



White Paper

Application of BOLD Technology in Assessing Biomarkers Within Neuroscience

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Application of BOLD Technology in Assessing Biomarkers Within Neuroscience

Biomarkers play a crucial role in understanding underlying mechanisms involved in a number of diseases, but also when setting a diagnosis of a given disease. To identify biomarkers and be able to with high precision and specificity assess biomarkers is today more or less mandatory already during the drug discovery process, while they during development are used to support outcome studies during clinical trials. In the area of neuroscience and neurodegenerative diseases, biomarkers are used to improve diagnostic precision in the clinic as well as to facilitate the development and monitoring of effective disease-modifying therapies.

Biomarkers aid in early diagnosis by identifying specific disease-related changes in for example protein aggregates and neuroinflammation even before clinical symptoms appear. They are also meant to allow tracking disease progression over time and provide insights into the underlying mechanisms. Examples of biomarkers of interest in diagnosis, disease progression and drug development include Amyloid- β and phosphorylated Tau in Alzheimer's disease, misfolded α -synuclein that forms Lewy bodies in Parkinson's disease and dementia with Lewy bodies, Neurofilament light (NfL) as a general marker of axonal degradation, neurogranin as a marker of postsynaptic degradation and for example SV2A, SNAP-25 and GAP-43 for presynaptic degradation. Taken together, biomarkers are essential tools for accurate diagnosis, disease monitoring, and advancing research and drug development in neurodegenerative diseases.

The identification and measuring of these biomarkers in neurodegenerative diseases is challenging due to several reasons. Relevant biomarkers are challenging to detect in blood due to the blood-brain-barrier (BBB), a protective structure that restricts the passage of large and/or charged molecules. Therefore, very few substances are able to cross the BBB, resulting in biomarkers from the brain or cerebrospinal fluid (CSF) often being present in blood in very low concentrations, making them difficult to identify and accurately quantify. Further, neurodegenerative diseases involve intricate processes, including proteins of various kinds in different states of aggregation and often modified by phosphorylation. Hence, identifying specific biomarkers related to these processes requires sophisticated techniques and very sensitive assays. Current imaging techniques (e.g., PET and MRI) provide valuable insights but have limitations in spatial resolution and sensitivity.

Ongoing research aims to refine diagnostic cut-offs and enhance sensitivity for these biomarkers with a constant search for innovative methods for biomarker detection, especially in the area of neuroscience. The vast majority of these methods, though, require costly automatic systems, specialized instrumentation, and software, as well as new protocols. This elevates costs and introduces logistical complexities that hinder adoption on a large scale. A new technology platform, BOLD, based on a molecular biology approach overcomes these barriers. It allows researchers to apply ultra-sensitive detection to virtually any immunodiagnostic assay and assay format.¹

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 has shown to increase sensitivity by up to 100x and the limit of detection by up to 50x.

To integrate BOLD into an 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 an explanation of steps 1-6 below. Note that no changes are needed regarding the original antibody pair in the ELISA.

¹ Biomarkers for neurodegenerative diseases, Nature Medicine volume 27, pages 954–963 (2021)

Illustration of BOLD implementation in a sandwich immunoassay

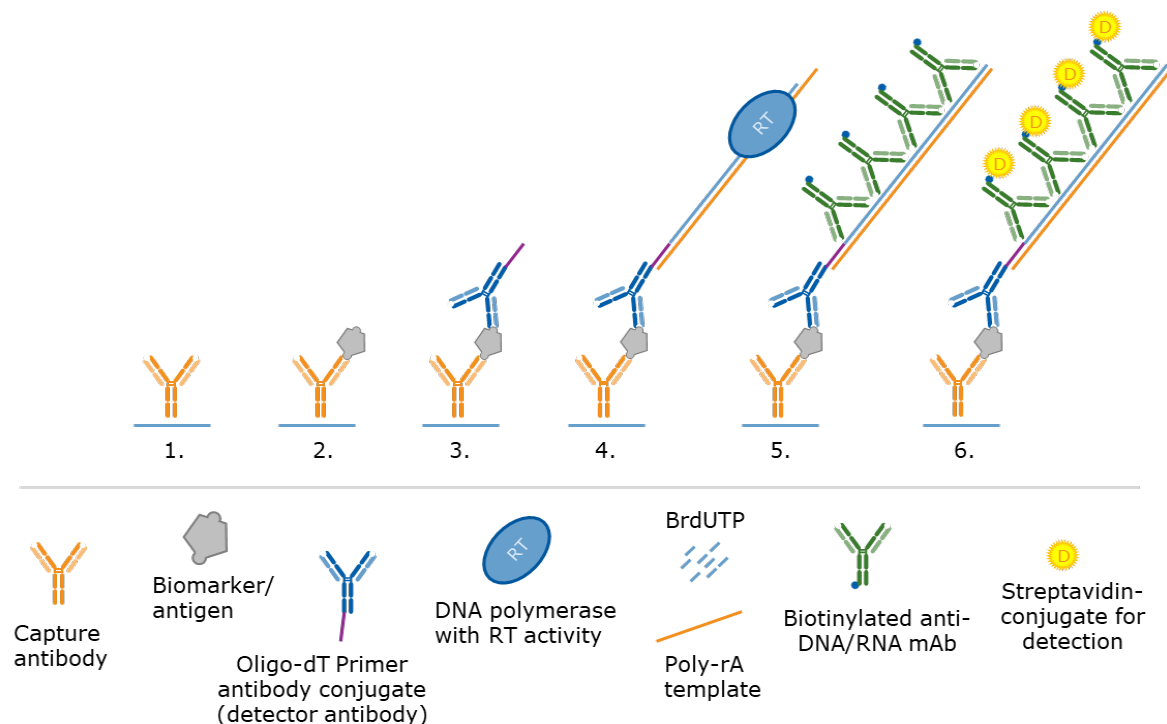


Figure 1. Schematic drawing of the steps in a BOLD amplified sandwich immunoassay.

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 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 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).

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

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 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. Early screening for AD 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 very low levels of biomarkers. Therefore, BOLD technology holds promise for facilitating the early detection and management of AD.

The below example describes the implementation of BOLD technology to a sandwich ELISA for quantitative in vitro determination of human pTau(181) in 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 polymerization 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.

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.

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

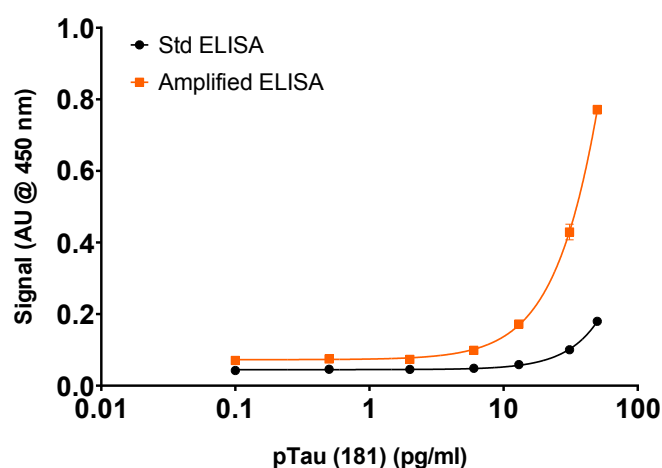


Figure 2. Calibration curves for pTau(181) antigen concentrations ranging from 0 to 50 pg/mL obtained for the Standard ELISA (black trace) and the amplified ELISA (orange trace). In amplified ELISA a concentration of 150 ng/mL of oligo-dT Primer conjugate, 0.5 U/mL polymerase and 0.3 µg/mL of the biotinylated 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 polymerizing DNA/RNA hybrid strands. The recommended polymerase concentration is 0.5 U/mL, with a polymerization 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 2.5 U/mL. As seen in Figure 3A, a five 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 polymerization times of 20, 40, and 60 minutes. As depicted in Figure 3B, a polymerization time between 20 to 60 minutes had a 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 polymerization time of 30 minutes which is a polymerization 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

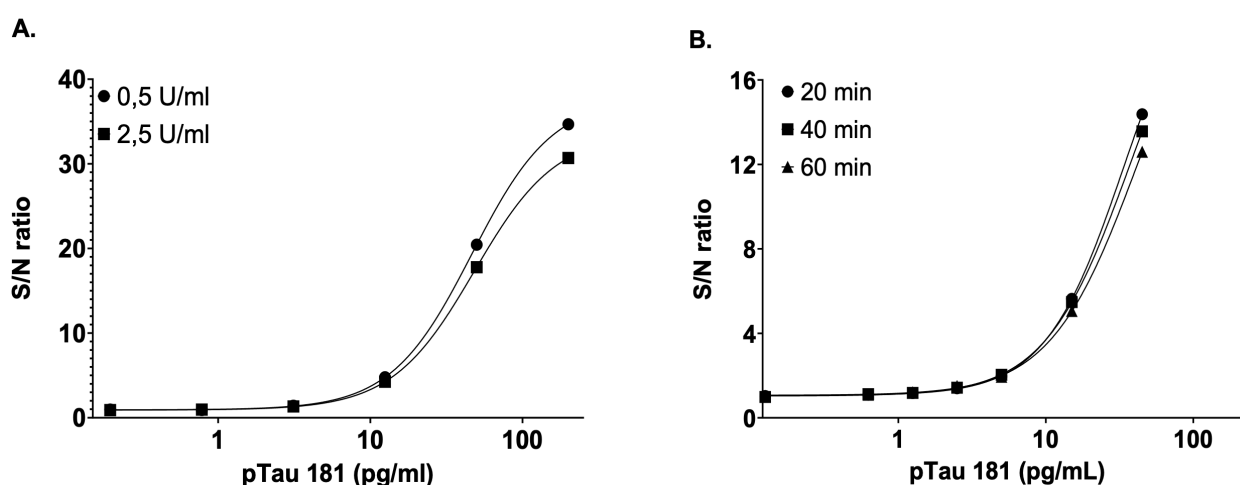


Figure 3.

- 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, included in Exazym® Biotin Detection Kit, is used for detecting the DNA/RNA hybrid strands that have been polymerized by a polymerase. 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 different concentrations of the biotinylated anti-DNA/RNA antibody. 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.

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

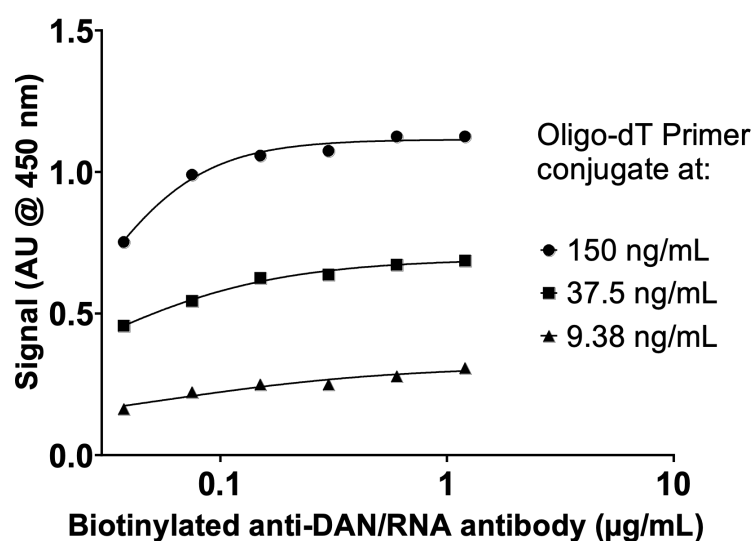


Figure 4. Absorbance signals obtained when using Biotinylated anti-DNA/RNA antibody concentrations ranging from 0.037 to 1.2 µg/mL obtained with the amplified ELISA. The pTau(181) concentration was 31 pg/mL for all curves and the concentrations of oligo-dT Primer conjugate were 9.38, 37.5 and 150 ng/mL. 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. In the comparison, we evaluated the two ELISAs with concentrations of pTau(181) ranging from 0 to 12 pg/mL.

As seen in Figure 5, a significant signal amplification was observed for all tested concentrations of pTau(181) for the amplified ELISA as compared to the standard ELISA. The Limit of Detection (LoD) in amplified ELISA was determined to be 0.08 pg/mL, while with standard ELISA, the LoD was determined to be 5.2 pg/mL.

Finally, we investigated the impact of human plasma on the performance of the BOLD ELISA. Calibrators from 0.5 to 12 pg/mL were prepared using standard ELISA sample buffer alone and supplemented with 25% human plasma. 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 $LoD (abs) = LOB + 1.645 \times SD$ 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

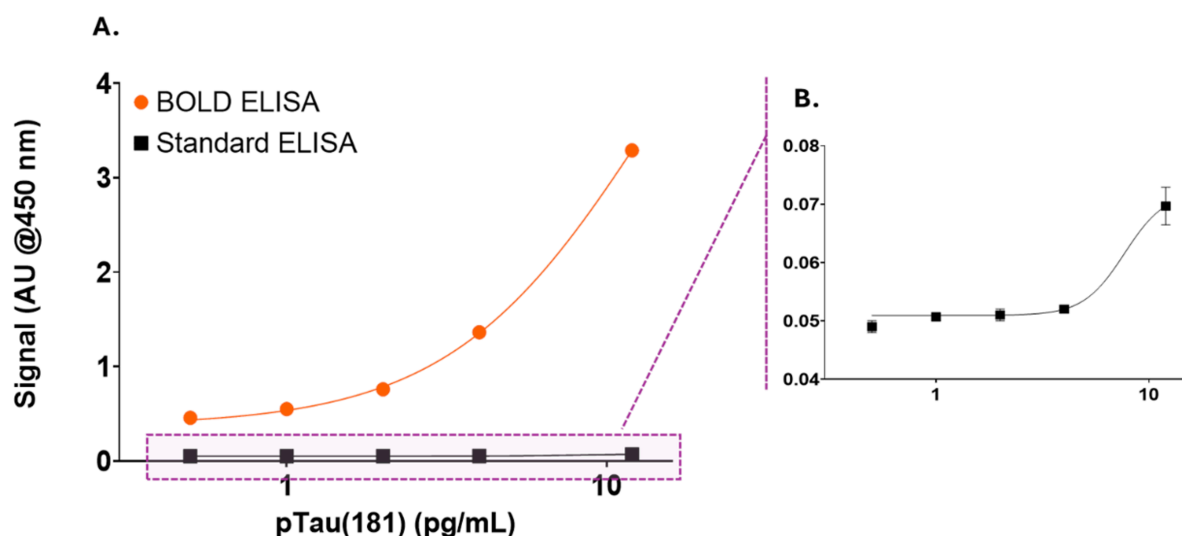


Figure 5.

- 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 μ g/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

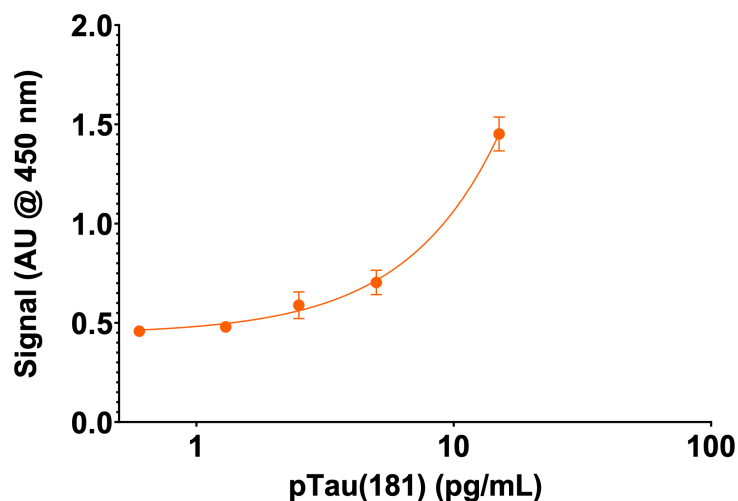


Figure 6. Amplified ELISA calibration curve for pTau(181) antigen concentrations ranging from 0.5 to 12 pg/mL prepared in 25% human plasma using ELISA sample buffer. The ELISA used 150 ng/mL of the oligo-dT Primer conjugate, 0.5 U/mL polymerase with a polymerisation time of 30 minutes, and 0.3 μ g/mL 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

Studying biomarkers in Alzheimer's disease (AD) is crucial to allow early diagnosis and thereby intervention, ideally even before clinical symptoms appear. A major challenge is the ability to reliably and accurately determine biomarker levels. Applying BOLD technology, as described above, provides a simple and cost-effective approach to significantly increase the ability to detect relevant biomarkers. 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. The prepared oligo-dT Primer conjugates successfully bridged the standard ELISA to the steps required for BOLD amplification. Note that 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 greatly improved. Yet, in the above example in applying BOLD to a standard ELISA, we obtained an approximately 60-fold lowering of LoD, as compared to the original ELISA.



About Exazym®

Exazym® is an add-on immunoassay reagent kit that provides attomole-level 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 Cavid

Cavid 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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