BOLD Signal Amplification of a Human pTau(181) Sandwich ELISA

EXAZYM
The BOLD Immunoassay Signal Amplifier

Application Note

Contents

CAVIDI

Objective

This application note describes the implementation of BOLD technology to a sandwich ELISA for quantitative in vitro determination of human pTau(181) in cerebrospinal fluid (CSF) and includes:

1. Conjugation of the oligo-dT Primer to the detector antibody of the standard ELISA and initial testing.
2. Evaluation of the effect of the assay concentration and polymerisation time on the performance of the polymerase.
3. Evaluation of the effect of assay concentration of the biotinylated anti-DNA/RNA antibody on the amplified signal.
4. Detection of pTau(181) in Human plasma.

Introduction

pTau(181) in Alzheimer's Diagnosis: Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by the accumulation of abnormal protein aggregates in the brain, leading to cognitive decline and memory loss. It is the most common cause of dementia in older adults. A well-established biological fluid biomarker for AD is increased levels of tau protein phosphorylated at position 181 (pTau(181)) in cerebrospinal fluid (CSF). Sample collection from CSF in elderly individuals is associated with inherent risks and complications, including the potential for infection, bleeding in patients with fragile blood vessels, and back pain. Additionally, early screening for AD risk and initiating treatment at an asymptomatic stage, prior to the onset of cognitive challenges, underscores the importance of easy and early detection of biomarkers. Given these considerations, there is a growing demand to detect these biomarkers in blood rather than CSF. However, measuring low abundance pTau(181) in blood presents a critical challenge in many pre-clinical, clinical, and diagnostic applications. Detection of this biomarker in blood requires a highly sensitive assay. Fortunately, the BOLD technology offers a solution to this issue. Its ultra-sensitive detection capabilities enable the detection of even the slightest trace of biomarkers. Therefore, BOLD technology holds promise for facilitating the early detection and management of AD.

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 antibody oligo-dT Primer conjugate serves as a crucial bridge for the addition of the BOLD amplification steps to the standard immunoassay, see Figure 1 with explanation of steps 1-6 below.

Steps of a BOLD Amplified ELISA

1. Coating with capture antibody: In this step, the capture 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 capture 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.

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

Illustration of BOLD implementation in a sandwich immunoassay

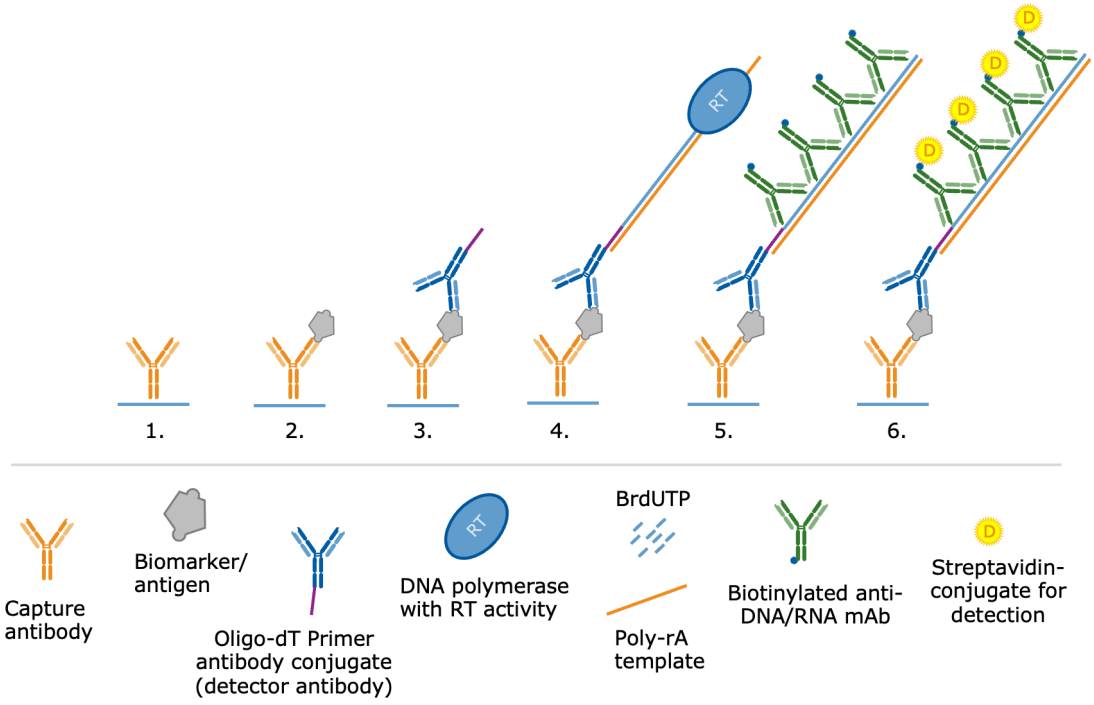

Figure 1 *Schematic drawing of the steps in a BOLD amplified sandwich immunoassay.*

4. DNA/RNA Hybrid Strand Generation: Next, a poly-rA template is introduced in the assay along with a polymerase with reverse transcriptase activity and BrdUTP. Through the action of the polymerase, a DNA/RNA hybrid strand is synthesized (BrdU/rA). The DNA/RNA hybrid strand

serves as a binding site for multiple anti-DNA/RNA antibodies (Exazym® Antibody Biotin). Note: This step is achieved with the **Exazym® Polymerase Reaction Kit**.

5. Detection of DNA/RNA Hybrid Strand: In this step, biotinylated anti-DNA/RNA mAbs are added, and they bind to the DNA/RNA hybrid strand. Note: This step is achieved with the **Exazym® Biotin Detection Kit**.
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

1. Conjugation of the oligo-dT Primer to the detector antibody of the standard ELISA and Initial Testing

To implement BOLD amplification to the standard pTau(181) sandwich ELISA, we conjugated the oligo-dT Primer to the unmodified detector anti-tau antibody of the standard ELISA. In Figure 2, we present results from an amplified ELISA using this anti pTau(181) oligo-dT Primer conjugate in comparison to the standard ELISA. As seen, a significant signal amplification was achieved in comparison to the standard pTau(181) ELISA. Consequently, we used this oligo-dT Primer conjugate for all subsequent experiments and tests presented in this poster.

Initial Comparison of Standard pTau(181) ELISA with Amplified ELISA

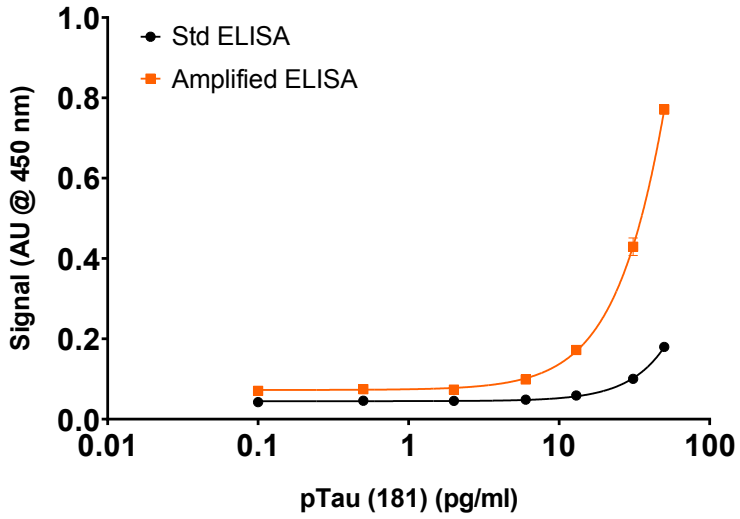

Figure 2 *Calibration curves for pTau(181) antigen concentrations ranging from 0 to 50 pg/mL obtained for the Standard ELISA (black trace) and the amplified ELISA (orange trace). In amplified ELISA a concentration of 150 ng/mL of oligo-dT Primer conjugate, 0.5 U/mL polymerase and 0.3 µg/mL of the biotinylated anti-DNA/RNA antibody were used. Detection was performed using Streptavidin-HRP and TMB substrate, with absorbance readings at 450 nm.*

2. Evaluation of the Effect of the Assay Concentration and Polymerization Time on the Performance of the Polymerase

The **Exazym® Polymerase Reaction Kit** includes polymerase which plays a crucial role in polymerising DNA/RNA hybrid strands. According to the provided Instruction for Use (IFU), the recommended polymerase concentration is 0.5 U/mL, with a polymerisation time of 10-30 minutes. In Figure 3A, we present the signal/background ratios (S/N ratios) obtained when using a polymerase concentration of 0.5 U/mL and of 2.5 U/mL. As seen in Figure 3A, 5 times increase in concentration did not improve the sensitivity of the amplified ELISA. Figure 3B presents S/N ratios when using 0.5 U/mL polymerase with polymerisation times of 20, 40 and 60 minutes. As depicted in Figure 3B, a polymerisation time between 20 to 60 minutes had low impact on the sensitivity or the signal of the amplified ELISA. For the final test of the BOLD amplified ELISA, with samples in plasma, we used 0.5 U/mL together with a polymerisation time of 30 minutes which is a polymerisation time within the tested range and suitable with respect to method execution considerations see Figure 6.

Impact of Polymerase Concentration and Time on the Amplified ELISA

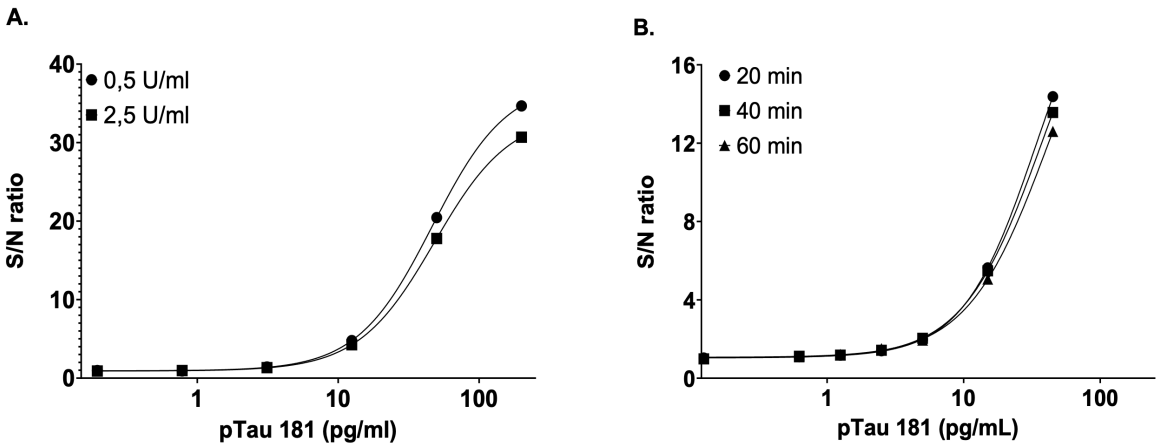

Figure 3

A. S/N ratios determined for the BOLD amplified pTau(181) ELISA when using different polymerase concentrations. The pTau(181) peptide dilutions spanned from 0 to 200 pg/ml.

B. S/N ratios determined for the BOLD amplified pTau(181) ELISA when using different polymerisation times. In both studies, 150 ng/mL of oligo-dT Primer conjugate and 0.3 µg/mL of biotinylated anti-DNA/RNA antibody were utilized. Streptavidin-HRP and TMB substrate were used for signal generation and absorbance was measured at 450 nm.

3. Evaluation of the Effect of Assay Concentration of the Biotinylated anti-DNA/RNA Antibody on the Amplified Signal

The biotinylated anti-DNA/RNA antibody, which is included in **Exazym® Biotin Detection Kit**, is used for detecting the DNA/RNA hybrid strands that have been polymerised by a polymerase. According to the IFU provided with the kit, a concentration of 0.3 µg/mL for the biotinylated anti-DNA/RNA antibody is a recommended starting point for detection. In this test we evaluated how different concentrations of the biotinylated anti-DNA/RNA antibody affect the absorbance signals. We combined it with varying the concentrations of the anti pTau(181) oligo-dT Primer conjugate. Figure 4 shows a plot of absorbance signals versus the concentration of the biotinylated anti-DNA/RNA antibody, using 9.38, 37.5 and 150 ng/mL of the oligo-dT Primer conjugate. As seen in Figure 4 all the curves reached a plateau when using an approximate concentration of 0.3 µg/mL of the biotinylated anti-DNA/RNA antibody. This is consistent with the concentration recommended in the IFU of the Exazym® Biotin Detection Kit, and a concentration of 0.3 µg/mL was selected together with 150 ng/mL of the oligo-dT conjugate when testing the amplified ELISA with pTau samples prepared in plasma, see Figures 5 and 6.

Impact of Biotinylated anti-DNA/RNA Antibody Concentration on the Amplified ELISA

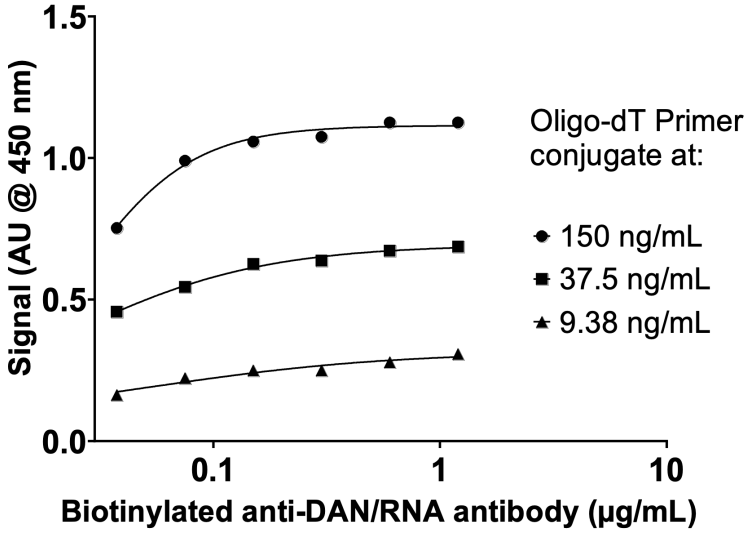

Figure 4 *Absorbance signals obtained when using Biotinylated anti-DNA/RNA antibody concentrations ranging from 0.037 to 1.2 pg/mL obtained with the amplified ELISA. The pTau(181) concentration was 31 pg/mL for all curves and the concentrations of oligo-dT Primer conjugate were 9.38, 37.5 and 150 ng/mL. Streptavidin-HRP and TMB substrate were used for signal generation and absorbance was measured at 450 nm.*

4. Detection of pTau(181) in Human Plasma

Based on the best-found conditions for the amplified ELISA reagents described above, including oligo-dT conjugate concentration, polymerase parameters, and biotinylated anti-DNA/RNA antibody concentration, we compared absorbance signals between the amplified ELISA and the standard ELISA, Figure 5. In the comparison we evaluated the two ELISAs with pTau(181) concentrations that ranged from 0 to 12 pg/mL. As seen in Figure 5, a significant signal amplification was observed for all tested pTau(181) concentrations for the amplified ELISA as compared to the standard ELISA. The limit of Detection (LoD) in amplified ELISA was determined to 0.08 pg/mL, while with standard ELISA, the LoD was determined to 5.2 pg/mL.

Additionally, we investigated the impact of human plasma on the performance of the amplified ELISA. Calibrators from 0.5 to 12 pg/mL were prepared using standard ELISA sample buffer alone and supplemented with 25% human plasma, Figure 6.

The results indicated that amplification was affected by plasma, resulting in a higher LOD of 2.4 pg/mL. However, the LOD determined in human plasma was still two times lower than the LOD in CSF declared by the supplier of the standard ELISA. LOD in these studies was calculated using the formula LOD (abs) = LOB + 1.645xSD Low conc. sample, and a sigmoidal 4PL curve fitting was used to convert the LOD signal to concentration unit.

pTAu(181) Antigen Calibration Curves for the Standard and Amplified ELISA

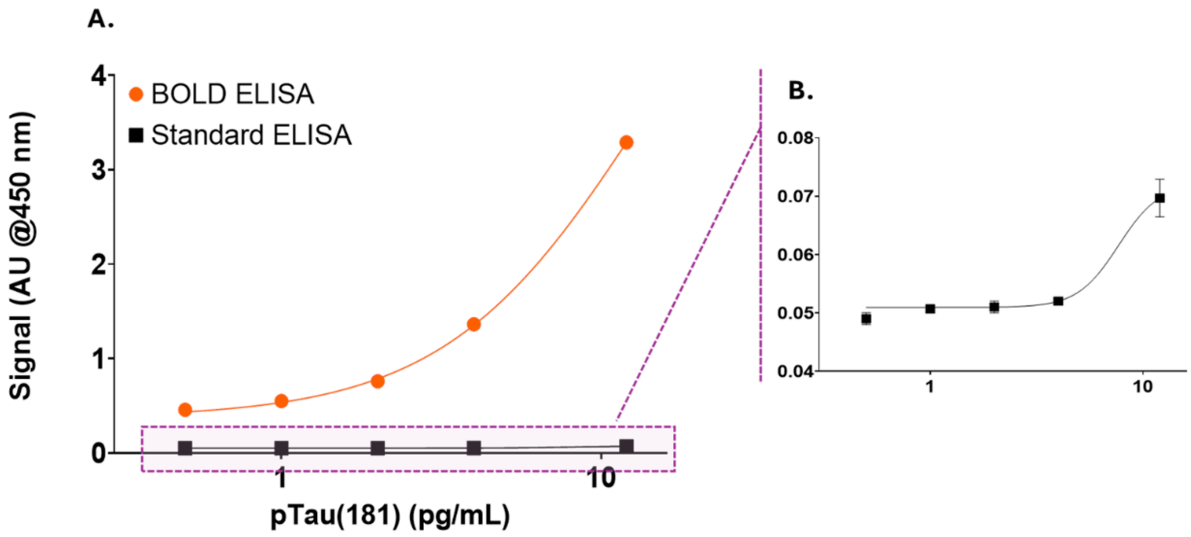

Figure 5

A. Calibration curves for pTau(181) antigen concentrations ranging from 0.5 to 12 pg/mL prepared in standard ELISA sample buffer. Black trace standard ELISA and orange trace amplified ELISA. In the amplified ELISA, 0.5 U/mL polymerase was used with a polymerisation time of 30 minutes, and 0.3 µg/mL of biotinylated anti-DNA/RNA antibody.

B. Zoom-in on calibration curve for pTau(181) obtained with the standard ELISA. Streptavidin-HRP and TMB substrate were used for signal generation and absorbance was measured at 450 nm.

Detection of pTau(181) in 25 % Human Plasma with Amplified ELISA

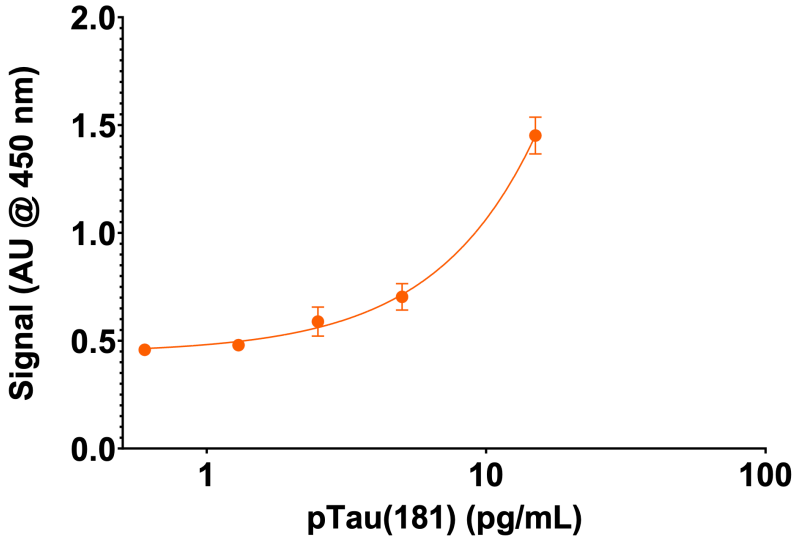

Figure 6 *Amplified ELISA calibration curve for pTau(181) antigen concentrations ranging from 0.5 to 12 pg/mL prepared in 25% human plasma using ELISA sample buffer. The ELISA used 150 ng/mL of the oligo-dT Primer conjugate, 0.5 U/mL polymerase with a polymerisation time of 30 minutes, and 0.3 µg/mL of biotinylated anti-DNA/RNA antibody. Streptavidin-HRP and TMB substrate were used for signal generation and absorbance was measured at 450 nm. Error bars show standard deviation, n=2.*

Conclusion

The prepared oligo-dT Primer conjugates successfully bridged the standard ELISA to the steps required for BOLD amplification. Note: we did not remove the unconjugated oligo-dT Primer (background signal) and unconjugated anti-pTau(181) antibody (competition). Consequently, the quality of both the conjugate and the test performance can be improved.

By exploring different assay parameters for the amplified ELISA we identified suitable conditions for an initial evaluation of the performance, i.e. LOD, for detecting pTau(181) in a standard ELISA buffer and in a 25 % plasma.

LOD of amplified ELISA in standard ELISA buffer: An LOD of 0.08 pg/mL was obtained for the amplified ELISA – approx. 60 times lower than the standard ELISA.

LOD of amplified ELISA in 25 % plasma: An LOD of 2.4 pg/mL was obtained for the amplified ELISA in plasma – more than 2 times lower than declared for the standard ELISA in CSF.

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-high 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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