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

EXAZYM
The BOLD Immunoassay Signal Amplifier

Application Note

Content

CAVIDI

1. Introduction

Sensitive immunoassays are highly important in many areas of biomedical research, clinical diagnostics, and therapeutic monitoring. They can be used, for example, in early detection of diseases, identification of low-abundance biomarkers, reduction of sample volume, and improved diagnostic accuracy. This Application Note will show an example of how easily and conveniently Cavidi's novel BOLD technology can be integrated into a sandwich ELISA with improved assay sensitivity and precision with low levels of analyte.

Tumour necrosis factor alpha (TNF-α) is a cytokine that plays an important role in inflammation and immune system regulation. It is expressed by various cell types, including phagocytes, lymphocytes, and other types of immune cells and non-immune cells (1). TNF-α exists in both transmembrane and soluble forms, and upon binding to its receptors, TNF-R1 and TNF-R2, it can trigger two distinct pathways: one leading to cell death and the other promoting cell survival (2).

Under normal conditions, TNF-α levels are typically around 0-2 pg/mL in plasma (3, 4) and 20-50 pg/mL in serum (5). TNF-α levels can rise significantly across various disease areas, including autoimmune and inflammatory diseases, severe infections, and cancer.

Conventional immunoassays on the market generally have a sensitivity around 1-15 pg/mL. Improved assay sensitivity would benefit the study of small changes from normal levels, or when dealing with limited sample volumes.

This paper will focus on how to develop an ultra-sensitive anti-TNF-α ELISA using Exazym@'s new BOLD technology, covering:

- The set up of a sandwich ELISA (section 2)·
- Application of BOLD technology (sections 3 and 4)
- Gathering of performance data (section 5)

2. 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.

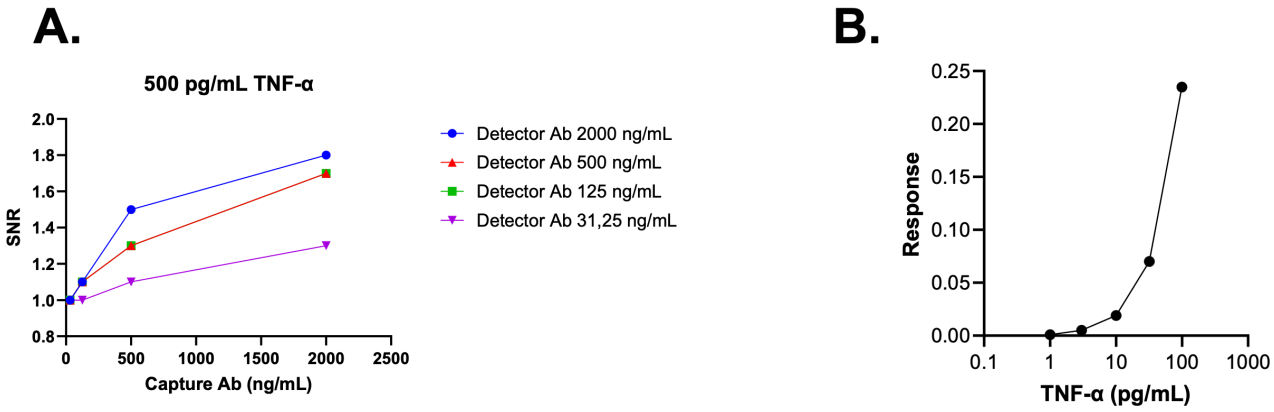


Figure 1. *Optimization and performance of an anti-TNF-α ELISA.*

(A) Signal-to-noise ratio (SNR) at 500 pg/mL TNF-α using cross titration of anti-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.

3. BOLD Technology

BOLD technology is highly versatile and can be applied to virtually any immunoassay. In this Application Note, a standard sandwich ELISA is used as the model assay, as shown in Figure 2.

To implement BOLD technology, an unmodified detector antibody is conjugated with an oligo-dT Primer, referred to here as the primer-modified detector. The assay follows the standard ELISA protocol, where a capture antibody first binds the target analyte (Figure 2.1 and 2.2). Next, the primer-modified detector binds to the analyte (Figure 2.3). DNA polymerase with reverse transcriptase (RT) activity, along with a specific reaction solution, is then added to synthesize a DNA/RNA hybrid strand (Figure 2.4). In the final steps, a biotinylated anti-DNA/RNA monoclonal antibody (tertiary antibody) is incubated to specifically bind the hybrid strand (Figure 2.5). Finally, streptavidin-HRP is added, and the signal is detected colorimetrically using a TMB substrate (3,3′,5,5′-Tetramethylbenzidine) and at 450 nm (Figure 2.6).

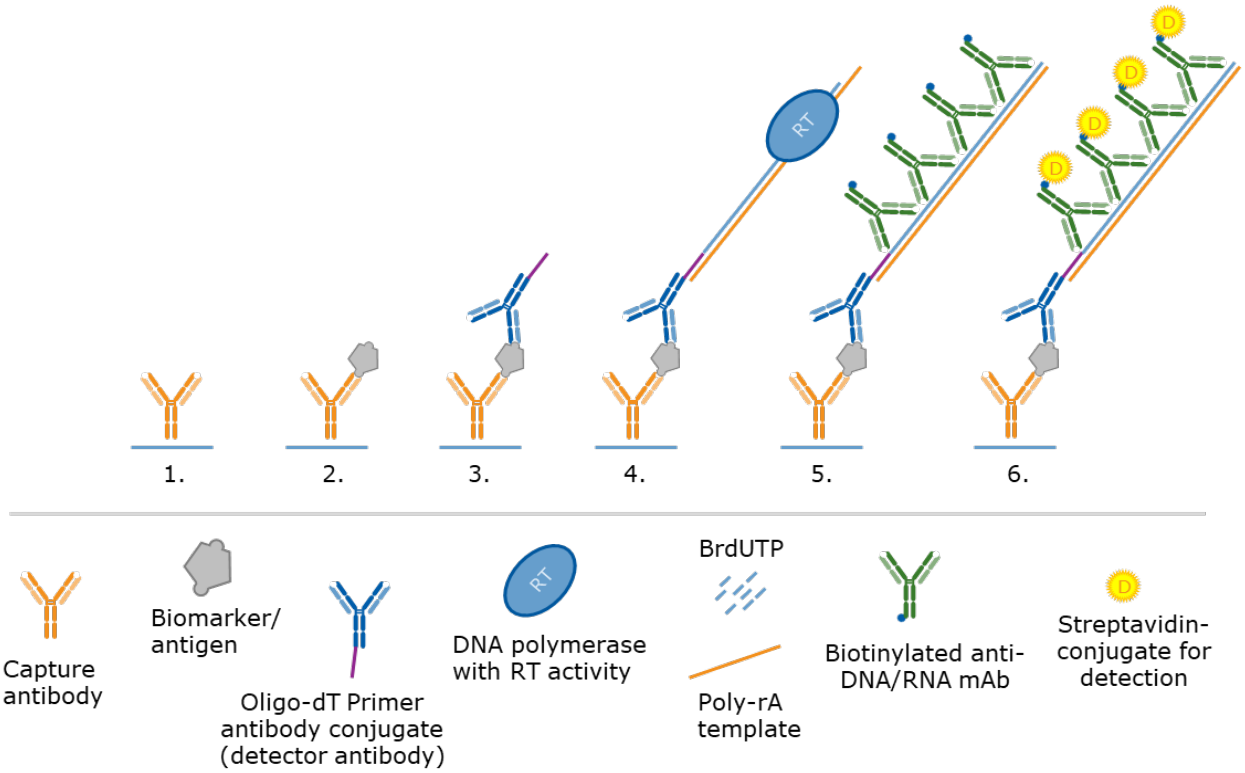


Figure 2. *Schematic drawing of the steps in a BOLD amplified sandwich immunoassay.*
(1) Coating with capture antibody.
(2) Capture of biomarker/antigen by capture antibody.
(3) Secondary binding of biomarker/antigen by oligo-dT Primer conjugated detector antibody ***(Exazym® ClickChem Conjugation Kit).***
(4) DNA/RNA Hybrid Strand Generation by DNA polymerase with RT activity ***(Exazym® Polymerase Reaction Kit).***
(5) Detection of DNA/RNA Hybrid Strand using biotinylated anti-DNA/RNA antibodies (tertiary antibodies) ***(Exazym® Biotin Detection Kit).***
(6) Signal generation using detection reagents, e.g., SA-HRP, TMB, and absorbance at 450 nm.

4. Application of BOLD technology to the Anti-TNF-α ELISA

The conjugation of the oligo-dT Primer to the unmodified anti-TNF-α detector antibody was achieved using click chemistry. Various molar excesses of ClickChem Label to detector antibody and oligo-dT Primer against the ClickChem-labeled detector antibody were tested. Specifically, ClickChem Label to detector antibody ratios ranged from 5x-20x, while oligo-dT Primer to ClickChem-labeled detector antibody ratios varied from 2x-20x. To optimize the BOLD amplified ELISA, different concentrations of these conjugates were evaluated to identify the combination that provided the best signal-to-noise ratio. Additionally, an Amicon filter (100 kDa cut-off) was used to remove free unconjugated primer from the primer-modified detector, ensuring a purified preparation and minimizing non-specific binding caused by free oligo-dT.

All BOLD incubation steps were performed according to the instructions for use (IFUs) of the Exazym® kits. The performance of the primer-modified detector was significantly influenced by its concentration in the assay (Figure 3). In contrast, the biotin-detector concentration (1-4 µg/mL) tested in the standard ELISA showed minimal influence on the assay, with optimal performance at 1 µg/mL (Figure 3).

As shown in 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). For optimal performance, an almost 8x lower concentration of the conjugates was required, significantly reducing the background signal while maintaining a 10x improved 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 the efficiency of BOLD technology in achieving significant signal amplification with a lower primer-modified detector concentration, thereby improving the background signal.

Additionally, the performance of the primer-modified detector, in terms of background and TNF-α signal levels, was influenced by molar conjugation ratios of the primer and detector and the removal of free and unreacted primer after conjugation, as exemplified by detector conjugates A-E in Figure 3. Optimizing these parameters, along with reducing the primer-modified detector concentration compared to the standard ELISA, allowed the background signals to be lowered to levels comparable to the standard ELISA while maintaining significant signal amplification.

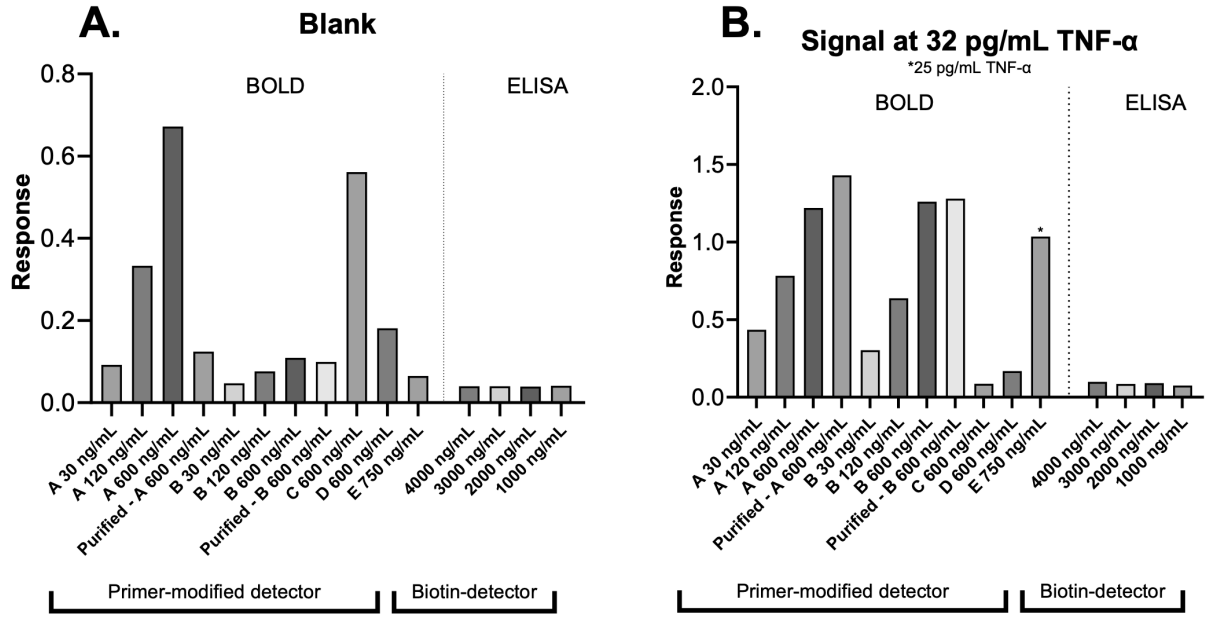


Figure 3. *Comparison of blank signals and TNF-α detection using BOLD amplified ELISA and standard ELISA.*

(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).*

5. 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 concentrations 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.

5.1. 6x 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.

Method	LoD BOLD pg/mL	LoD ELISA pg/mL	LoD Improvement
$LoD = Mean_{blank} + 3xSD_{blank}$	0.25	1.39	6x
$LoD = Mean_{blank} + 2xSD_{blank}$	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**

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

5.2. 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).

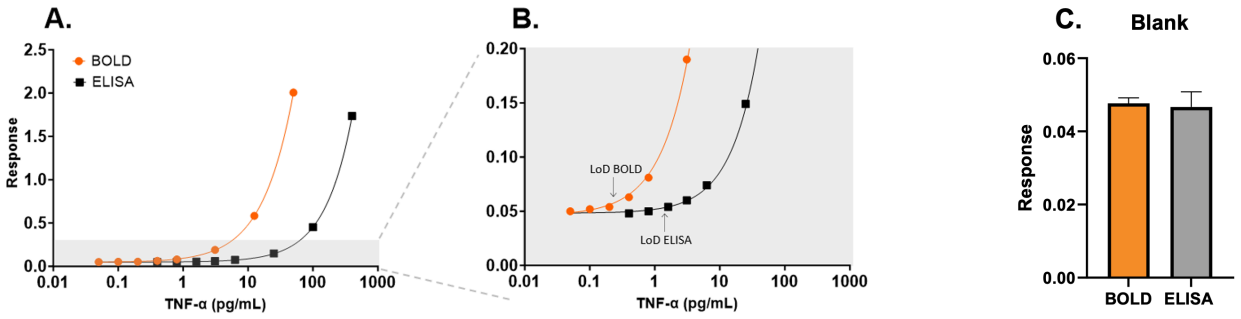
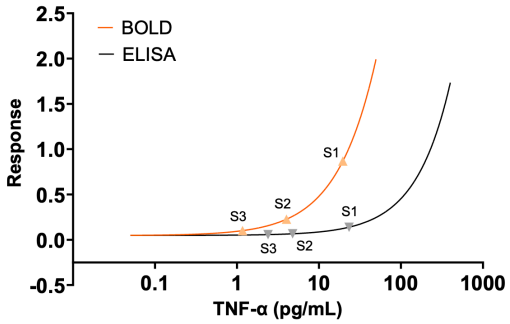


***Figure 4.** Enhanced detection of TNF-α using BOLD technology compared to standard ELISA.*
(A) Comparison of signal response over a range of TNF-α concentrations between the BOLD amplified ELISA (0.05–50 pg/mL) and the standard ELISA (0.4–400 pg/mL).
(B) Zoom-in view of the lower TNF-α responses (0.05-25 pg/mL).
(C) Comparison of blank signals (absence of antigen) between BOLD and standard ELISA.

5.3. 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.*

6. Conclusion

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.

7. References

1. Kany, Shinwan, Jan Tilmann Vollrath, and Borna Relja. "Cytokines in Inflammatory Disease." *International Journal of Molecular Sciences* 20, no. 23 (November 28, 2019): 6008. https://doi.org/10.3390/ijms20236008.
2. Zelová, Hana, and Jan Hošek. "TNF-α Signalling and Inflammation: Interactions between Old Acquaintances." *Inflammation Research* 62, no. 7 (July 2013): 641–51. https://doi.org/10.1007/s00011-013-0633-0.
3. Li, Yingkai, John S. Yi, Melissa A. Russo, Marilyn Rosa-Bray, Kent J. Weinhold, and Jeffrey T. Guptill. "Normative Dataset for Plasma Cytokines in Healthy Human Adults." Data in Brief 35 (April 2021): 106857. https://doi.org/10.1016/j.dib.2021.106857.
4. Koelman, Liselot, Olga Pivovarova-Ramich, Andreas F. H. Pfeiffer, Tilman Grune, and Krasimira Aleksandrova. "Cytokines for Evaluation of Chronic Inflammatory Status in Ageing Research: Reliability and Phenotypic Characterisation." *Immunity & Ageing* 16, no. 1 (December 2019): 11. https://doi.org/10.1186/s12979-019-0151-1.
5. Kleiner, Giulio, Annalisa Marcuzzi, Valentina Zanin, Lorenzo Monasta, and Giorgio Zauli. "Cytokine Levels in the Serum of Healthy Subjects." *Mediators of Inflammation* 2013 (2013): 434010. https://doi.org/10.1155/2013/434010.

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EXAZYM

The BOLD Immunoassay Signal Amplifier

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 its Exazym® range of products.

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Cavidi AB

Virdings Allé 2

754 50 Uppsala, Sweden

Tel: +46 18 55 20 40

CAVIDI