Signal Amplification of a Human IL-4 Sandwich ELISA

EXAZYM
The BOLD Immunoassay Signal Amplifier

Application Note

Content

CAVIDI

Introduction

This application note describes the implementation of BOLD technology to a human IL-4 sandwich ELISA including:

1. Conjugation of Exazym® oligo-dT Primer to the secondary antibody of the standard immunoassay.
2. Testing and comparing two oligo-dT Primer conjugates in a BOLD amplified ELISA.
3. Evaluating the assay concentration of the biotinylated tertiary antibody.
4. Evaluating the assay concentration and polymerization time for the reversed transcriptase.
5. Comparison of human IL-4 standard sandwich ELISA with BOLD amplified ELISA

Cytokines

Cytokines, including interleukins, interferons, tumor necrosis factors, and chemokines, play central roles in immune responses and various physiological processes. These small proteins act as messengers, orchestrating communication between immune cells and influencing inflammation, cell growth, and tissue repair. Among the myriad of cytokines, several stand out as valuable biomarkers for various diseases. Interleukin-4 (IL-4) for example, is an important cytokine that is secreted by activated T cells, basophils, and mast cells. It has both pro- and anti-inflammatory effects, depending on the context. IL-4 is a key regulator of immune responses, particularly in allergic reactions. Therefore, IL-4 is involved in some allergic diseases where IL-4 plays a role in the pathogenesis of allergic asthma and other atopic conditions. IL-4 also plays a role in autoimmune disorders where dysregulation of IL-4 is associated with autoimmune diseases. Consequently, detecting cytokines at low concentrations is critical for understanding disease mechanisms, monitoring treatment efficacy, and identifying potential therapeutic targets. Many cytokines, IL-4 included, are present in trace amounts, especially during early disease stages. Therefore, ultra-sensitive assays are essential to accurately quantify these molecules and provide valuable insights into disease progression.

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 simple add-on process that can be used with any immunoassay. Integration of BOLD into an immunoassay can 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; see Figure 1 for the principle of click chemistry. The conjugation is performed with the Exazym® ClickChem Conjugation kit.

Schematic drawing of the steps involved in the conjugation of the oligo-dT Primer to a secondary antibody

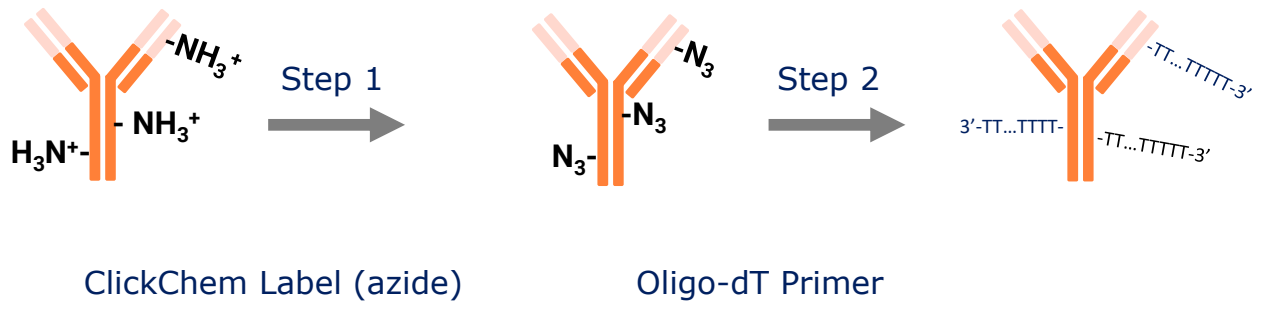

***Figure 1**. Step 1: Amine coupling of azide tags to lysine residues on the mAb using NHS-PEG4-azide. Step 2: Azides are reacting with DBCO-TEG modified oligo-dT Primer.*

This oligo-dT Primer antibody conjugate serves as a crucial bridge, connecting the standard immunoassay with the necessary subsequent steps involved in BOLD to achieve ultra-sensitive detection. Below, using a sandwich immunoassay set-up, all important steps of BOLD technology are presented. Also, see the visualization of BOLD when implemented in a sandwich immunoassay in Figure 2.

1. Coating with primary capture antibody: In this step, the primary antibody is added and coated to the surface type used in the immunoassay.
2. Capture of biomarker/antigen: In this step, the biomarker or antigen sample is added and bound by the immobilized primary antibody.
3. Secondary binding of biomarker/antigen: In this step, the oligo-dT Primer antibody conjugate is added and binds specifically to the captured biomarker/antigen. Note: The conjugation of the oligo-dT Primer to the antibody is performed with the Exazym® ClickChem Conjugation Kit.

Typically steps 1 and 2 can be performed as the standard immunoassay. For step 3, the concentration of the oligo-dT Primer antibody conjugate often can be decreased compared to the standard immunoassay.

4. DNA/RNA Hybrid Strand Generation: Next, a poly-rA template is introduced in the assay along with a polymerase containing reverse transcriptase activity and BrdUTP. Through the action of the reverse transcriptase polymerase, a DNA/RNA hybrid strand is synthesized (BrdU/rA). The DNA/RNA hybrid strand serves as a binding site for multiple tertiary antibodies (Exazym® Antibody Biotin). Note: This step is achieved with the Exazym® Polymerase Reaction Kit.
5. Tertiary Antibody Binding: In this step, biotinylated tertiary mAbs are added, and they bind to the DNA/RNA hybrid strand. Note: This step is achieved with the Exazym® Biotin Detection Kit.

Illustration of BOLD implementation in a sandwich immunoassay

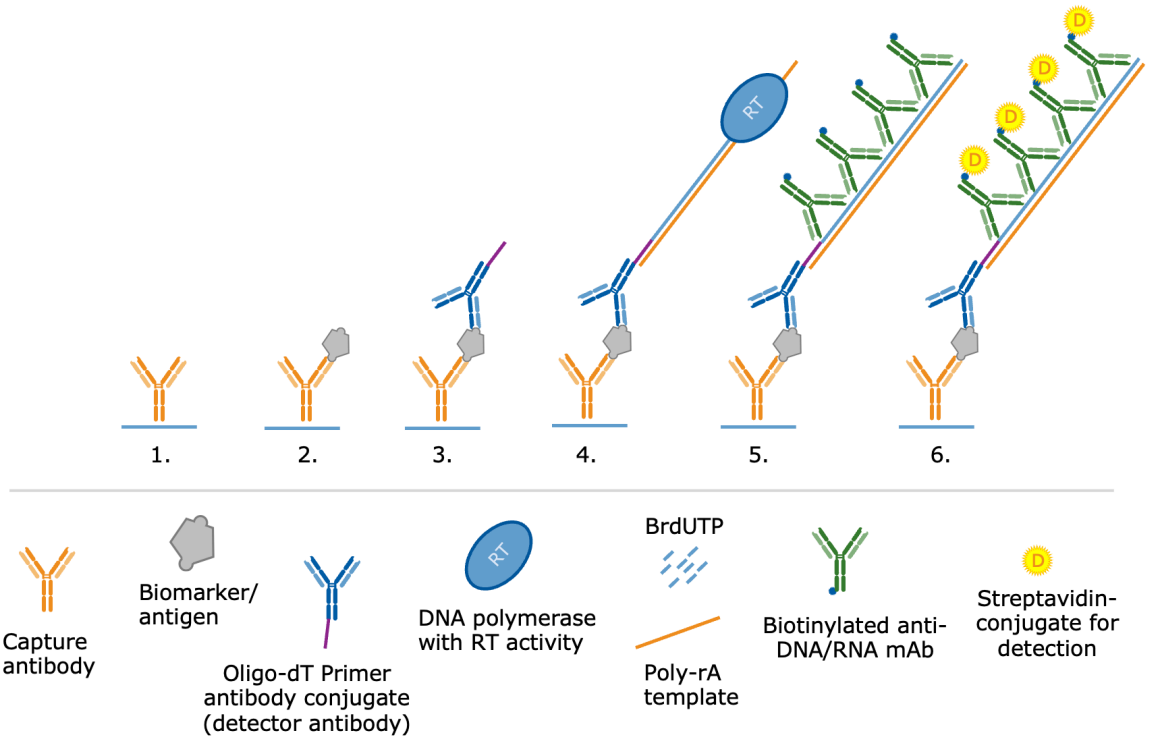

Figure 2. *Schematic drawing of the steps in a BOLD amplified sandwich immunoassay.*

6. Signal Generation: Finally, in this step, the amplified signal is generated by adding Streptavidin labeled with a suitable reporter molecule (fluorescence or luminescence) or a Streptavidin-enzyme conjugate, such as Streptavidin-HRP, together with a suitable substrate system (same as standard ELISA).

Methods and Results

Conjugation of Exazym® oligo-dT Primer to the secondary antibody of the immunoassay

The conjugation of the oligo-dT Primer to the unmodified secondary antibody of the IL-4 sandwich ELISA was performed with click chemistry (Exazym® ClickChem Conjugation Kit).

Two conjugates were prepared:

1. **Anti IL-4 oligo-dT Primer conjugate 6x:5x**

   Azide labeling of the IL-4 antibody was performed using a 6 times molar excess of the ClickChem label.

   Conjugation of the oligo-dT Primer to the azide labeled IL-4 antibody was performed using a 5 times molar excess of the oligo-dT Primer.

2. **Anti IL-4 oligo-dT Primer conjugate 20x:30x**

   Azide labeling of the IL-4 antibody was performed using a 20 times molar excess of the ClickChem label.

   Conjugation of the oligo-dT Primer to the azide labeled IL-4 antibody was performed using a 30 times molar excess of the oligo-dT Primer.

Successful conjugation was evaluated using SDS-PAGE and prior to functional testing of the conjugate in a BOLD amplified ELISA, the concentration was determined using Pierce™ BCA Protein Assay (Cat No: 23227). As visualized by the SDS-PAGE analysis in Figure 3, both tested molar excess combinations of ClickChem Label and oligo-dT Primer resulted in the conjugation of the oligo dT-Primer to the secondary antibody. It is also clear and expected that an increase in conjugation level was observed with increasing molar excess of the ClickChem Label and oligo-dT Primer (6x:5x < 20x:30x).

SDS-PAGE analysis of prepared anti-IL-4 oligo-dT Primer conjugates

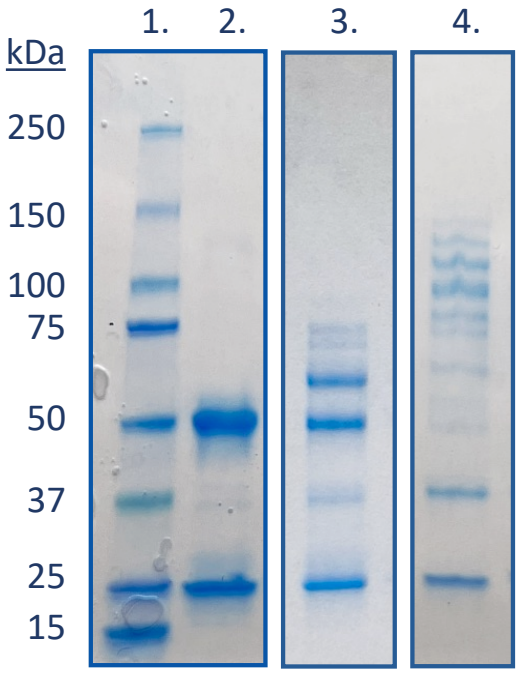

Figure 3. SDS-PAGE analyses of the unmodified anti-IL-4 antibody and the anti-IL-4 oligo-dT Primer conjugates were performed on a 4–15% precast polyacrylamide gel, 8.6 × 6.7 cm (W × L). All antibodies were analyzed at an amount of 5 µg and under reducing conditions.
Lane 1: Protein Standards.
Lane 2: 5 µg unmodified anti-IL-4 antibody
Lane 3: 5 µg anti-IL-4 oligo-dT Primer conjugate (6x:5x)
Lane 4: 5 µg anti-IL-4 oligo-dT Primer conjugate (20x:30x).

Testing and comparing two oligo-dT Primer conjugates in a BOLD amplified ELISA.

The function of the two oligo-dT Primer conjugates, 6x:5x and 20x:30x were tested in a BOLD amplified ELISA (Figure 2), using components of the Exazym® Polymerase Reaction Kit and Exazym® Biotin Detection Kit. Signal generation was achieved with Streptavidin-HRP, which binds to the biotinylated tertiary antibodies bound to the DNA/RNA hybrid strands. Detection of bound biotinylated tertiary antibodies was performed through colorimetric detection using Streptavidin-HRP and TMB substrate (absorbance at 450 nm). As seen in Figure 4 both oligo-dT Primer conjugates were successful in bridging the standard sandwich ELISA to the BOLD amplified ELISA. Compared to the standard sandwich ELISA, a much lower concentration of both conjugates was needed, 15.63-125 ng/mL versus 1 µg/mL recommended for the standard ELISA. Using 1 µg/mL of the oligo-dT Primer conjugates as recommended for the standard ELISA resulted in absorbance signals outside the dynamic range of the plate reader, results not shown. The highest absorbance signals, using the same concentrations, were observed for the 20x:30x oligo-dT Primer conjugate, Figure 4A.

However, comparing the Signal-to-Noise (S/N) ratios, Figure 4B, the two conjugates were more comparable, with slightly higher S/N ratios for the 20x:30x oligo-dT conjugate at low human IL-4 concentrations (0.1-1 pg/mL), Figure 4B. The highest S/N ratio was observed for the 20x:30x oligo-dT Primer conjugate at a concentration of 31.25 µg/mL, and this concentration for the oligo-dT conjugate was used when comparing the final BOLD amplified ELISA with the standard human IL-4 sandwich ELISA, Figure 7.

Testing the performance of the anti-IL-4 oligo-dT Primer conjugates in a BOLD amplified ELISA

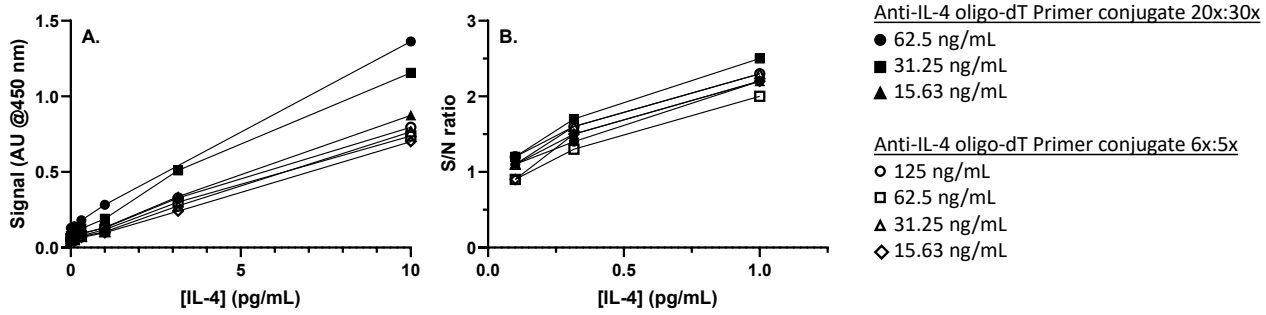

Figure 4*. Evaluation of the anti-IL-4 oligo-dT Primer conjugates. A. Absorbance signals for IL-4 antigen concentrations ranging from 0 to 10 pg/mL. B. Signal-to-Noise (S/N) ratios for IL-4 antigen concentrations 0.1, 0.316 and 1.0 pg/mL (data obtained from A.). In this BOLD amplified ELISA oligo-dT Primer conjugate 20x:30x at a concentration of 15.63, 31.25 and 62.5 ng/mL were tested and compared to oligo-dT Primer conjugate 6x:5x tested at concentrations 15.63, 31.25, 62.5 and 125 ng/mL. The concentration of the biotinylated tertiary antibody was 0.3 µg/mL.*

Evaluating the assay concentration of the biotinylated tertiary antibody

The biotinylated tertiary antibody, which comes with the Exazym® Biotin Detection Kit, is utilized for detecting the DNA/RNA hybrid strands that have been polymerized by reverse transcriptase. As per the Instruction for Use (IFU) provided with the kit, a concentration of 0.3 µg/mL for the biotinylated tertiary antibody is a recommended starting point for detection. In Figure 5, we present the absorbance signals (A.) and S/N ratios (B.) obtained when testing concentrations of 0.1, 0.3, and 0.6 µg/mL of the biotinylated tertiary antibody (oligo-dT Primer conjugate 20x:30x was utilized). As seen, there were minimal differences observed among the tested concentrations, which is favorable from a method robustness perspective. For the comparison between the final BOLD amplified ELISA and the standard human IL-4 sandwich ELISA, a concentration of 0.3 µg/mL was used (Figure 7).

Impact of biotinylated tertiary antibody concentration on BOLD amplified ELISA

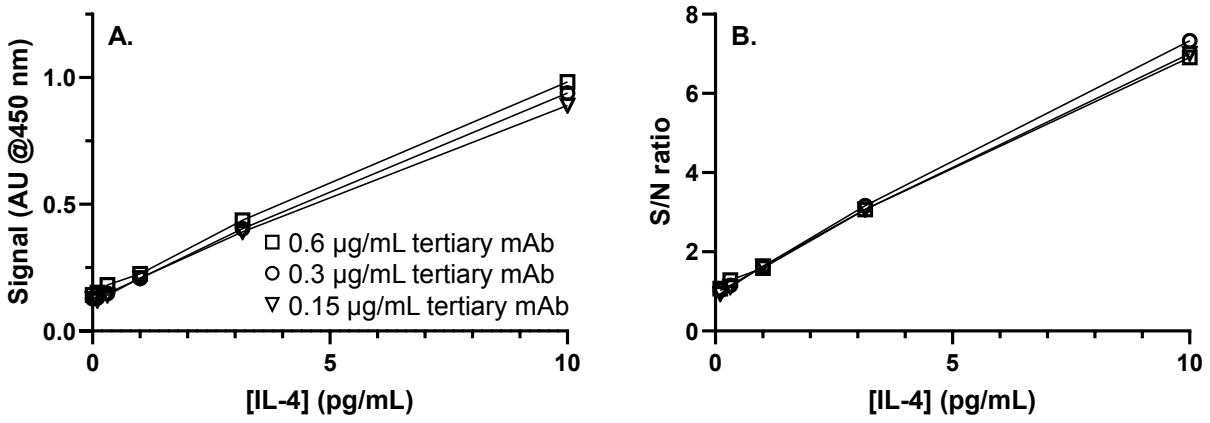

Figure 5. *A. Absorbance signals for IL-4 antigen concentrations ranging from 0 to 10 pg/mL. B. Signal-to-Noise (S/N) ratios corresponding to the IL-4 antigen concentrations in A. In this BOLD amplified ELISA oligo-dT Primer conjugate 20x:30x at a concentration of 250 ng/mL was used and concentration of the biotinylated tertiary antibody were tested at 0.15, 0.3 and 0.6 µg/mL.*

Evaluating the assay concentration and polymerization time for the reversed transcriptase.

The Exazym® Polymerase Reaction Kit includes reverse transcriptase, which plays a crucial role in polymerizing DNA/RNA hybrid strands. According to the provided Instruction for Use (IFU), it is recommended to use a well concentration of 0.5 U/mL and incubate/polymerize for 10-30 minutes. In Figure 6, we present absorbance signals (A.) and S/N ratios (B.) obtained from testing reverse transcriptase concentrations of 0.1 and 0.5 U/mL, along with polymerization times of 30 and 60 minutes (using oligo-dT Primer conjugate 20x:30x). As seen, higher reverse transcriptase concentrations yielded higher absorbance signals, and longer polymerization times as well (Figure 6A). However, when comparing S/N ratios across different combinations, the highest S/N ratio was observed with 0.5 U/mL reverse transcriptase and a 30-minute polymerization time (Figure 6B). Apparently, longer polymerization time also led to increased background/blank signals. For the comparison between the final BOLD amplified ELISA and the standard human IL-4 sandwich ELISA, a well concentration of 0.5 U/mL and a polymerization time of 30 minutes were used (Figure 7).

Impact of reverse transcriptase concentration and time on BOLD amplified ELISA

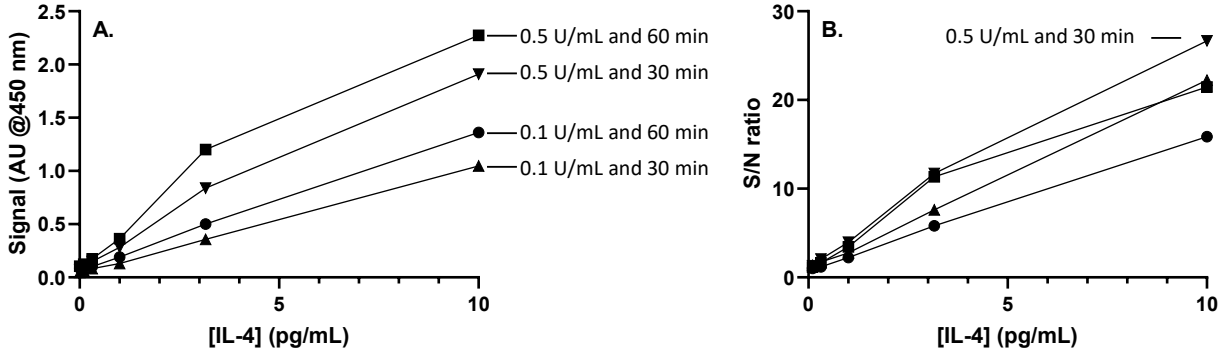

Figure 6. *A. Absorbance signals for IL-4 antigen concentrations ranging from 0 to 10 pg/mL. B. Signal-to-Noise (S/N) ratios corresponding to the IL-4 antigen concentrations in A. In this BOLD amplified ELISA oligo-dT Primer conjugate 20x:30x at a concentration of 62.5 ng/mL was used and concentration of the biotinylated tertiary antibody was 0.3µg/mL.*

Comparison of human IL-4 standard sandwich ELISA with BOLD amplified ELISA

In the final test of the BOLD amplified ELISA, we used the best conditions identified and presented above. Specifically, we used the best-performing combination concerning the oligo-dT Primer conjugate (20x:30x), the concentration of the biotinylated tertiary antibody, and the concentration and polymerization time of the reverse transcriptase. Simultaneously, the standard human IL-4 sandwich ELISA was executed under its optimized parameters. For both ELISA systems, Streptavidin-HRP and TMB substrate were used for signal generation, and absorbance was measured at 450 nm.
In Figure 7A-C, we present the IL-4 antigen calibration curves obtained using both the standard sandwich ELISA and the BOLD amplified ELISA. As seen, the absorbance signals corresponding to the tested IL-4 concentrations were significantly enhanced with the BOLD amplified ELISA compared to the standard ELISA (Figure 7A- C). Specifically, the limit of detection (LOD) for the standard human IL-4 ELISA was calculated to be 1.9 pg/mL, based on the concentration derived from the background signal (blank) plus twice the standard deviation of the blank (using a 4-parameter logistic fit). The LOD for the BOLD amplified ELISA was significantly lower, 0.040 pg/mL—nearly 50 times better than the standard ELISA.

Comparison of standard human IL-4 ELISA and BOLD amplified human IL-4 ELISA

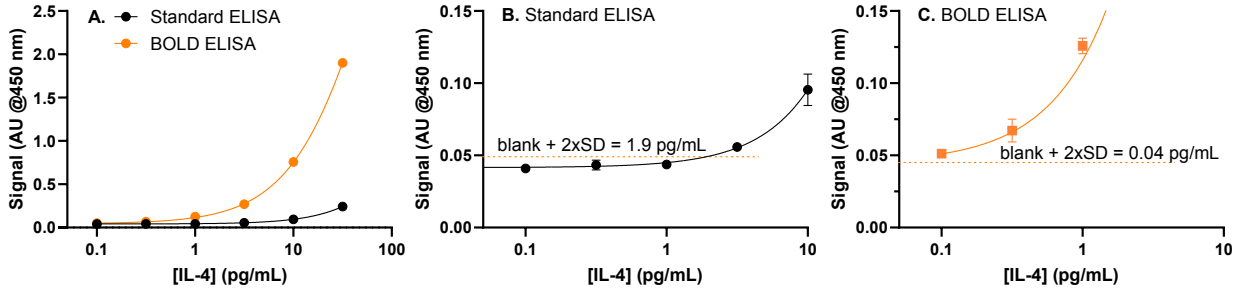

Figure 7. *IL-4 Antigen Calibration Curves. A. Calibration curves for IL-4 antigen concentrations ranging from 0.1 to 31.6 pg/mL are shown. The black trace represents the standard ELISA, while the orange trace corresponds to the BOLD amplified ELISA. B. Zoomed-in view of the IL-4 calibration curve within the range of 0.1 to 10 pg/mL obtained for the standard ELISA. Notably, the biotinylated secondary anti-IL-4 monoclonal antibody (mAb) was used at a concentration of 1 µg/mL. C. Zoomed-in view of the IL-4 calibration curve within the range of 0.1 to 1 pg/mL obtained for the BOLD amplified ELISA. In the BOLD amplified ELISA, the oligo-dT Primer conjugate 20x:30x was used at a concentration of 31.6 ng/mL and the concentration of the biotinylated tertiary antibody was 0.3 µg/mL. The orange broken lines in panels B and C represents the LOD as determined from background signal (blank) plus 2 times the standard deviation (SD).*

Conclusion

Both tested oligo-dT Primer conjugates successfully bridged the standard ELISA to the subsequent steps required for BOLD amplification and we selected the oligo-dT Primer conjugate 20x:30x for the final comparison of the BOLD amplified ELISA and the standard ELISA. In this application, we did not perform any purification of the conjugate. Specifically, we did not remove the unconjugated oligo-dT Primer (background signal) and unconjugated anti-IL-4 antibody (competition). Consequently, there exists an opportunity to improve the overall quality of the conjugate.

Variation in the concentration of the biotinylated tertiary antibody had little effect on the absorbance signals obtained in the BOLD amplified assay. A well concentration of 0.3 µg/mL, recommended in the IFU of the Exazym® Biotin Detection Kit, was selected when comparing the BOLD amplified ELISA with the standard ELISA.

The concentration and reaction time of the reverse transcriptase had an effect on the signals obtained with the BOLD ELISA. A reverse transcriptase well concentration of 0.5 U/mL and a polymerization time of 30 minutes yielded the best results. These parameters align with the recommended starting points provided in the IFU of the Exazym® Polymerase Reaction Kit.

Finally, after applying the best conditions found for the BOLD amplified ELISA, we compared it to the standard human IL-4 sandwich ELISA run under its optimized parameter. The BOLD amplified ELISA demonstrated amplified absorbance signals for all tested IL-4 concentrations, achieving a significantly lower limit of detection (LOD) of 0.040 pg/mL—nearly 50 times better than the standard ELISA.

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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