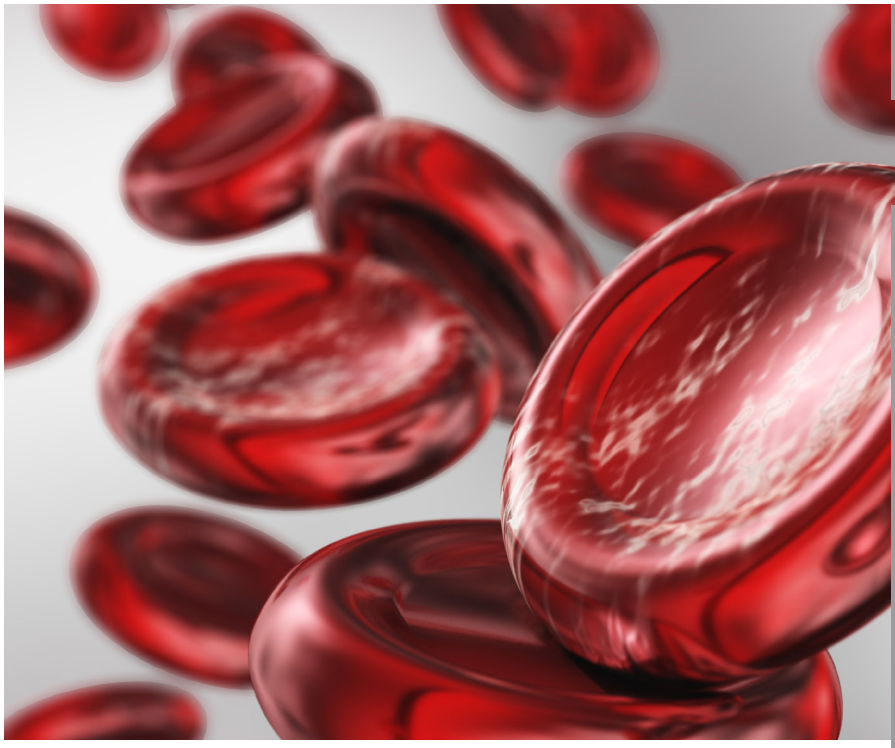

APPLICATION NOTE

DELFIA TRF

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Applicability of DELFIA Fluorescence-Based Immunoassay Technology to Detect and Quantitate Biomarkers Within a Broad Array of Complex Biological Samples

Introduction

ELISA has commonly been used for quantitation of biomarkers and other analytes in a variety of sample types. In an ELISA, separation of the target analyte from other proteins and biomolecules in the sample is accomplished via repeated, stringent wash steps within the workflow. While wash-based ELISAs provide excellent quantitation of the target biomolecule, there are several limitations. Colorimetric-based ELISAs have a small dynamic assay range that can sometimes require extensive dilution for samples to fall within the measurable window of the assay. Chemiluminescent-based ELISAs have improved dynamic range performance over colorimetric-based ELISAs; however, assay signal must be detected rapidly within a narrow window of time.

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In contrast, DELFIA® time-resolved fluorescence (TRF) technology provides a wash-based assay with a workflow similar to ELISA, but with a wide dynamic range and stable signal that can be measured from overnight to days, months or even years post-assay with proper storage of plates. DELFIA assays can be used to detect and quantitate biomolecules of interest in complex sample types, including tissue, serum, plasma, whole blood, cerebrospinal fluid, and other biological samples. In a DELFIA analyte detection assay, capture antibody is directly or indirectly coated to a microplate. Sample or standard is added, followed by a series of wash steps. Following the first series of washes, Europium-labeled detection antibody (or a combination of primary antibody and Europium-labeled secondary antibody or an alternate Europium-labeled tracer) is added. The plate is washed in a final step, followed by addition of the Enhancement Solution, which releases the Europium from its chelate, allowing the formation of a new, highly fluorescent chelate in solution. Excitation at 320 or 340 nm leads to detectable emission at 615 nm (Figure 1). The amount of analyte is proportional to the emission signal which can be quantitated by interpolation from a standard curve. The time-resolved nature of the detection step minimizes background signal, resulting in a stable, maximized signal-to-background.

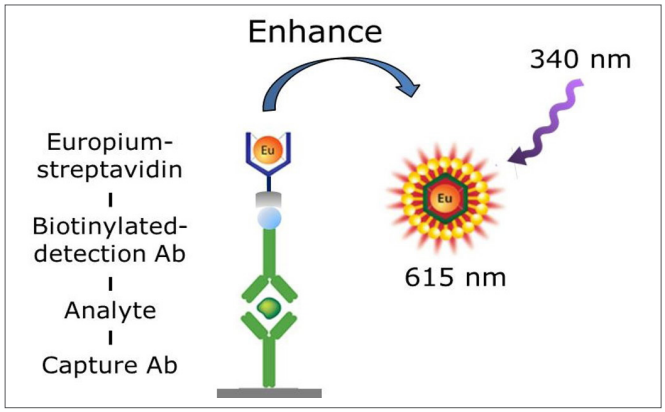

Figure 1. Assay principle for DELFIA TRF analyte detection assay. As illustrated, in this sandwich immunoassay format a biotinylated detection antibody is used in conjunction with Europium-streptavidin tracer.

We developed and validated a DELFIA assay for detection of mouse MMP12 with various biological samples using spike-and-recovery and linearity experiments. Though the target analyte for the model assay was a mouse protein (mouse MMP12), porcine tissue, human urine, human serum, human plasma, and sheep blood were used considering commercial availability. DELFIA proved to be a robust assay with high sensitivity, superior signal stability, and wide dynamic range, compatible with a variety of complex biological samples with minimal sample dilution required.

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)
- DELFIA Eu-N1-streptavidin (PerkinElmer, #1244-360)
- DELFIA Assay Buffer (PerkinElmer, #1244-106)
- DELFIA Wash Concentrate (PerkinElmer, #1244-114)
- DELFIA Enhancement Solution (PerkinElmer, #1244-105)
- 96-well SpectraPlate™ HB (PerkinElmer, #6005600)
- DPBS, no calcium, no magnesium (ThermoFisher, #14190-114)
- BSA (PerkinElmer, #CR84-100)

Samples

- Cell Lysates: HCT116 cell line (ATCC), lysed with 1X AlphaLISA immunoassay buffer
- Supernatant: RPMI-1640 (ATCC, #30-2001) + 10% FBS (ThermoFisher, #26140079)
- 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)
- Blood: Sodium Heparin Sheep Whole Blood (BioIVT custom)

Diluents for Standard Curves

- Lysis buffer (PerkinElmer, #AL000C)
- RPMI + 10% FBS
- DELFIA assay buffer
- RIPA buffer (ThermoFisher, #89900)
- Fetal bovine serum (FBS)

DELFIA Assay Development

A sandwich assay was developed using the same polyclonal anti-mouse MMP12 antibody as both the capture and biotinylated detection antibody. High bind SpectraPlates™ were coated with capture antibody using the recommended protocol.[1] Biotinylated anti-mouse MMP12 antibody was prepared using the ChromaLink® biotinylation reagent as described in the ChromaLink® manual. The concentration of biotinylated antibody, concentration of Europium-streptavidin, order of addition, and incubation times were optimized as described.[1] The final, optimized protocol is shown in Figure 2.

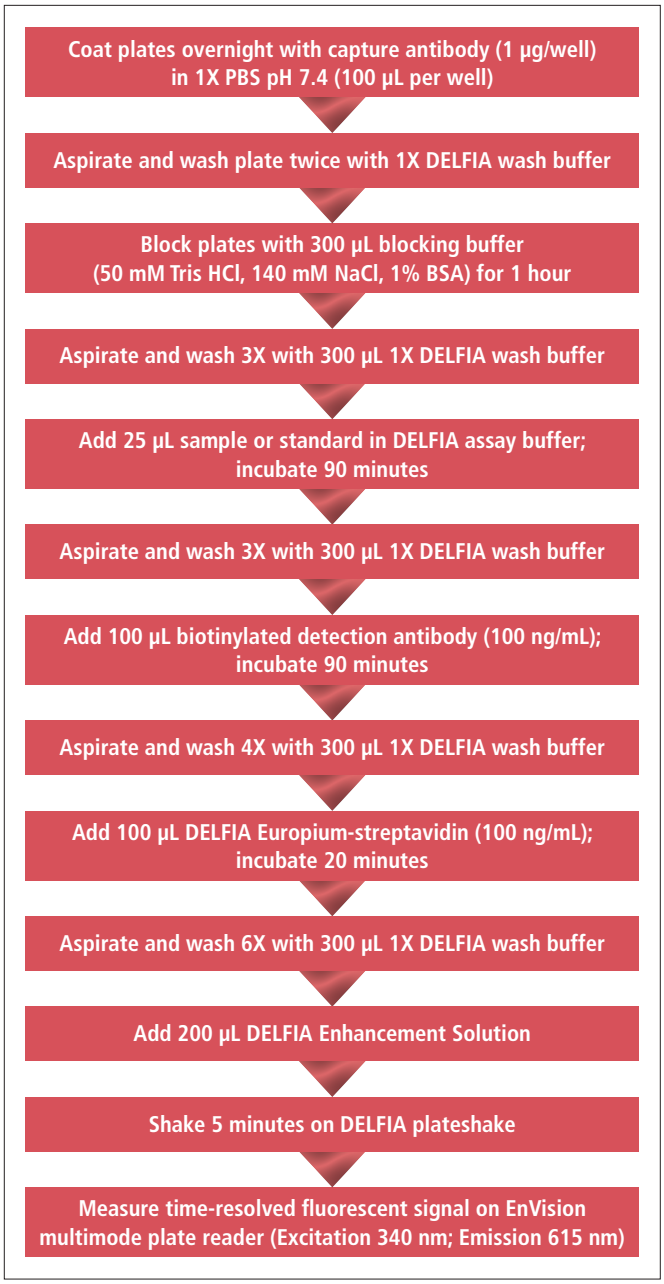

Figure 2. Optimized protocol for DELFIA mouse MMP12 detection assay. All steps were performed at room temperature. Plates were washed using a BioTek plate washer.

Instrumentation and Data Analysis

The assay signal was measured using a PerkinElmer EnVision® multimode plate reader using default values for standard DELFIA TRF detection. Standard curves for the DELFIA assay were performed in indicated sample diluents using recombinant standards, and the data plotted in GraphPad Prism®. A nonlinear regression using the four-parameter logistic equation (sigmoidal dose-response curve with variable slope) and $1/Y^2$ data weighting was used to analyze the data. The lower detection limit (LDL) of the assay was calculated by multiplying 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 initially determine an appropriate diluent for each sample type. A known amount of standard analyte was spiked into each sample, followed by two-fold dilution increments in the chosen diluents. Dilutions of the standard analyte in each proposed diluent were set up in a separate set of tubes. The optimized DELFIA assay protocol was run using spiked samples and standards in proposed diluent. To assess linearity, the concentrations of each spike and spiked 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 10,000 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. The percent recovery was calculated using the following equation:

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

Results

Linearity and spike-and-recovery experiments were performed to determine the optimal diluent for each sample type and assess overall sample compatibility. Criteria for sample compatibility included a linearity correlation coefficient (R^2) >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 diluent are shown in Table 1. The plasma sample required a four-fold dilution in 100% FBS for an acceptable linearity, indicating that the plasma samples would need to be diluted four-fold in 100% FBS prior to running the assay. All other sample types did not require dilution for linearity (data not shown), hence could be used undiluted. Suitable recoveries were obtained for all samples.

Table 1. Recovery for optimal diluent for each sample type, in DELFIA mouse MMP12 assay. Average recovery from four spiked samples is shown.

Sample Type	% Recovery
Cell Lysate (Diluent: Lysis Buffer)	103
RPMI + 10% FBS (Diluent: RPMI + 10% FBS)	102
Urine (Diluent: DELFIA Assay Buffer)	113
Tissue (Diluent: RIPA Buffer)	113
Serum (Diluent: 100% FBS)	91
Plasma (Diluent: 100% FBS)	90
Blood (Diluent: 100% FBS)	83

From the standard curve for each diluent (Figure 3), the sensitivity, dynamic range, and signal-to-background were calculated. As observed in Table 2, the DELFIA assay maintained excellent sensitivity with broad dynamic range and high signal-to-background ratio for all sample types.

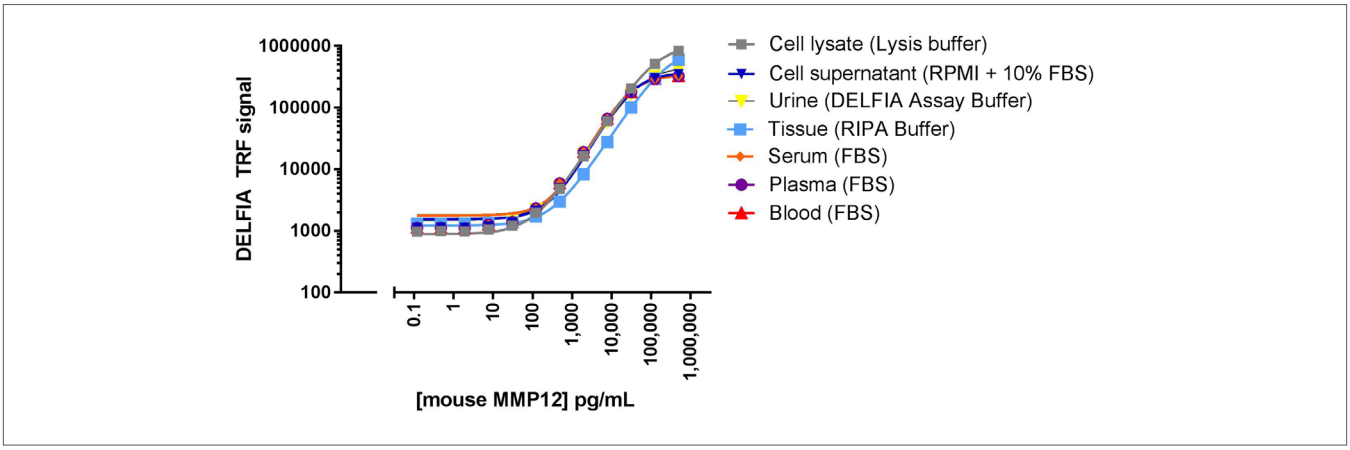

Figure 3. Standard curves for DELFIA mouse MMP12 assay in the final diluent for each sample matrix.

Table 2. Compiled results for DELFIA assay performance for all sample types.

Sample Type	Compatible with DELFIA?	Diluent for Standard Curve	Assay Sensitivity (LDL)	Dynamic Range	Signal-to-Background
Cell Lysate	✓	Lysis Buffer	47 pg/mL	5 log	769
Supernatant (RPMI + 10% FBS)	✓	RPMI + 10% FBS	19 pg/mL	5 log	303
Urine	✓	DELFIA Assay Buffer	18 pg/mL	5 log	395
Tissue	✓	RIPA Buffer	55 pg/mL	5 log	470
Serum	✓	100% FBS	10 pg/mL	5 log	311
Plasma	✓ (4-fold Sample Dilution Was Required for Linearity)	100% FBS	41 pg/mL*	5 log	311
Whole Blood	✓	100% FBS	26 pg/mL	5 log	235

**Sample required dilution (determined by linearity experiments) – value indicates calculated sensitivity in undiluted sample, as corrected by sample dilution factor.*

Summary

A DELFIA time-resolved fluorescence (TRF) assay for detection of mouse MMP12 was developed and used as a model to assess compatibility of DELFIA technology with various biological sample types. Linearity and spike-and-recovery experiments were used to determine appropriate diluents for the standard curve and overall sample compatibility. The DELFIA assay was compatible with complex sample matrices, including cell lysates, cell supernatants, urine, tissue, serum, plasma, and whole blood. Assay performance was also measured by evaluating the sensitivity, dynamic range, and signal-to-background ratio for each sample type. DELFIA proved to be a highly sensitive assay with a wide dynamic range and excellent signal-to-background across various sample types with minimal, if any, sample dilution, and hence can be used as a wash-based assay that offers significant signal stability improvement over traditional colorimetric and chemiluminescent ELISA.

References

1. DELFIA Immunoassay Development Guide, PerkinElmer, 2019.

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