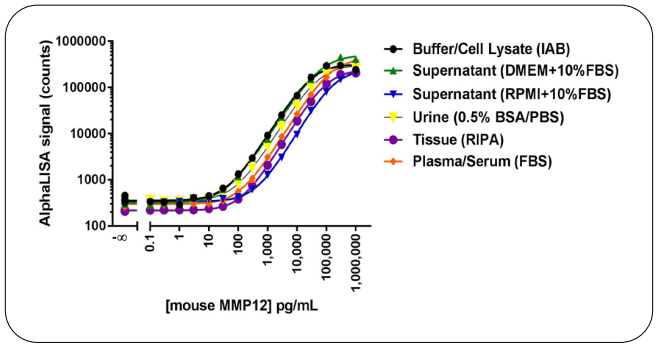


Figure 3: Standard curves for AlphaLISA mouse MMP12 assay in final diluent for each sample matrix.

Table 2: Compiled results for AlphaLISA assay performance for all sample types.

Sample type	Compatible with AlphaLISA?	Diluent for standard curve	Assay sensitivity (LDL)	Dynamic range	Signal-to-background
Buffer	✓	AlphaLISA Immunoassay Buffer	5 pg/mL	5 log	1269
Cell Lysate	✓	AlphaLISA Immunoassay Buffer	10 pg/mL	5 log	1493
Supernatant (DMEM + 10% FBS)	✓	DMEM + 10% FBS	13 pg/mL	5 log	1438
Supernatant (RPMI + 10% FBS)	✓	RPMI + 10% FBS	26 pg/mL	4.5 log	675
Urine	✓	PBS + 0.5% BSA	15 pg/mL	5 log	796
Tissue	✓	RIPA Buffer	31 pg/mL	5 log	990
Serum	✓ (2-fold sample dilution was required for linearity)	100% FBS	*77 pg/mL	4 log	1313
Plasma	✓	100% FBS	17 pg/mL	5 log	1108

*Sample required dilution (determined by linearity experiments) – value indicates calculated sensitivity in undiluted sample, as corrected by sample dilution factor. Standard curves in FBS performed on different days for serum and plasma samples.

Summary

AlphaLISA can be used as a convenient, fast, no-wash assay for the detection and quantitation of target analyte in a variety of complex sample matrices. Using an AlphaLISA assay for detection of mouse MMP12, AlphaLISA technology was shown to be compatible with both simple and complex sample matrices, including cell lysates, cell supernatants, urine, tissue, serum, and plasma. Linearity and spike-and-recovery experiments were used to determine appropriate diluents for the standard curve and overall sample compatibility. Assay performance was also measured by evaluating the sensitivity, dynamic range, and signal-to-background ratio for each sample type, and the AlphaLISA format provided a highly sensitive assay with large dynamic range and excellent signal-to-background values.

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