Development of an Ultra-Sensitive Anti-TNF-α ELISA Using BOLD Technology

Johanna Hedin, Jessica Pettersson, Veronika Nordal, Maryam Mirzaei, and Peter Stenlund
Cavidi AB, Virdings Allé 2 SE-754 50 Uppsala Sweden

EXAZYM
The BOLD Immunoassay Signal Amplifier

Poster

OBJECTIVE

Sensitive immunoassays are crucial for early disease detection, identifying low-abundance biomarkers, and improving diagnostic accuracy. This poster demonstrates the integration of Cavidi's BOLD technology into an anti-TNF-α sandwich ELISA, enhancing sensitivity and precision, particularly at low analyte levels. The focus will be on developing an ultra-sensitive anti-TNF-α ELISA using BOLD, detailing the setup, application, and gathering of performance data.

INTRODUCTION

Sensitive immunoassays are crucial in biomedical research, clinical diagnostics, and therapeutic monitoring for early disease detection, identifying low-abundance biomarkers, reducing sample volume, and enhancing diagnostic accuracy.

Tumour necrosis factor alpha (TNF-α), a cytokine involved in inflammation and immune regulation, is found in both transmembrane and soluble forms. It triggers pathways that can lead to cell death or survival (1). TNF-α levels are usually 0-2 pg/mL in plasma (2, 3) and 20-50 pg/mL in serum (4) but can increase in various diseases such as autoimmune disorders, severe infections, chronic inflammation and cancer. Conventional immunoassays typically have a sensitivity of 1-15 pg/mL, and enhancing this sensitivity could improve the detection of small changes and enable testing with limited sample volumes. This could easily be achieved by the integration of BOLD technology to the immunoassay.

BOLD TECHNOLOGY OVERVIEW

Cavidi has developed a new technology, BOLD, which is based on a molecular biology approach to bring ultra-sensitive detection to virtually any immunodiagnostic assay. BOLD stands for Binding Oligo Ladder Detection and is a process that can be added to any immunoassay. Integration of BOLD into an immunoassay will improve the sensitivity and the limit of detection by up to 50x.

To integrate BOLD into the immunoassay, an oligo-dT Primer is conjugated, using click chemistry, to an unmodified detector antibody of the standard immunoassay. The conjugation is performed with the **Exazym® ClickChem Conjugation Kit**. This oligo-dT Primer detector antibody conjugate serves as a crucial bridge for the addition of the BOLD amplification steps to the standard immunoassay. The **Exazym® Polymerase Reaction Kit** includes the polymerase and essential building blocks for DNA/RNA hybrid strand synthesis. This facilitates enhanced detection through the binding of multiple biotinylated anti-DNA/RNA tertiary antibodies from the **Exazym® Biotin Detection Kit** to each analyte. Subsequently, a streptavidin-conjugate (or other detection label) is used for final detection. Figure 1 illustrates all the steps of an amplified sandwich ELISA.

Illustration of BOLD Implementation in a Sandwich Immunoassay

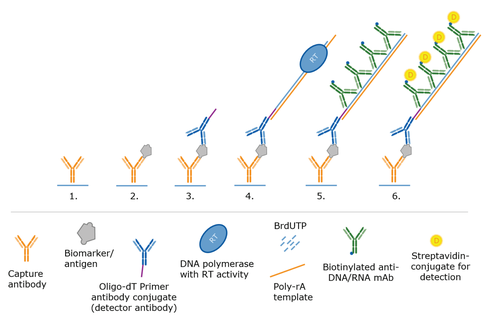


Figure 1. *Schematic drawing of the steps in an amplified sandwich immunoassay.*

METHODS AND RESULTS

1. Development of the Anti-TNF-α ELISA

The ELISA was set up in a 96-well microtiter plate format with the antigen, a matching antibody pair with a biotinylated detector, streptavidin-HRP and colorimetric detection. Initially, the capture and the biotinylated detector antibody was cross-titrated and assessed with regards to the signal-to-noise ratio at 500 pg/mL (Figure 1A) and 50 pg/mL (not shown) TNF-α. The assay development process further continued with the optimization of various parameters, including further titration of the capture antibody, evaluation of different coating buffers, adjustment of the standard curve range, and fine-tuning of the streptavidin-HRP concentration. These refinements led to an assay sensitivity of approximately 1 pg/mL (Figure 1B), placing it within the sensitivity range of commercially available ELISA kits. All steps of the ELISA were performed at room temperature (~22°C) with colorimetric detection.

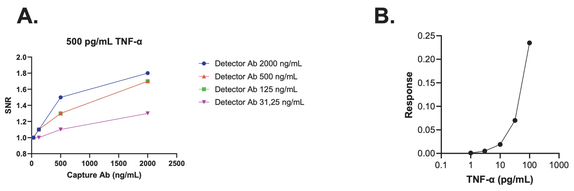


Figure 2. Optimization and performance of an anti-TNF-α ELISA.
(A) *Signal-to-noise ratio (SNR) at 500 pg/mL TNF-α capture antibody and biotin-labeled detector antibody. The SNR was evaluated at four different concentrations of the detector antibody: 2000 ng/mL, 500 ng/mL, 125 ng/mL, and 31.25 ng/mL.*
(B) *ELISA standard curve demonstrating the response of the assay to varying concentrations of TNF-α after optimization of assay parameters.*

2. BOLD Application to Standard ELISA

The oligo-dT primer was conjugated to the unmodified anti-TNF-α detector antibody using click chemistry, testing various molar ratios of ClickChem label to antibody (5x-20x) and oligo-dT primer to ClickChem-labeled antibody (2x-20x). To optimize the BOLD amplified ELISA, different concentrations were tested for the best signal-to-noise ratio. Free unconjugated primer was removed using an Amicon filter (100 kDa cut-off) to minimize non-specific binding.

All BOLD incubation steps were performed according Exazym® kit instructions. The concentration of the primer-modified detector significantly impacted assay performance (Figure 3). In contrast, the biotin-detector concentration (1-4 µg/mL) in the standard ELISA had minimal effect, with optimal performance at 1 µg/mL (Figure 3).

The concentrations of the primer-modified detector conjugates, exemplified by conjugates A and B, greatly influenced both background and TNF-α signals in the BOLD amplified assay (Figures 3A and 3B). Optimal performance required an 8x lower concentration of the conjugates, reducing background while maintaining a 10x stronger TNF-α signal compared to the standard ELISA (Figure 3A and 3B: 120 ng/mL primer-modified detector and 1000 ng/mL biotin-detector). This demonstrates BOLD technology's efficiency in amplifying signals with lower detector concentrations, thereby improving background signal.

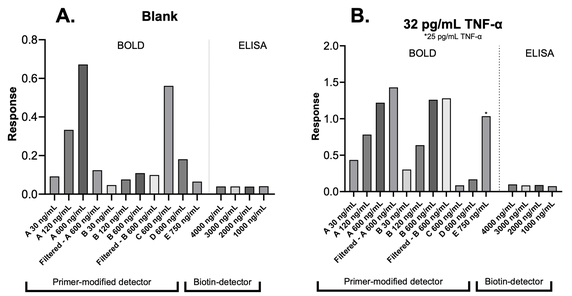


Figure 3. Comparison of blank signals and TNF-α detection using BOLD amplified ELISA and standard ELISA.
(A) *Blank signals generated using the BOLD amplified ELISA with various primer-modified detectors (Conjugates A-E) and the standard ELISA with a biotinylated detector.*
(B) *Blank-corrected signal responses at a TNF-α concentration of 32 pg/mL for all conjugates except conjugate E* (25 pg/mL).*

The performance of the primer-modified detector, including background and TNF-α signal levels, was influenced by molar conjugation ratios of the primer and detector, as well as the removal of unreacted primer (Figure 3). Optimizing these parameters, along with reducing the primer-modified detector concentration compared to the standard ELISA, lowered background signals to levels similar to the standard ELISA while maintaining significant signal amplification.

3. Performance Data

Performance data was collected across three runs to evaluate the Limit of Detection (LoD), intra-assay precision and inter-assay precision. Both the standard ELISA and the BOLD amplified ELISA were prepared on strip-based microtiter plates using identical methods and working solutions. Two separate TNF-α antigen concentration series were prepared: 0.05–50 pg/mL for the BOLD amplified ELISA and 0.4–400 pg/mL for the standard ELISA. Each of the concentration in the series was analyzed in quadruplicate. In addition, high, medium, and low concentration samples were prepared and run in triplicate, alongside seven blank samples.

Detection in the BOLD amplified ELISA was performed using a primer-modified detector (Conjugate E) at 150 ng/mL, while the standard ELISA utilized a biotin-detector at 1000 ng/mL. The standard ELISA was completed with streptavidin-HRP and a colorimetric readout, while the BOLD amplified ELISA followed the Exazym® kit protocols before being read with the same streptavidin-HRP and same colorimetric detection. Substrate incubation times were consistent between the two assays, and data were fitted using a four-parameter logistic (4PL) curve.

3.1 Six-Fold Increased Sensitivity

The LoD was determined using three different methods as shown in Table 1. BOLD amplification of the standard ELISA demonstrated a mean LoD of 0.24 pg/mL, representing a 6-fold improvement over the standard ELISA's mean LoD of 1.38 pg/mL.

Table 1. *Comparison of the LoD obtained for BOLD and the standard TNF-α ELISA.*

Method	LoD BOLD pg/mL	LoD ELISA pg/mL	LoD improvement
LoD = $Mean_{blank}$ + 3xSD	0.25	1.39	6x
LoD = $Mean_{blank}$ + 2xSD	0.18	1.01	6x
LoB = $Mean_{blank}$ + 1,645(SD_{blank}) LoD = LoB + 1,645($SD_{low\ concentration\ sample}$)	0.30	1.74	6x
Mean LoD	**0.24**	**1.38**	**6x**

3.2 Enhanced Sensitivity with Low Background

The sensitivity of the standard ELISA was significantly enhanced by BOLD amplification (Figure 4A and 4B), while maintaining a low background (Figure 4C).

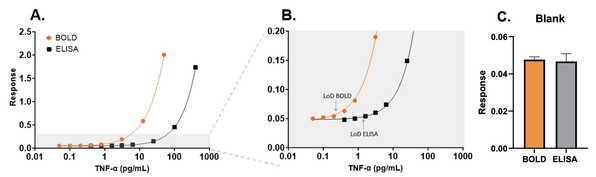
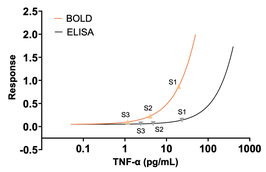


Figure 4. Enhanced detection of TNF-α using BOLD technology compared to standard ELISA.
(A) *Comparison of signal response over a range of TNF-α concentrations between the BOLD amplified ELISA (0.05–25 pg/mL) and the standard ELISA (0.4–400 pg/mL).*
(B) *Zoom-in view of the lower TNF-α responses (0.05-0.25 pg/mL).*
(C) *Comparison of blank signals (absence of antigen) between BOLD and standard ELISA.*

3.3 Reliable and Reproducible Results

The repeatability (triplicate) and intermediate precision (three runs) were evaluated for the high, medium and low samples (Figure 5). The BOLD amplified ELISA consistently delivers reliable results across a broad range of sample concentrations, both within and between runs. In contrast, the standard ELISA showed lower precision at the lower end of the curve, where it failed to reliably detect TNF-α.



	Intra-assay precision (Repeatability)*		Inter-assay precision (Intermediate precision)	
Sample	**BOLD**	**ELISA**	**BOLD**	**ELISA**
S1	<7%	<4%	10 %	6 %
S2	<8%	<9%	10 %	16 %
S3	<8%	<35%	5 %	30 %

*Highest obtained value

Figure 5. *Intra-assay precision (Repeatability) and inter-assay precision (Intermediate precision) of the BOLD amplified ELISA versus standard ELISA.*

CONCLUSIONS

The integration of BOLD technology into the standard ELISA protocol has significantly enhanced the sensitivity of TNF-α detection, achieving a six-fold lower LoD compared to the standard ELISA. This improvement allows for more accurate detection of low-abundance analytes, which is particularly critical in applications where sample concentrations or volumes are minimal or where detecting small changes is crucial. Additionally, the BOLD amplified ELISA maintained low background signals and demonstrated consistent reliability and precision across various sample concentrations, outperforming the standard ELISA at the lower detection levels. These results highlight the potential of BOLD technology to expand the capabilities of traditional immunoassays, making it a valuable tool for sensitive biomarker detection in both research and clinical settings.

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ABOUT EXAZYM®

Exazym® is an add-on immunoassay reagent kit that provides ultra-sensitive detection of low-abundance biomarkers using standard immunoassay workflows. It is based on a new detection method called Binding Oligo Ladder Detection, or BOLD for short.

ABOUT CAVIDI

Cavidi is a Swedish Biotech company with 40 years of experience working with the detection of low-abundance biomarkers requiring ultra-sensitivity with low-background noise. Our first products were in the fields of oncology and infectious diseases. Today, the company is focused exclusively on the Exazym® products.