APPLICATION NOTE

A novel DELFIA time-resolved fluorescence biomarker detection assay with superior performance to conventional ELISA.

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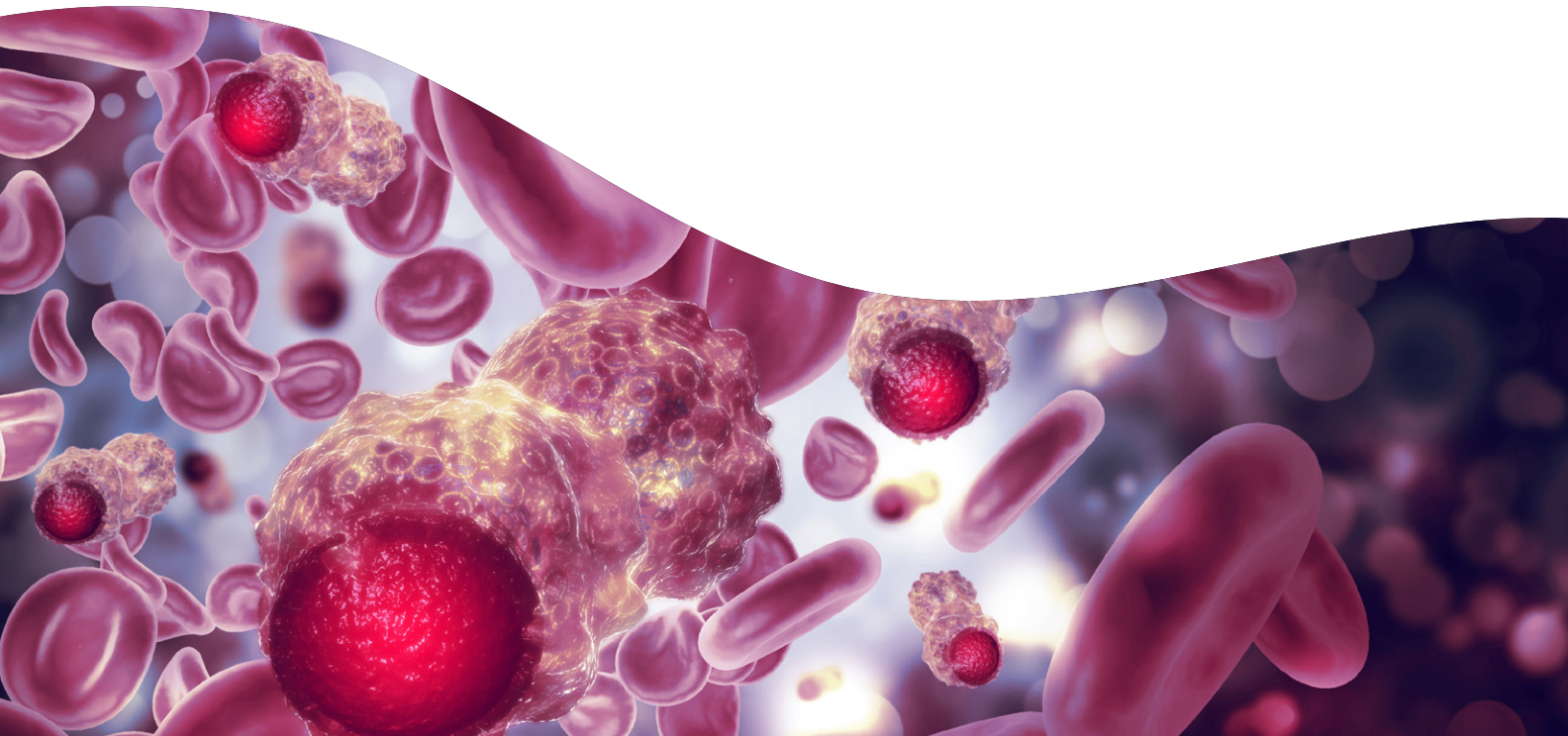
Introduction

ELISAs (enzyme-linked immunosorbent 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. While wash-based ELISAs provide excellent quantitation of the target biomolecule, there are several limitations. Colorimetric-based ELISAs have a narrow 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 over colorimetric-based ELISAs, however, assay signal must be detected rapidly within a narrow window of time.

In contrast, DELFIA® time-resolved fluorescence technology provides a wash-based assay based on a principle and workflow similar to ELISA (see Figure 1 and Table 1), but with a stable signal that can be measured from overnight to days, months or years post-assay with proper storage of plates.



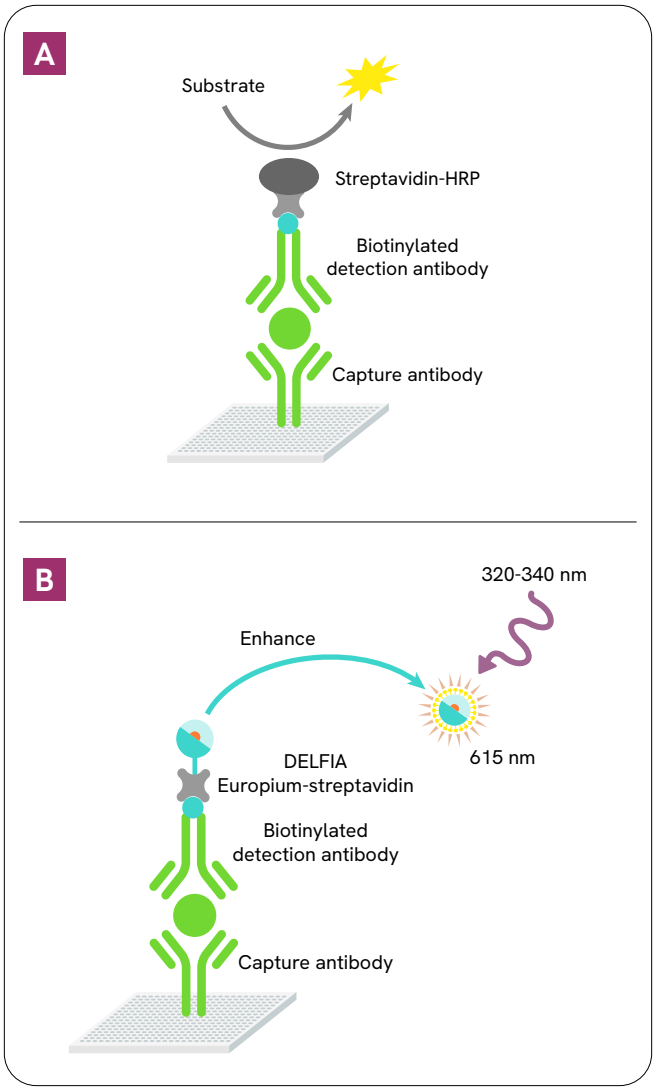


Figure 1: A) Assay principle for an ELISA biomarker detection assay. Capture antibody is directly coated to a microplate. Target analyte is detected using biotinylated antibody in conjunction with streptavidin-horseradish peroxidase (streptavidin-HRP). Upon addition of an appropriate HRP substrate, a chemiluminescent or colorimetric signal is produced. The amount of analyte bound is proportional to the signal. B) Assay principle for DELFIA time-resolved fluorescence (TRF) biomarker detection. In the DELFIA assay, streptavidin-HRP is replaced with a DELFIA Europium-streptavidin tracer. Upon addition of the Enhancement Solution, a highly fluorescent Europium chelate is released into the solution. Excitation at 320 or 340 nm leads to detectable emission at 615 nm. The amount of bound analyte is proportional to the signal emitted which can be quantitated by interpolation from a standard curve.

Table 1: Comparison of workflows for ELISA and DELFIA biomarker detection assays.

ELISA	DELFIA
Coat plate with capture antibody; block and wash	
Add sample	
Incubate; wash	
Add biotinylated antibody	
Incubate; wash	
Add streptavidin-HRP	Add DELFIA Europium-streptavidin
Incubate; wash	
Add substrate; monitor closely	Add Enhancement Solution
Stop reaction	Measure time-resolved fluorescence up to 24 hours post-assay or after plate storage
Read signal promptly	

Here we developed a DELFIA biomarker detection assay for mouse MMP12 as a model for comparison with the performance of the corresponding chemiluminescent and colorimetric ELISAs utilizing the same capture and detection antibodies. DELFIA outperformed as a robust assay with high sensitivity, superior signal stability, wide dynamic range, and compatible with the use of a wide spectrum of complex biological samples as starting material requiring minimal sample dilution.

Materials and methods

Sandwich immunoassays were developed for detection of mouse MMP12 using each assay technology. The same polyclonal anti-mouse MMP12 was used as both capture and detection antibody for each assay. Aliquots from the same vial of anti-mouse MMP12 antibody were used for all assays. High bind clear SpectraPlates™ or white OptiPlates™ (Revvity, Inc., Waltham, MA) were coated with capture antibody using the recommended protocol (Revvity). Biotinylated anti-mouse MMP12 antibody was prepared using the ChromaLink® biotinylation reagent as described in the ChromaLink® manual (TriLink Biotechnologies, San Diego, CA). The same batch of biotinylated antibody was used for all assays. For each assay technology, the concentration of biotinylated antibody, concentration of streptavidin-HRP or DELFIA Europium-streptavidin, order of addition, and incubation times were optimized.

Chemiluminescent ELISA

- Anti-mouse MMP12 antibody, polyclonal (R&D Systems, #AF3467)
- Mouse MMP12 recombinant analyte (R&D Systems, #3467-MPB-020)
- ChromaLink® biotinylation reagent (TriLink Biotechnologies, #B1001-105)
- Zeba desalting columns (ThermoFisher Scientific, #89889)
- Streptavidin-HRP (ThermoFisher Scientific, #21130)
- SuperSignal ELISA Pico Substrate (ThermoFisher Scientific, #37070)
- Blocking Buffer: 50 mM Tris HCl, 140 mM NaCl, 1% BSA
- ELISA Assay Buffer: Blocking Buffer with 0.05% Tween-20
- 96-well white OptiPlate HB (Revvity, #6005500)
- DPBS, no calcium, no magnesium (ThermoFisher Scientific, #14190-114)
- BSA (Revvity, #CR84-100)

All steps were performed at room temperature. Wash steps were performed using a BioTek plate washer (BioTek Instruments, Winooski, VT). The DELFIA PlateShake was used for all incubations except coating and blocking. To coat the plates, 100 µL of 1 µg/mL antibody in 1X PBS pH 7.4 was added to each well and incubated overnight. Buffer was aspirated, and the plates were washed twice with 300 µL of 1X DELFIA wash buffer. Plates were then blocked for one hour with 300 µL of blocking buffer, followed by aspiration. For the chemiluminescent ELISA assay, 75 µL of sample or standard was first added to the wells followed by a 90-minute incubation. Plates were washed 3X with 300 µL of 1X DELFIA wash buffer. Biotinylated antibody (100 µL at 0.1 µg/mL in ELISA Assay Buffer) was then added to each well and incubated for 1.5 hours. After four additional wash steps, 100 µL of streptavidin-HRP (100 µL of a 1:50,000 dilution in ELISA Assay Buffer) was added to each well and incubated 20 minutes. Plates were washed 4X, followed by the addition of 75 µL of a 1:1 mix of ELISA Pico Substrate. Plates were briefly mixed by shaking, followed by immediate measurement of the luminescent signal using the EnVision® multimode plate reader (Revvity).

Colorimetric (traditional) ELISA

- Anti-mouse MMP12 antibody, polyclonal (R&D Systems, #AF3467)
- Mouse MMP12 recombinant analyte (R&D Systems, #3467-MPB-020)
- ChromaLink® biotinylation reagent (TriLink Biotechnologies, #B1001-105)
- Zeba desalting columns (ThermoFisher Scientific, #89889)
- Streptavidin-HRP (ThermoFisher Scientific, #21130)
- TMB substrate (ThermoFisher Scientific, #002023)
- Blocking Buffer: 50 mM Tris HCl, 140 mM NaCl, 1% BSA
- ELISA Assay Buffer: Blocking Buffer with 0.05% Tween-20
- 96-well SpectraPlate HB (Revvity, #6005600)
- DPBS, no calcium, no magnesium (ThermoFisher Scientific, #14190-114)
- BSA (Revvity, #CR84-100)

All steps were performed at room temperature. Wash steps were performed using a BioTek plate washer (BioTek Instruments, Winooski, VT). The DELFIA PlateShake was used for all incubations except coating and blocking. To coat the plates, 100 µL of 1 µg/mL antibody in 1X PBS pH 7.4 was added to each well and incubated overnight. Buffer was aspirated, and the plates were washed twice with 300 µL of 1X DELFIA wash buffer. Plates were then blocked for one hour with 300 µL of blocking buffer, followed by aspiration. For the colorimetric ELISA assay, 75 µL of sample or standard was first added to the wells followed by a 90-minute incubation. Plates were washed 3X with 300 µL of 1X DELFIA wash buffer. Biotinylated antibody (100 µL at 0.25 µg/mL in ELISA Assay Buffer) was then added to each well and incubated for 1.5 hours. After four additional wash steps, 100 µL of streptavidin-HRP (100 µL of a 1:50,000 dilution in ELISA Assay Buffer) was added to each well and incubated 20 minutes. Plates were washed 4X, followed by the addition of 100 µL of TMB substrate. Signal was developed for 15 minutes, followed by the immediate addition of stop solution (100 µL of H_2SO_4). Absorbance at 450 nm was measured using the monochromator on the EnVision multimode plate reader.

DELFIA TRF assay

- Anti-mouse MMP12 antibody, polyclonal (R&D Systems, #AF3467)
- Mouse MMP12 recombinant analyte (R&D Systems, #3467-MPB-020)
- ChromaLink® biotinylation reagent (TriLink Biotechnologies, #B1001-105)
- Zeba desalting columns (ThermoFisher Scientific, #89889)
- DELFIA Eu-N1-streptavidin (Revvity, #1244-360)
- DELFIA Assay Buffer (Revvity, #1244-106)
- DELFIA Wash Concentrate (Revvity, #1244-114)
- DELFIA Enhancement Solution (Revvity, # 1244-105)
- 96-well SpectraPlate HB (Revvity, #6005600)
- DPBS, no calcium, no magnesium (ThermoFisher Scientific, #14190-114)
- BSA (Revvity, #CR84-100)

All steps were performed at room temperature. Wash steps were performed using a BioTek plate washer (BioTek Instruments, Winooski, VT). The DELFIA PlateShake was used on slow speed for all incubations except coating and blocking. To coat the plates, 100 µL of anti-mouse MMP12 antibody (1 µg/mL) in 1X PBS pH 7.4 was added to each well and incubated overnight. Buffer was aspirated, and the plates were washed twice with 300 µL of 1X DELFIA wash buffer. Plates were then blocked for one hour with 300 µL of blocking buffer (50 mM Tris HCl, 140 mM NaCl, 1% BSA), followed by aspiration of the blocking buffer. For the DELFIA assay, 25 µL of sample or standard was first added to the wells followed by a 90-minute incubation. Plates were washed 3X with 300 µL of 1X DELFIA wash buffer. Biotinylated antibody (100 µL at 0.1 µg/mL in DELFIA Assay Buffer) was then added to each well and incubated for 1.5 hours. After four additional wash steps, 100 µL of Europium-streptavidin (100 ng/mL in DELFIA Assay Buffer) was added to each well with a 20-minute incubation. Plates were washed 6X, followed by the addition of 200 µL DELFIA Enhancement Solution. Plates were then shaken at slow speed for 5 minutes. Time-resolved fluorescence signal was measured on the EnVision multimode plate reader.

Samples

All samples were aliquoted and stored for use. Aliquots from the same dispensed lots were used for all technologies.

- Buffer: 1X AlphaLISA Immunoassay Buffer (Revvity, #AL000C)
- Cell Supernatant: DMEM + 10% FBS
- Cell Supernatant: RPMI + 10% FBS
- Cell Lysates: HCT116 cell line, lysed using AlphaLISA ImmunoAssay Buffer
- 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, Novi, MI)
- Serum: Normal Human Pooled Serum, sterile filtered (Sigma, #H4522)
- Plasma: K3EDTA Pooled Human Plasma, no filtration (BioIVT custom, BioIVT, Westbury, NY)

Diluents for standard curves

- AlphaLISA immunoassay buffer (for cell lysis)
- DMEM + 10% FBS
- RPMI + 10% FBS
- 0.5% BSA in PBS
- RIPA buffer (ThermoFisher Scientific, #89900)
- Fetal bovine serum (FBS) (ThermoFisher Scientific, #26140079)
- ELISA assay buffer

Data analysis

Standard curves for all assays were performed in indicated diluents using recombinant mouse MMP12 standard. For the colorimetric ELISA assay, data was plotted in GraphPad Prism® with linear fitting. For the chemiluminescent ELISA and DELFIA assays, 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 each assay was calculated by multiplying three times the standard deviation of the average background values and interpolating concentration from the standard curve. %CV was calculated using 12 data points at a single concentration of analyte in the specified diluent, using the following equation:

$$\%CV = (\text{Standard Deviation} / \text{Mean}) \times 100$$

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 serial dilutions in the chosen diluents. Dilutions of the standard analyte in each proposed diluent were set up in a separate set of tubes. Each optimized 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. Criteria for sample compatibility included a linearity correlation coefficient (R^2) >0.995.

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

Criteria for sample compatibility included spike-and-recovery values within the range of 70-130%.

Results

Sample compatibility

Determining the appropriate diluent that matches the biological sample of interest is critical for accurate detection in any assay. Substances within the sample such as organic compounds, proteins, lipids and salts, as well as sample viscosity and pH, can interfere with the chemistry or binding interactions involved in the assay itself, or 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 quantitation.

Linearity and spike-and-recovery experiments were performed to determine the optimal diluent for each sample type and assess overall sample compatibility. The identified optimal diluents for each sample type are listed in Table 2. Linearity results are summarized in Table 3.

A suitable diluent was identified for each sample type for all technologies. However, for some technologies, certain samples required dilution to meet linearity requirements (R^2 >0.995). For both the chemiluminescent and colorimetric ELISA mouse MMP12 assays, serum and plasma samples required an 8-fold dilution of sample in 100% FBS (or FBS/ELISA Assay Buffer, for the plasma samples) for linearity. For the chemiluminescent ELISA, cell lysate samples required a 4-fold dilution in immunoassay buffer, while whole blood required an 8-fold dilution in 100% FBS diluent. For the colorimetric ELISA, cell lysate samples required a 2-fold dilution in immunoassay buffer, while whole blood samples required a 2-fold dilution in 100% FBS diluent. However, the DELFIA mouse MMP12 assay did not require any dilution for most sample types, except for plasma samples (which required 4-fold sample dilution in 100% FBS for good linearity). Therefore, the DELFIA mouse MMP12 assay required the least amount of sample preparation (pre-dilution), regardless of sample type, which enables significant time savings and ease of use further enhancing productivity.

Table 2: Identified diluents for each sample type, for ELISA and DELFIA mouse MMP12 assays.

Sample type	Chemiluminescent ELISA	Colorimetric ELISA	DELFIA TRF
Buffer	ELISA Assay Buffer	ELISA Assay Buffer	DELFIA Assay Buffer
Cell lysate	Lysis Buffer	Lysis Buffer	Lysis Buffer
Supernatant (DMEM + 10% FBS)	ELISA Assay Buffer	ELISA Assay Buffer	DELFIA Assay Buffer
Supernatant (RPMI + 10% FBS)	RPMI + 10% FBS	RPMI + 10% FBS	RPMI + 10% FBS
Urine	ELISA Assay Buffer	ELISA Assay Buffer	DELFIA Assay Buffer
Tissue	RIPA	RIPA	RIPA
Serum	100% FBS	100% FBS	100% FBS
Plasma	FBS/ELISA Assay Buffer (1:1)	FBS/ELISA Assay Buffer (1:1)	100% FBS
Whole Blood	100% FBS	100% FBS	100% FBS

Table 3: Linearity results for mouse MMP12 assays across various sample types. The checkmarks indicate good linearity was observed (R^2 >0.995) with the diluent indicated in Table 2. If sample dilution was required for good linearity, the dilution factor is indicated in parentheses.

Sample type	Chemiluminescent ELISA	Colorimetric ELISA	DELFIA TRF
Buffer	✓	✓	✓
Cell lysate	✓ (4-Fold Sample Dilution)	✓ (2-Fold Sample Dilution)	✓
Supernatant (DMEM + 10% FBS)	✓	✓	✓
Supernatant (RPMI + 10% FBS)	✓	✓	✓
Urine	✓	✓	✓
Tissue	✓	✓	✓
Serum	✓ (8-Fold Sample Dilution)	✓ (8-Fold Sample Dilution)	✓
Plasma	✓ (8-Fold Sample Dilution)	✓ (8-Fold Sample Dilution)	✓ (4-Fold Sample Dilution)
Whole Blood	✓ (8-Fold Sample Dilution)	✓ (2-Fold Sample Dilution)	✓

Samples were pre-diluted as indicated in linearity results, when applicable. All samples exhibited appreciable recovery (recovery between 70-130%) with the chosen diluents (data not shown).

Sensitivity

Sample matrix effects can sometimes negatively impact the sensitivity of the assay by affecting antibody binding to target analyte. To assess the effect of sample matrix on assay sensitivity, standard curves of recombinant mouse MMP12 were performed in each diluent for each assay technology and the lower limit of detection was calculated as described in Materials and Methods.

Results are shown in Table 4. Because any required sample dilution also impacts the detectable limit in undiluted sample, the sensitivities indicated below are corrected for any sample dilution factor required, as derived from linearity experiments (see Table 3). The chemiluminescent ELISA, colorimetric ELISA, and DELFIA TRF mouse MMP12 assays had approximately the same calculated lower limit of detection for each sample type; however, due to required sample dilutions for serum, plasma, and whole blood, the detectable limit of mouse MMP12 in undiluted sample is poorer for chemiluminescent and colorimetric ELISA, compared to the DELFIA assay.

Table 4: Sensitivity of the mouse MMP12 assay, for each assay technology and sample type. The lower limit of detection is indicated in pg/mL, as calculated for undiluted sample. Samples that required dilution for linearity are shown with LDLs corrected by the required dilution factor.

Sample type	Sensitivity (LDL) (pg/mL)		
	Chemiluminescent ELISA	Colorimetric ELISA	DELFIA TRF
Buffer	26	36	18
Cell lysate	89*	94*	47
Supernatant (DMEM + 10% FBS)	26	36	18
Supernatant (RPMI + 10% FBS)	14	28	19
Urine	26	36	18
Tissue	48	85	55
Serum	280*	276*	10
Plasma	200*	299*	41*
Whole Blood	280*	69*	26

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

Signal-to-background

Sample matrix effects can also decrease overall signal-to-background of the assay by interfering with the detection signal. Each assay technology evaluated here utilized a different detection method: luminescence for chemiluminescent ELISA, absorbance at 450 nm for colorimetric ELISA, and time-resolved fluorescence for the DELFIA TRF assay. In theory, sample matrix effects could affect each assay technology to a different degree. The signal-to-background for each assay and sample type was derived from the standard curve of recombinant mouse MMP12 prepared in the identified diluent (Table 5). The chemiluminescent ELISA exhibited the best signal-to-background across all sample types. The DELFIA TRF assay also showed excellent signal-to-background which was stable overnight. The colorimetric ELISA delivered good signal-to-background across all sample types, though the signal-to-background was significantly lower compared to chemiluminescent ELISA and DELFIA. For the chemiluminescent and colorimetric ELISA, the choice of substrate for the HRP-catalyzed detection step also likely has a strong impact on signal-to-background. Interestingly, the DELFIA TRF assay and colorimetric ELISA assay exhibited stable signal-to-background across all sample types (except for the cell lysate sample using DELFIA technology). This implies that the sample matrix had less of an impact on the overall signal-to-background for these assays. While the chemiluminescent ELISA had superior signal-to-background, the signal window had greater fluctuation depending on sample type. This implies that the sample matrix had a greater impact on the chemiluminescent ELISA assay.

Dynamic range

As shown in earlier experiments, certain sample types (particularly for the chemiluminescent and colorimetric ELISAs) required pre-dilution to achieve good linearity for the mouse MMP12 assay. Sample dilution may also be required to ensure the concentrations of experimental samples fall within the detectable range of the assay. This is referred to as the “dynamic range” of the assay. In general, the wider the dynamic range of the assay, the less likely it is that sample dilution will be required. Because sample dilution requires an extra preparation step, this makes the assay more tedious. The dynamic range of each assay was determined from a standard curve of recombinant mouse MMP12 prepared in the corresponding diluent for each sample type. Results are shown in Table 6. The DELFIA TRF assay showed the widest dynamic range, suggesting that sample pre-dilution would likely not be required. The chemiluminescent ELISA also had an excellent dynamic range. The colorimetric ELISA had poor dynamic range, which suggests that samples would need to be tested undiluted and with dilution to ensure the concentration of mouse MMP12 was within the dynamic range of the assay, and not past the upper detection limit which leads to inaccurate interpolations of unknown concentrations.

Table 5: Signal window for the mouse MMP12 assay, for each assay technology and sample type.

Sample type	Signal-to-background		
	Chemiluminescent ELISA	Colorimetric ELISA	DELFIA TRF
Buffer	1061	45	395
Cell lysate	931	50	769
Supernatant (DMEM + 10% FBS)	1061	45	395
Supernatant (RPMI + 10% FBS)	455	44	303
Urine	1061	45	395
Tissue	763	34	470
Serum	449	40	311
Plasma	745	40	311
Whole Blood	449	40	235

Table 6: Dynamic range for the mouse MMP12 assay, for each assay technology and sample type.

Sample type	Dynamic range		
	Chemiluminescent ELISA	Colorimetric ELISA	DELFIA TRF
Buffer	4 log	1.5 log	5 log
Cell lysate	4 log	1.5 log	5 log
Supernatant (DMEM + 10% FBS)	4 log	1.5 log	5 log
Supernatant (RPMI + 10% FBS)	4 log	1.5 log	5 log
Urine	4 log	1.5 log	5 log
Tissue	4 log	1.5 log	5 log
Serum	4 log	1.5 log	5 log
Plasma	4 log	1.5 log	5 log
Whole Blood	4 log	1.5 log	5 log

Reproducibility

Lastly, the reproducibility of each assay was evaluated by calculating the coefficient of variation, expressed as %CV. Because all tested assay technologies were wash-based assays, there was concern that the reproducibility of the assays would be impacted by repeated washes. All wash steps were performed using a plate washer to make washing steps consistent across each technology. Reproducibility for each assay and sample type is indicated in Table 7. All assays exhibited similar, excellent reproducibility.

Table 7: Reproducibility for the mouse MMP12 assay, for each assay technology and sample type.

Sample type	Reproducibility (%CV)		
	Chemiluminescent ELISA	Colorimetric ELISA	DELFIA TRF
Buffer	5%	3%	4.5%
Cell lysate	3%	5%	3%
Supernatant (DMEM + 10% FBS)	5%	3%	4.5%
Supernatant (RPMI + 10% FBS)	2.5%	3%	5.5%
Urine	5%	3%	4.5%
Tissue	2.5%	3.5%	3%
Serum	3%	3%	4%
Plasma	2.5%	3%	4%
Whole Blood	3%	3%	4%

Conclusions

Three different assay technologies (chemiluminescent ELISA, colorimetric ELISA, and DELFIA TRF) were evaluated in a biomarker detection assay for mouse MMP12 that utilized the same capture and detection antibodies. Sample compatibility, sensitivity, signal-to-background, dynamic range, and assay reproducibility were assessed. Each assay technology was compatible with each sample type tested, including serum, plasma, and blood. The DELFIA TRF mouse MMP12 assay required no sample dilution for linearity, except for plasma, which required a 4-fold sample dilution. The chemiluminescent and colorimetric mouse MMP12 ELISAs required significant dilution of cell lysate, serum, plasma, and blood samples. While calculated lower limits of detection (LDL) were similar across all technologies evaluated per given sample type, the DELFIA TRF assay exhibited the best sensitivity in undiluted samples since all but one sample type could be used without sample pre-dilution. The DELFIA TRF mouse MMP12 assay also showed best dynamic range, suggesting that pre-dilution of samples would also not be required for unknown sample concentrations to fall within the detectable range of the assay. The colorimetric ELISA showed the narrowest dynamic range, suggesting that samples would need to be tested by dilution to ensure biomarker concentrations fall within the detectable range of the assay. Though all assay technologies tested were wash-based assays, each assay exhibited excellent reproducibility as indicated by %CV.

In addition, the DELFIA TRF assay provided superior signal stability, allowing the assay plate to be measured after overnight incubation, as well as enabling storage of the assay plates for future measurements. In contrast, the chemiluminescent ELISA had to be measured within 10 minutes of substrate addition, while the colorimetric ELISA required addition of stop solution within 30 minutes of substrate addition. We therefore conclude that DELFIA TRF assays provide an excellent alternative to ELISA for detection of biomarkers in complex matrices and deliver superior or similar assay performance.

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