Efficient Oligo-dT Primer Conjugation for BOLD Amplified ELISA

Introduction

In this short application note we describe a streamlined process for the implementation of Binding Oligo Ladder Detection (BOLD) signal amplification into a sandwich ELISA. The implementation process is straightforward and uncomplicated. Below, we have outlined the first steps in the implementation process, specifically the conjugation of the oligo-dT Primer to the secondary antibody, and recommended initial evaluation of the conjugate.

1. Conjugation of the oligo-dT Primer to the secondary antibody used in the standard ELISA with click chemistry.
2. Evaluation of the conjugation level using SDS-PAGE.
3. Testing the function and performance of the oligo-dT Primer conjugate in a BOLD amplified ELISA.

Workflow for conjugating Exazym® oligo-dT Primer to a secondary antibody with click chemistry.

Step 1

Label the secondary antibody with azide tags using the ClickChem label.

Step 2

Conjugate the oligo-dT Primer to the azide-labeled secondary antibody.

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

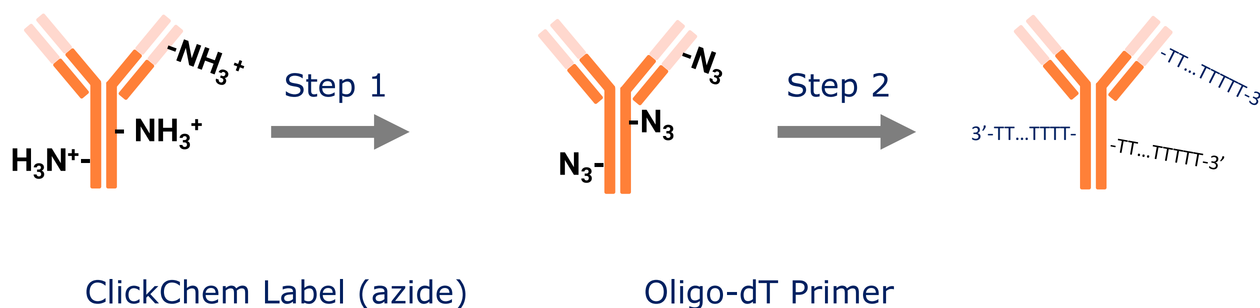

***Figure 1**. Simplified overview of the steps involved in conjugation of oligo-dT Primer to the secondary antibody to set-up the standard ELISA for signal amplification using BOLD technology.*

1. Conjugation of the oligo-dT Primer to the secondary antibody used in the standard ELISA with click chemistry.

In this example, three different azide-labeled secondary antibodies were prepared with different molar excess levels of the Exazym® ClickChem label with respect to the secondary antibody (5, 20, and 40 times excess). Typically, a molar excess of 5-20 is sufficient to achieve a conjugation level of the oligo-dT Primer that gives a significant signal amplification in your ELISA. In short, the azide labeling of the secondary antibody was performed as follows:

Step 1

Label the secondary antibody with azide tags using the ClickChem label.

1. The secondary antibody was labeled with the ClickChem label (NHS-PEG4-azide) at a 5, 20, and 40 times molar excess. Incubation time is 30 minutes at room temperature.
2. The labeling reaction was stopped with Exazym® quenching buffer. Incubation time is 5 minutes at room temperature.
3. The uncoupled/unreacted ClickChem label was removed by a spin column (provided with the kit).

In the second step, the oligo-dT Primer is conjugated to the azide-labeled secondary antibody. In the Exazym® conjugation kit, a molar excess of 5-20 times versus the azide-labeled secondary antibody is recommended; in fact, 20 times excess is the maximum molar excess that can be achieved with the supplied oligo-dT Primer. In this conjugation experiment, a 40 times molar excess of the oligo-dT Primer was tested for informative purposes.

Step 2

Conjugate the oligo-dT Primer to the azide-labeled secondary antibody.

Three different oligo-dT Primer antibody conjugates were prepared.

1. Oligo-dT Primer was added with a molar excess of 5, 20, and 40 times compared to the ClickChem labeled secondary antibody.
2. The mixture was incubated overnight at room temperature.

This resulted in three different antibody oligo-dT Primer conjugates, named 5x:5x, 20x:20x, and 40x:40x molar excess of the ClickChem label and the oligo-dT Primer with respect to the antibody, clickchem label:oligo-dT primer. Before proceeding to evaluate the various conjugates with SDS-PAGE, we recommend determining the concentration of each conjugate (Pierce™ Micro BCA Protein Assay Kit, Cat No: 23235 or BCA Protein Assay (Cat No: 23227).

2. Evaluation of the conjugation level using SDS-PAGE

SDS-PAGE was used to evaluate the conjugation levels of the prepared oligo-dT Primer conjugates. As visualized by the SDS-PAGE analysis in Figure 2 below, all the tested molar excess of ClickChem Label and oligo-dT Primer resulted in the conjugation of the oligo dT-Primer to the secondary antibody. Of the molar excess combinations tested, an increase in conjugation level was observed with increasing molar excess of the ClickChem Label and oligo-dT Primer (5:5 < 20:20 < 40:40).

SDS-PAGE analysis of oligo-dT Primer conjugates

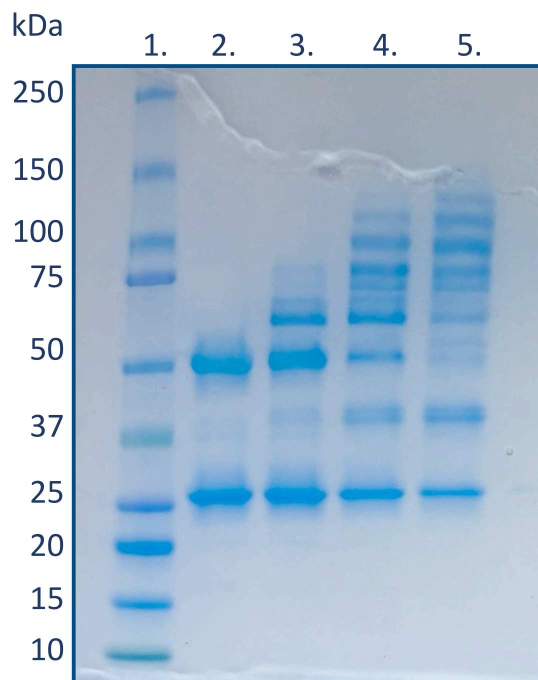

Figure 2. *Evaluation of conjugated and unmodified secondary antibody using SDS-PAGE analysis. The analysis was performed on a 4–15% precast polyacrylamide gel, 8.6 × 6.7 cm (W × L). All antibodies were analyzed at an amount of 5 µg and under reducing conditions. Lane 1: 5 µL Protein Standards. Lane 2: unmodified secondary antibody, Lane 3: Oligo-dT Primer conjugated secondary antibody (5:5), Lane 4: Oligo-dT Primer conjugated secondary antibody (20:20), Lane 5: Oligo-dT Primerconjugated secondary antibody (40:40).*

A higher conjugation level does not necessarily mean that the conjugate will work better in the BOLD amplified ELISA. Therefore, it is important to evaluate the function of the conjugates in a BOLD amplified ELISA; see the section below.

3. Testing the function and performance of the oligo-dT Primer conjugate in a BOLD amplified ELISA.

Each of the three oligo-dT Primer conjugated secondary antibodies was evaluated in a BOLD amplified ELISA and compared to the original standard ELISA. Two concentrations of each oligo-dT Primer secondary antibody conjugate were tested, 62.5 ng/mL and 500 ng/mL. A higher concentration,
2 µg/mL, of the corresponding secondary antibody was used in the standard ELISA. For both BOLD amplified and standard ELISA, the well surfaces were coated with the same primary antibody at a concentration of 2 µg/mL in PBS.

As depicted in Figure 3 below, all the prepared oligo-dT Primer conjugates exhibited signal amplification with BOLD, surpassing the standard ELISA. A much lower concentration, 62.5 ng/mL of the oligo-dT Primer conjugates was needed in the BOLD ELISA to achieve a significant signal amplification as compared to the signal observed for the standard ELISA, which used 2000 ng/mL of biotinylated secondary antibody. Besides comparing signals obtained for different oligo-dT conjugates binding to the biomarker, it is also important to evaluate the background signal, i.e., the signal achieved with no biomarker present, and compare it to the signal observed when the biomarker is present.

Comparing standard curves obtained with oligo-dT Primer conjugates and Standard ELISA

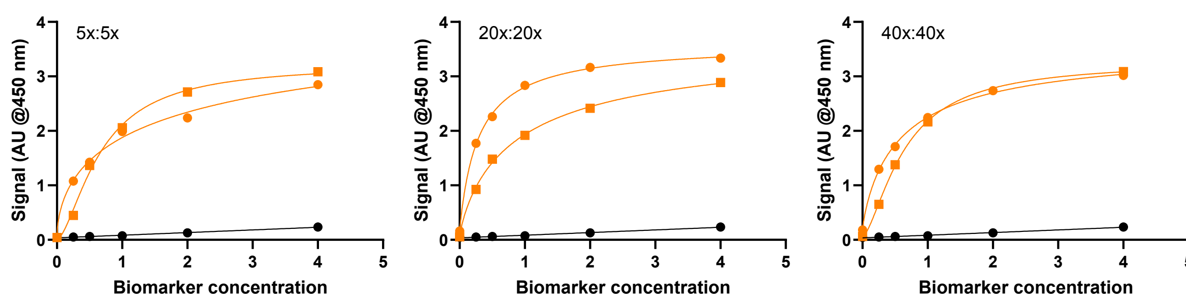

Figure 3. *ELISA standard curves for the BOLD amplified ELISAs (orange trace) and the standard ELISAs (black trace). Orange closed square 62.5 ng/mL of oligo-dT Primer conjugated secondary antibody. Orange closed circle 500 ng/mL oligo-dT Primer conjugated secondary antibody. Black closed circle 2 µg/mL of biotinylated secondary antibody. Left graph: conjugate prepared with 5 times molar excess of ClickChem Label and oligo-dT Primer compared to secondary antibody. Middle graph: conjugate prepared with 20 times molar excess of ClickChem Label and oligo-dT Primer compared to secondary antibody. Right graph: conjugate prepared with 40 times molar excess of ClickChem Label and oligo-dT Primer compared to secondary antibody. Colorimetric detection using SA-HRP conjugate and TMB as substrate.*

Figure 4 below depicts all background signals obtained for the prepared oligo-dT Primer conjugates at two different concentrations, one high, 500 ng/mL, and one low, 62.5 ng/mL. Based on the results shown in Figure 4 below, it is evident that increasing the concentration of oligo-dT primer conjugate leads to a higher background signal. The signals obtained when using a concentration of 62.5 ng/mL of the oligo-dT conjugates were further evaluated, with results presented in Figure 5 below.

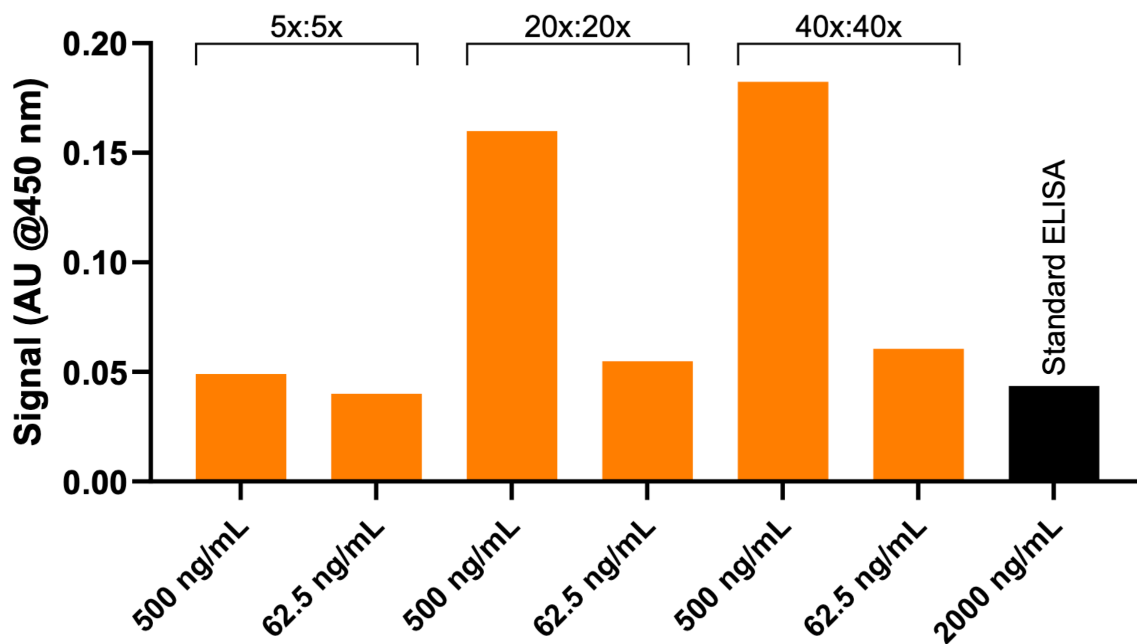

***Figure 4**. Illustration of the background signals (blanks) obtained for the different oligo-dT Primer conjugated secondary antibodies compared to the standard ELISA. Text above brackets indicate the molar excess of ClickChem Label versus antibody and the molar excess of the oligo-dT Primer versus ClickChem labeled antibody.*

Figure 5 depicts the S/N ratios of the oligo-dT Primer conjugates, i.e., the signals obtained for the lowest biomarker concentration tested versus the background signal (S/N ratio). Based on this result when using the lowest oligo-dT Primer conjugate concentration, 62.5 ng/mL, the best S/N ratio, 17, was observed for the 20x:20x oligo-dT Primer conjugate. The S/N ratio of the standard ELISA was 1.1.

Comparing S/N ratios obtained with oligo-dT Primer conjugates and Standard ELISA

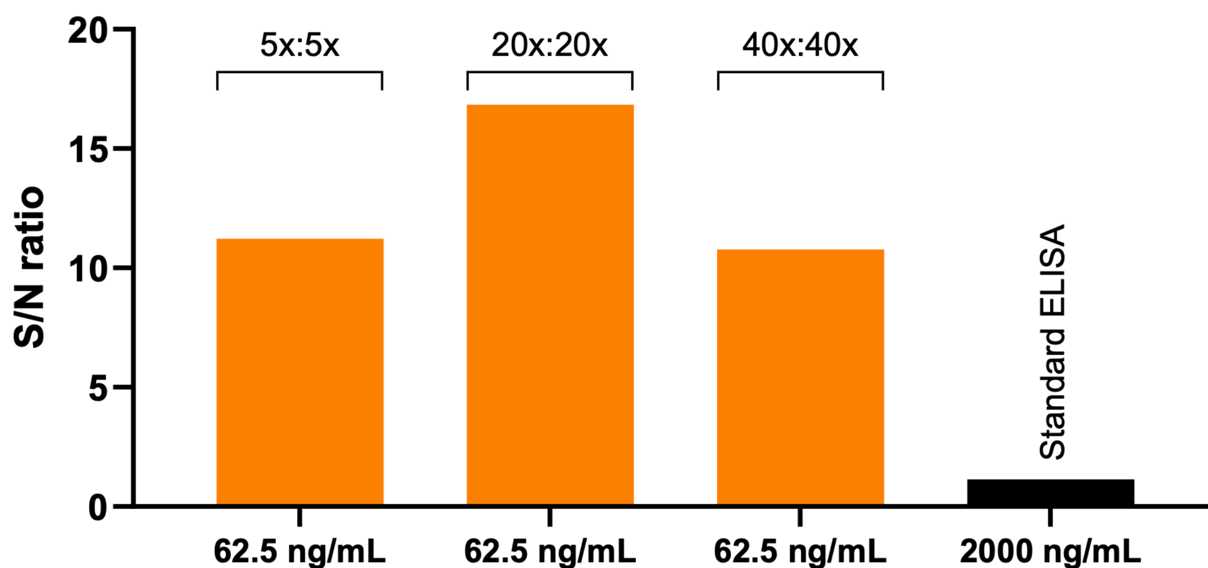

***Figure 5**. Illustration of the S/N ratio obtained for the different oligo-dT Primer conjugated secondary antibodies (orange) compared to the standard ELISA (black). Detection was performed identically for the BOLD and Standard ELISA. The concentrations below the columns represent the concentration of the oligo-dT Primer conjugate and the same but biotinylated secondary antibody of the standard ELISA. Text above brackets indicate the molar excess of ClickChem Label versus antibody and the molar excess of the oligo-dT Primer versus ClickChem labeled antibody.*

Conclusion

In this short application note, we demonstrate a straightforward method for successfully conjugating the oligo-dT Primer to an unmodified secondary antibody of a sandwich ELISA using click chemistry (Exazym® ClickChem Conjugation Kit). The choice of molar excess of both the ClickChem Label and the oligo-dT Primer impacted the signal amplification when used in the BOLD amplified ELISA.
Our experimental approach involved testing various molar excess ratios, specifically, 5x, 20x, and 40x, for both the ClickChem Label and the oligo-dT Primer in comparison to the secondary antibody.
Through these simple tests, we discovered that the 20x:20x ratio yielded the best results in the BOLD amplified ELISA, with a signal-to-noise ratio 15 times better than the standard sandwich ELISA.
It's worth considering that exploring additional ratio combinations of the ClickChem Label and the oligo-dT Primer could potentially enhance the signal amplification even more in the BOLD amplified ELISA. Researchers should fine-tune the conjugation conditions, specifically the ratios, based on their evaluation of the conjugate using SDS-PAGE and further testing in the BOLD amplified ELISA.
As a starting point, we recommend utilizing a molar excess of 5-20 times for the ClickChem Label, combined with a 5-20 times excess of the oligo-dT Primer compared to the secondary antibody.
This recommendation aligns with the Exazym® ClickChem Conjugation Kit.

About Exazym®

Exazym® is an add-on ELISA reagent kit that provides attomole-level detection of low-abundance biomarkers using standard ELISA 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 firm with 30 years of experience working with the detection of low-abundance biomarkers requiring ultra-high sensitivity with low-background noise. The company's 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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