

Enhanced Sensitivity Detection of Low Concentration Analytes Using Enzymatic Amplification



Technical Note

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Octet®, Biolayer Interferometry (BLI), cytokine detection, sensitivity, enzymatic amplification, horseradish peroxidase (HRP)

Abstract

This document presents the development and optimization of a multi-step Octet® Biolayer Interferometry (BLI) assay for the detection of low abundant molecules, using the cytokine TNF α as the primary example. The study introduces a combined-step workflow involving HRP-conjugated antibodies and AEC amplification, which significantly enhances detection sensitivity and signal magnitude compared to traditional single-step assays. Key optimization strategies, including adjustments in antibody concentration, choice of enzymatic reagent and shake speeds, were employed to achieve reliable quantitation of low cytokine concentrations. The research demonstrates the robustness of the assay setup, while also highlighting the necessity for parameter fine-tuning to accommodate different molecular targets. Promising results from IL-6 detection experiments suggest the assay's applicability to a wider range of molecules. This methodology establishes a foundation for improving detection speed and sensitivity in the quantification of low abundant molecules using BLI.

Introduction

Octet® Biolayer Interferometry (BLI) allows direct detection of specific proteins or drug molecules - even in complex mixtures and unpurified samples, such as cell culture supernatants and lysates. When quantifying high concentrations of molecules or large molecules, such as antibodies, a simple 1-step reaction between ligand and analyte is often sufficient for high magnitude detection of the analyte of interest. However, when the analyte concentration in the sample is low and/or the analyte is small, the sensitivity of the BLI instrument can limit the ability to detect the analyte of interest. The Octet® R8e System has major improvements in sensitivity compared to traditional BLI instruments, with greatly enhanced detection of small molecules (see **application note 'How Low Can You Go? A New Era of Sensitivity in Octet® BLI'** for more information).¹ However, even with this increased sensitivity, a sufficient lower limit of detection may not be achieved for some analytes in a 1-step assay.

To improve detection of low concentration analytes, the observed binding signal can be increased by introducing a multi-step assay workflow and enzymatic amplification, for example using antibodies conjugated to horseradish peroxidase (HRP). The analyte bound HRP conjugate reacts with an HRP substrate resulting in the formation of a precipitate, in a quantity directly related to the amount of analyte present on the biosensor. Currently, this is utilized in the Octet® Anti-CHO Host Cell Protein (HCP) kit for detection of low concentrations of HCP contaminants in products from CHO cell culture (see **technical note 'CHO Host Cell Protein Detection' or more information**).²

This technical note walks through the development of a multi-step Octet® BLI assay that deploys an enzyme reaction step for signal amplification for the detection of low abundant analytes, using the cytokines TNFα and IL-6 as examples. Detection of cytokines is critical in *in vitro* research for characterization of immune cell function, therapeutic evaluation and predicting inflammatory responses.^{3,4} The described multi-step assay increases detection sensitivity and the magnitude of binding signals compared to a single-step assay. This workflow has the potential to speed up the detection of low abundant cytokines compared to traditional ELISA methods.

Amplification Assay Format

The multi-step sandwich format of the assay (Figure 1A) involves the capture of a biotinylated monoclonal antibody, specific to the analyte of interest, onto streptavidin-coated biosensors. This is followed by a dip of the biosensors into the sample of interest, in this case TNFα. A matched pair monoclonal HRP-conjugated detection antibody (i.e. binding to a different epitope on the analyte than the capture antibody) is then bound. Next, biosensors are dipped into acetate buffer, as provided with the AEC staining kit, followed by AEC (3-amino-9-ethylcarbazole, Sigma #AEC101), both of which are used at the concentrations recommended in the product information sheet. AEC is then oxidized in the presence of HRP to produce an insoluble precipitate. This results in amplification of the signal in proportion to the level of HRP, and therefore the concentration of TNFα, that is present.

The possibility of including an additional, hapten-conjugated antibody step, prior to the addition of HRP (as in the **technical note 'CHO Host Cell Protein Detection'**) was evaluated, but this was found to provide little to-no improvement in sensitivity or signal window compared to addition of the HRP antibody in isolation, and so was not explored further (data not shown). This difference could be due to the use of monoclonal rather than polyclonal antibodies or the type of molecule being detected.

Previous work showed that combining the sample and HRP-conjugated antibody steps in the sample plate prior to assessment on the Octet® R8e can lead to enhanced detection sensitivity compared to running the two steps sequentially on the instrument. Plate maps in Figures 1B and 1C demonstrate these two assay configurations.

Figures 1D and 1E show the detection of a range of TNFα concentrations (375 to 1,500 pg/mL) using the fully separated assay and the combined sample-HRP antibody assay, respectively. There was both a higher maximal signal and window between the top concentration of TNFα tested and the 0 pg/mL reference using the combined-step workflow. There was also greater separation of the lower TNFα concentrations, indicating increased detection sensitivity. This suggests that the anti-TNFα-HRP antibody binds more readily to TNFα in solution, compared to when it is bound to the capture antibody immobilized on the sensor. Moreover, the reduced number of steps decreased the time taken to acquire data on the instrument. For these reasons the combined workflow was taken forward for further optimization. In both the separated and combined-step workflows, there was minimal separation between the traces for each concentration of TNFα prior to the final AEC step, confirming the need for enzymatic amplification for detection of these low cytokine concentrations.

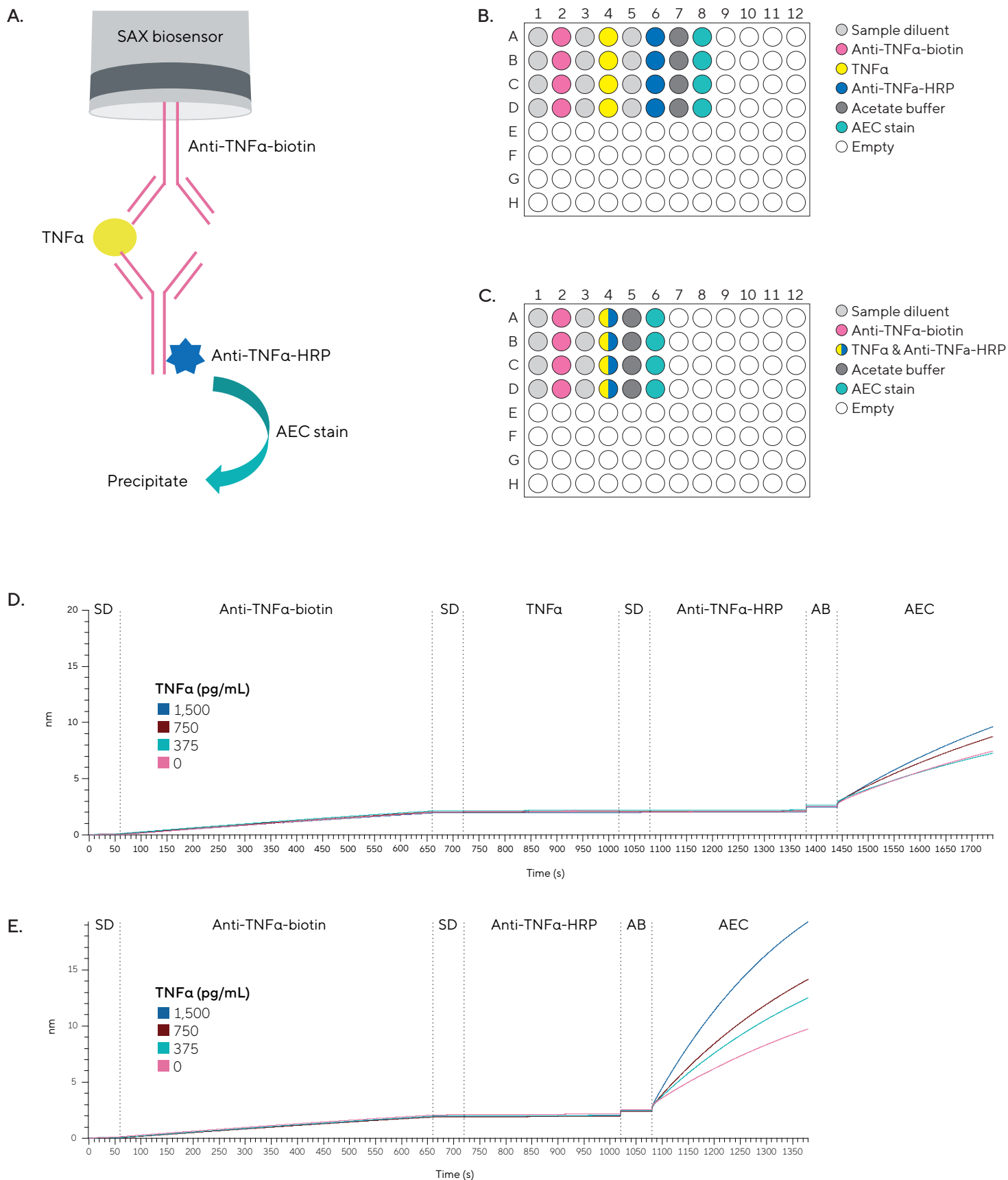


Figure 1. Combining sample and HRP conjugated antibody steps increases signal window and improves assay sensitivity

(A) Schematic of multi-step method used for amplification of signals for detection of low concentrations of TNFα using BLI. (B) Plate map for multi-step detection of TNFα (fully separated workflow). (C) Plate map for multi-step TNFα detection with sample (i.e. TNFα) and anti-TNFα-HRP steps pre-combined in plate before running on instrument. Traces show detection of a range of concentrations of TNFα (375 to 1,500 pg/mL) using (D) fully separated and (E) combined sample and HRP antibody steps. SD = Octet® Sample Diluent, AB = Acetate Buffer.

Antibody Selection

As mentioned in the previous section, this assay used matched antibody pairs known to bind distinct epitopes on the molecule of interest. This ensures that sequential binding required for the sandwich format can be achieved. Matched pairs can be purchased from vendors, or the BLI platform can be used to evaluate epitope complementarity of non matched pairs.⁵ Assessment of multiple antibody pairs, for example targeting different clones or with different isotypes, can show differences in terms of biosensor loading and subsequent analyte detection. For the TNF α experiments, matched ELISA antibody pairs from SinoBiological yielded optimal results and are used in the experiments shown (capture antibody #10602-MM01 and detection antibody #10602-MM08).

Antibody Conjugation

The unconjugated capture antibody required biotinylation to facilitate loading on to streptavidin-based biosensors. The optimal molar coupling ratio (MCR) for biotinylation for kinetics applications on BLI is considered to be a 1:1 biotin-to-ligand ratio. However, recent work (technical note: 'Optimizing Protein-Streptavidin Interactions: Effects of Biotinylation Ratios Across Biosensor Types') revealed that for quantitation applications, increasing the MCR can lead to increased loading of capture ligand and increased subsequent binding to the analyte.⁶ The risk of cross-linking molecules on the sensor surface with a higher MCR needs to be avoided for kinetics as it can lead to inaccurate data, however this is not a concern for quantitation and, instead, particularly when detecting low abundance analytes, the aim is to boost the sensitivity and signal window as much as possible. For this reason, a biotinylation MCR of 5:1 was used throughout these experiments.

Biosensor Selection

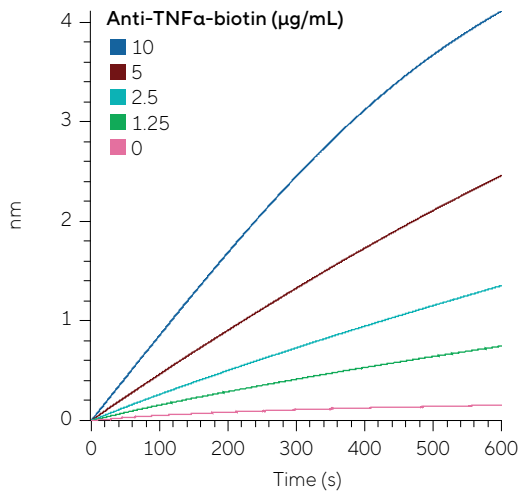
A variety of streptavidin biosensors have been developed at Sartorius for the capture of biotinylated ligands. While these biosensors (Octet® SA, SAX, SAX2 and SSA) are all coated with streptavidin molecules and intended for use with biotinylated ligands, they differ in the intended applications. Comparison between streptavidin-based biosensors for antibody-based quantitation applications was investigated in the technical note 'Optimizing Protein-Streptavidin Interactions: Effects of Biotinylation Ratios Across Biosensor Types'. These experiments found SAX biosensors to be optimal, due to highest dynamic range and signal magnitude, and, consequently, these were used for cytokine quantitation experiments.⁶

Capture and Detection Antibody Concentration Determination

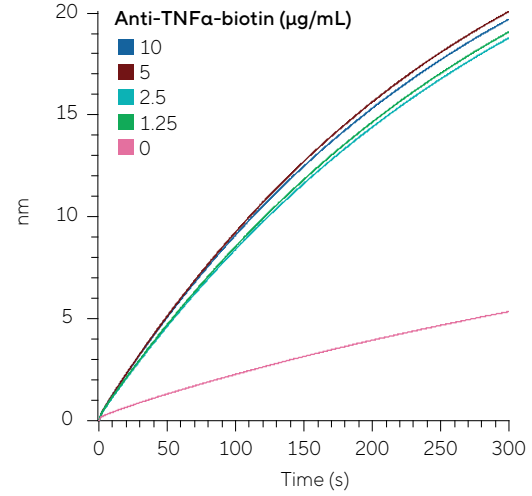
Binding of biotinylated capture antibody to streptavidin biosensors can vary depending on factors such as the antibody isotype or MCR. Therefore, it can be useful to optimize antibody loading concentration for each new capture antibody. Figure 2A shows the response for a range of concentrations of biotinylated anti-TNF α (1.25 to 10 μ g/mL, 5:1 MCR) binding to SAX biosensors. There is a clear increase in loading with increasing antibody concentration. Loaded sensors were used in the multi-step assay for TNF α detection, with the final AEC step shown in Figure 2B. All four loaded biosensors showed comparable responses for detection of 10,000 pg/mL TNF α , approximately 4 times larger than the signal for the unloaded reference sensor. The 5 μ g/mL load concentration was used due to the marginally higher binding response to TNF α .

The effect of titrating the HRP-conjugated detection antibody was also tested by comparing the signal window in the presence and absence of TNF α . Figure 2C shows that at a high detection antibody concentration of 10 μ g/mL, the signal for the biosensor in the presence of 1,500 pg/mL TNF is high, with a binding rate of 0.088, however the background signal for the 0 pg/mL reference biosensor is also very high, with a binding rate of 0.083, resulting in a very small signal window. With 5 μ g/mL detection antibody (Figure 2D), there is a much larger signal window between the 1,500 pg/mL and the reference sensor, with binding rates of 0.086 and 0.032, respectively. Figure 2E displays a low signal magnitude and signal window when 2.5 μ g/mL detection antibody is used, with a binding rate of 0.030 for the 1,500 pg/mL TNF α biosensor and 0.018 for the reference. The graph in Figure 2F shows an overlay of the reference subtracted signals at each detection antibody concentration, confirming that 5 μ g/mL is optimal.

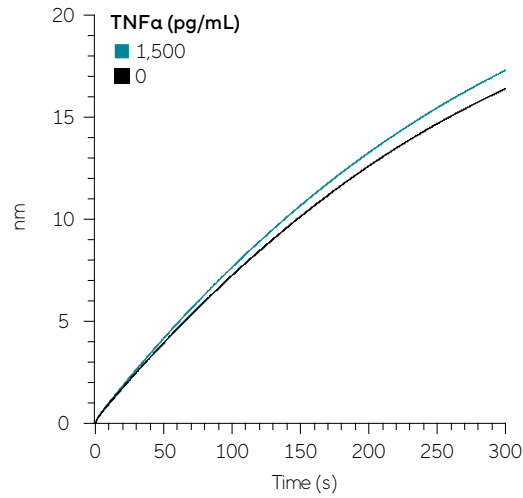
A. Anti-TNF α -biotin



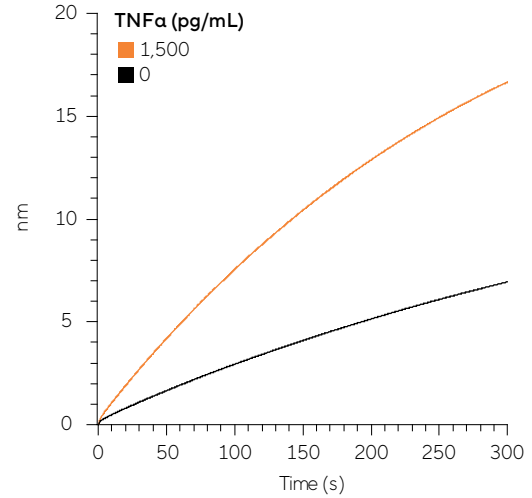
B. AEC step: Capture antibody titration



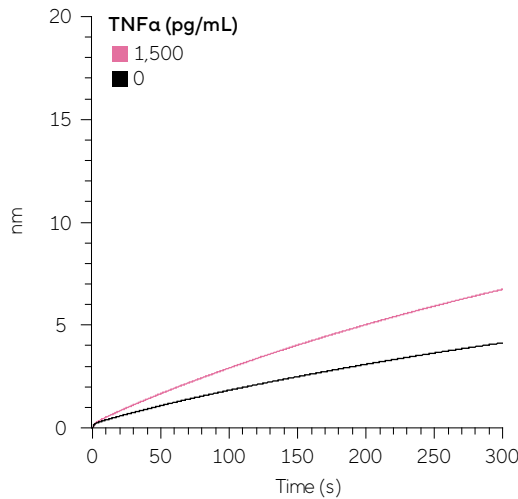
C. AEC step: 10 μ g/mL detection antibody



D. AEC step: 5 μ g/mL detection antibody



E. AEC step: 2.5 μ g/mL detection antibody



F. AEC step: Detection antibody titration overlay

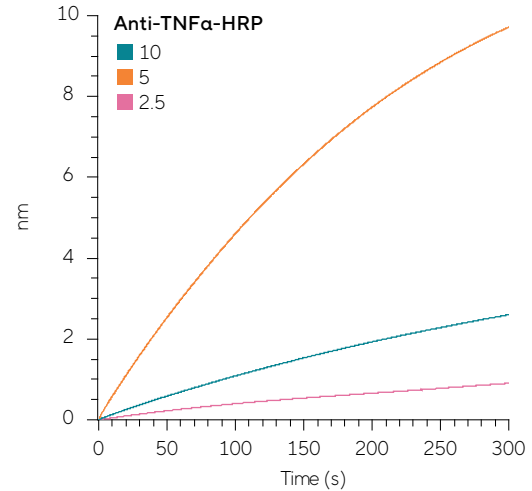


Figure 2. Titrations to determine optimal concentrations of load and capture antibodies

(A) Loading of a range of concentrations of capture antibody (anti-TNF α -biotin, 1.25 to 10 μ g/mL) to SAX biosensors. (B) Signal for AEC amplification step following detection of a single, high concentration of TNF α (10,000 pg/mL) and subsequent amplification with biosensors loaded in panel A. To assess the optimal concentration of detection antibody (anti-TNF α -HRP (μ g/mL)), the signal window in the final AEC step between detection of 1,500 pg/mL TNF α and a 0 pg/mL reference sensor was compared with (C) 10 μ g/mL, (D) 5 μ g/mL and (E) 2.5 μ g/mL of anti-TNF α -HRP (μ g/mL). (F) Overlay of reference subtracted signals for detection of 1,500 pg/mL TNF α with each anti-TNF α -HRP (μ g/mL) concentration.

Shake Speed Evaluation

The speed at which the plate is shaken whilst data is being acquired on the Octet® BLI System can have a large effect on molecular interactions, impacting the dynamic range, sensitivity and reproducibility of the assay. For kinetic assays 1,000 RPM is standard, whilst 400 RPM is the default for quantitation assays. However, optimal shake speeds can vary between assays and can be a critical parameter to optimize. For these assays, a shake speed of 1,000 RPM for the loading step maximized the amount of capture antibody leading to increased signal magnitude. For the analyte association and AEC steps, reducing the shake speed to 400 RPM led to more reproducible signals and improved detection of the lowest concentrations of analyte.

Assay Reproducibility

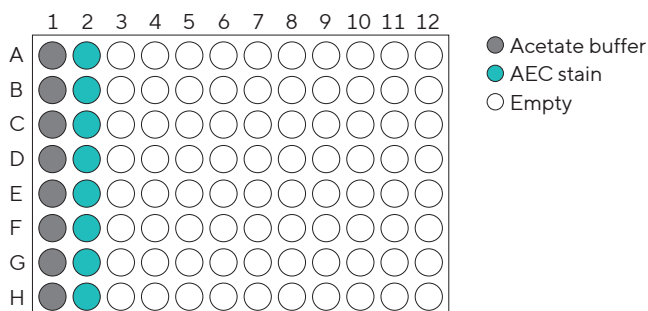
When developing an assay for analyte quantitation, it is important to ensure the reproducibility of the measurements taken. To explore this, three replicate columns of biosensors were loaded with anti-TNFα-biotin and incubated with a range of concentrations of analyte (TNFα, 69 to 50,000 pg/mL) pre-mixed with HRP-conjugated antibody (combined-step assay format). A 0 pg/mL reference sensor was included in each column. These incubation steps were carried out offline using the Octet® AS Offline Biosensor Immobilization Station, which can shake and heat the plate (30 °C used throughout) to support offline ligand immobilization and analyte binding. This means assays are not limited to only a single plate in terms of the number of wells used for samples, reagents and buffers. This allows for many more samples to be tested in a single assay. The final enzyme amplification step is carried out online, as shown in the plate maps in Figures 3A and 3B. This greatly speeds up data acquisition and improves reproducibility compared to sequential running of multiple columns of sensors, for example across multiple plates. The use of 384-well plates, for example with the Octet® R8e, or, additionally with higher-throughput instrumentation, with the Octet® RH16 or RH96 systems can further increase the number of samples run simultaneously or on a single plate.

Two different HRP substrates, AEC and metal enhanced DAB, were evaluated for amplification of the signals for TNFα detection. The binding rate equation which resulted in an optimal fit was selected for each reagent, with initial slope used for AEC and R equilibrium used for metal enhanced DAB. Binding responses shown in Figures 3C and 3D show three replicates at each concentration of TNFα with the 0 pg/mL reference subtracted. These data show that the maximum signal for the top concentrations of TNFα is higher with metal enhanced DAB than with AEC.

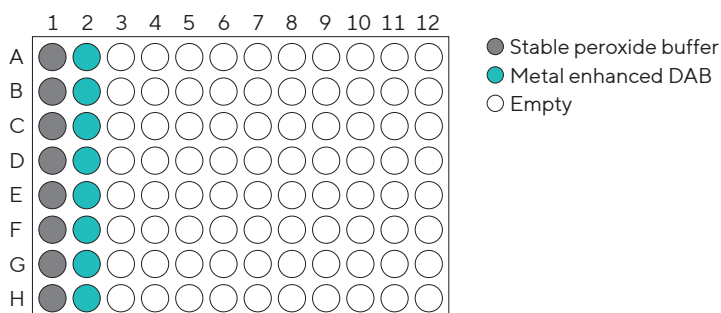
However, further inspection of the curve fits in the concentration-response curves in Figures 3E and 3F shows a better-quality fit, particularly for the lower concentrations of TNFα (69 to 1,852 pg/mL), with the AEC reagent.

Improved sensitivity detection of low TNFα concentrations was reinforced with the metrics in the tables in Figures 3G and 3H. With AEC, CVs at each concentration were <5% showing highly reproducible data across the concentration range. At lower concentrations of DAB, the CV was higher, particularly at the 69 pg/mL TNFα concentration, which gave a CV of 49% (<10% is a good threshold for reproducible quantitation in BLI). The percentage recovery metric showed the calculated concentration as a percentage of the known concentration, to give a measure of how predictive the standard curve is, with a value between 90% and 110% generally considered acceptable for BLI quantitation assays. Most values for AEC were within this range with only the 206 and 617 pg/mL values reaching slightly higher at 111% and 112%, respectively. In contrast, for DAB, at the same concentrations, the percentage recoveries were much higher at 147% and 143%. Both the residuals and R2 values at low concentrations showed better fits for the AEC amplified data and provided further evidence for improved sensitivity detection of TNFα using this reagent. The AEC also has an added benefit of reduced toxicity compared to the highly toxic metal enhanced DAB which can improve safety and ease of use.

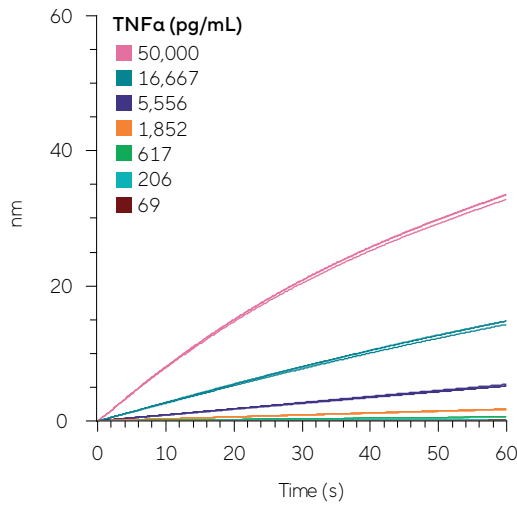
A. Online steps (AEC)



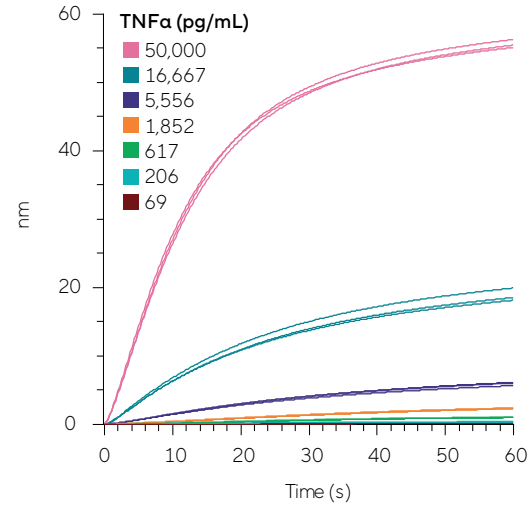
B. Online steps (metal enhanced DAB)



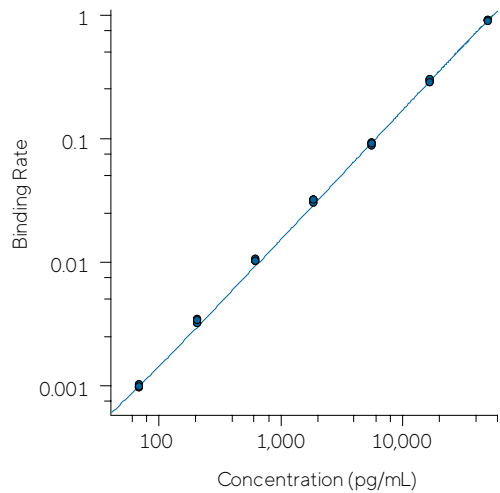
C. AEC



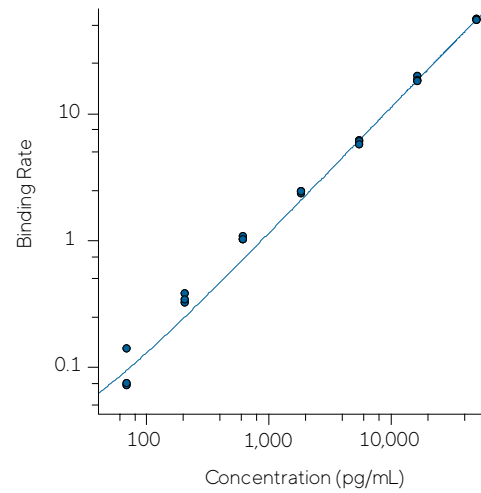
D. Metal enhanced DAB



E. Dose Response - 5PL (Unweighted) (AEC)



F. Dose Response - 5PL (Unweighted) (metal enhanced DAB)



G. AEC reproducibility and fit parameters

Known Concentration (pg/mL)	Calculated Concentration CV (%)	Recovery (%)	Residuals	R ²
50,000	1.4	100.0	8.55	1.000
16,667	2.6	100.1	11.37	1.000
5,556	2.7	98.4	7.39	0.998
1,852	3	106.6	2.30	0.992
617	2.4	110.7	1.80	1.000
206	4.2	111.6	9.67	1.000
69	3.5	99.4	2.53	0.994

H. Metal enhanced DAB reproducibility and fit parameters

Known Concentration (pg/mL)	Calculated Concentration CV (%)	Recovery (%)	Residuals	R ²
50,000	1.3	100.0	0.94	0.997
16,667	5.1	100.3	3.76	0.999
5,556	4.4	95.4	4.63	0.998
1,852	1.9	113.9	13.93	0.996
617	2.8	146.7	46.65	0.988
206	9.2	142.9	43.10	0.968
69	49	99.4	43.45	0.921

Figure 3. Sensitivity of TNFα detection was higher using AEC compared to metal enhanced DAB for enzymatic amplification

Initial loading and combined sample and HRP antibody steps were carried out offline using the Octet® AS Offline Biosensor Immobilization Station. Three replicate columns of biosensors were incubated with a range of concentrations of TNFα (69 to 50,000 pg/mL). A 0 pg/mL reference sensor was included in each column. Plate maps show online amplification steps for (A) AEC stain and (B) metal enhanced DAB (buffer and reagent wells were re-used for each column of sensors). Overlay of reference subtracted signals for (C) AEC and (D) DAB (n=3). Concentration-response curves with a 5PL unweighted fit for (E) AEC (generated using initial slope binding rate equation) and (F) metal enhanced DAB (generated using R equilibrium binding rate equation). Tables show CVs, % recovery (calculated concentration as a percentage of known concentration), residuals and R² values for (G) AEC and (H) DAB.

Applicability for Other Molecules

To determine the applicability of this assay set-up for detection of other cytokines, experiments were conducted using an anti-IL6 matched antibody pair from SinoBiological (capture antibody #10602-MM01 and detection antibody #10602-MM08). Most steps were transferred directly from the TNF α assay, however, the antibody titration experiments were repeated to ensure optimal concentrations were used for this pair. In this instance, much lower concentrations of 0.625 $\mu\text{g/mL}$ and 2.5 $\mu\text{g/mL}$ (compared to 5 $\mu\text{g/mL}$ for the TNF α assay) were found to be optimal for the biotinylated antibody and HRP-conjugated antibody, respectively. The time for the AEC step was also extended to 300 seconds for the IL-6 assay, as the traces still increased linearly by the end of the 60 second timeframe used in Figure 3 for TNF α experiments.

Figure 4A shows the AEC amplification step for detection of a range of concentrations of IL-6 (156 to 10,000 pg/mL) using optimized conditions. An initial slope binding rate equation (Figure 4B) was applied and used to generate the concentration response-curve in Figure 4C (5PL unweighted). R2 values for this curve were all > 0.999.

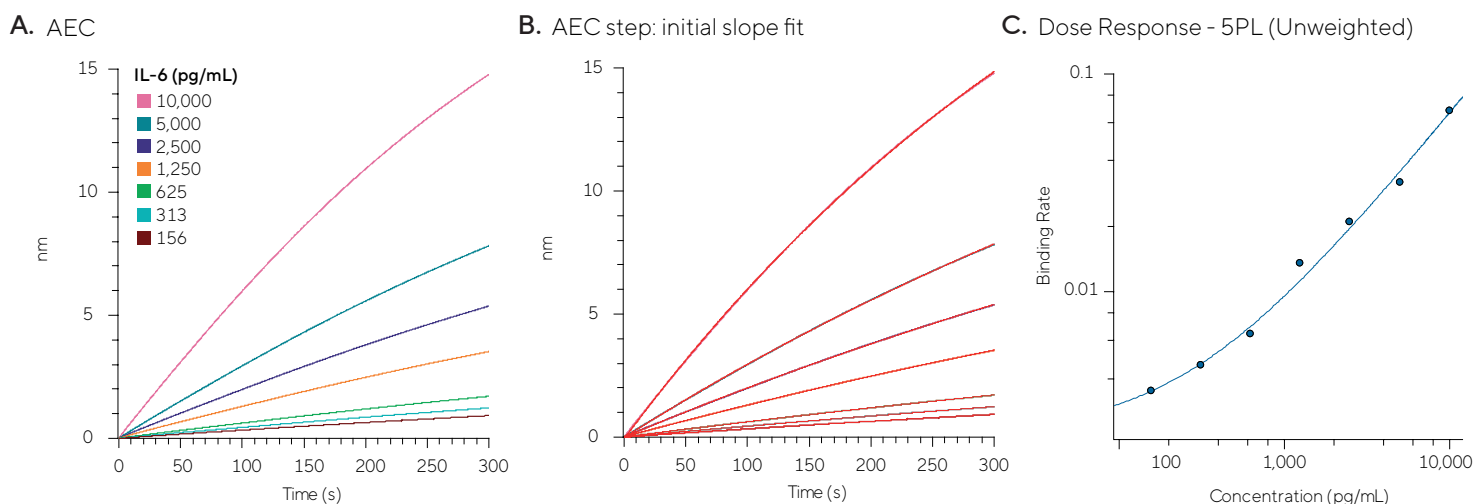


Figure 4. Amplification workflow can be applied to other molecules, such as IL-6

(A) Reference subtracted signals for AEC amplification step showing detection of a range of concentrations of IL-6 (156 to 10,000 pg/mL). (B) Initial slope binding rate equation applied to the AEC step. (C) Concentration response-curve calculated by applying initial slope binding rate equation to A and fitting with 5PL unweighted curve.

Conclusion

This document outlines the development and optimization of a multi-step Octet® Biolayer Interferometry (BLI) assay with enzyme amplification for the detection of cytokines, using TNF α as the primary example. The introduction of a combined sample and HRP-conjugated antibody step alongside AEC amplification, resulted in enhanced sensitivity and signal magnitude compared to traditional single-step assays. The optimization of various parameters, including antibody concentration, conjugation, biosensor selection, and shake speeds, contributed to reproducible quantitation of low cytokine concentrations.

Furthermore, the applicability of this assay setup for detecting other molecules was explored, showing promising results for IL-6 detection when translated directly from the TNF α assay. Adjustments in antibody concentrations and assay timings were necessary to achieve optimal results, suggesting that while the core workflow is robust, individual assay parameters may require fine-tuning for different targets.

Overall, the enhanced sensitivity and reproducibility of this multi-step assay workflow offer a valuable tool for *in vitro* research, therapeutic evaluation, and predicting inflammatory responses. The methodology described in this document provides a foundation for further exploration and application to a broader range of molecules beyond cytokines, potentially accelerating advancements in biomedical research and diagnostics.

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