



White Paper

Application of Exazym® and BOLD Amplification in Immunoassays

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Exazym® Enables Attomole-Level Detection of Low-Abundance Biomarkers Using Standard ELISA Workflows.

Cavidi has launched the Exazym® signal amplification technology to bring ultra-sensitive detection levels to conventional immunodiagnostic assays. Intended applications for this technology include translational research, health screens, and diagnostics testing.

The BOLD Science Behind Exazym®

Measurements of low-abundance biomolecules, including proteins and nucleic acids, remain a critical challenge in many clinical and diagnostic applications due to insufficient sensitivity. While some clinical diagnostic measurement methods have made significant advances in sensitivity, there are still many potential disease biomarkers that exist in accessible biofluids at levels below the detection limits of these techniques or where increased precision would be desirable. Furthermore, in a majority of cases, they require specialized instruments, greatly increasing the cost and logistical complexity of large-scale adoption.

The aim of the BOLD technology is to provide greater access to high-sensitivity assays. We have accomplished this with a novel approach that uses conventional assay instrumentation found in most labs. Tests have shown that this approach is capable of achieving levels of sensitivity that exceed those produced by expensive closed systems while maintaining selectivity.

A schematic drawing of the principles underlying the BOLD technology is shown in Figure 1.

In short, in a standard ELISA the detector antibody carries a signal-generating enzyme or has a molecular tag that allows it to bind such a signal-generating moiety, e.g., a biotin tag that allows attachment of a streptavidine-enzyme conjugate. The BOLD technology expands the number of signal-generating enzyme molecules that the detector antibody will carry. Instead of a few enzyme molecules, a “ladder” containing a stretch of many antigenic sites is added to the detector antibody by the Exazym® process. The “ladder” is generated by conjugating a short DNA primer to the detector antibody, which after addition of a RNA template allows a polymerase with reverse transcriptase activity to generate a DNA strand. The DNA strand of the DNA/RNA hybrid strand enables a multitude of tertiary signaling antibodies to bind. The net result is a significant increase in the number of signaling antibodies per detector antibody, which ultimately increases the signal yield from each bound analyte molecule, a signal amplification technology called Binding Oligo Ladder Detection (BOLD). See Figure 1 for details.

Binding Oligo Ladder Detection (BOLD)

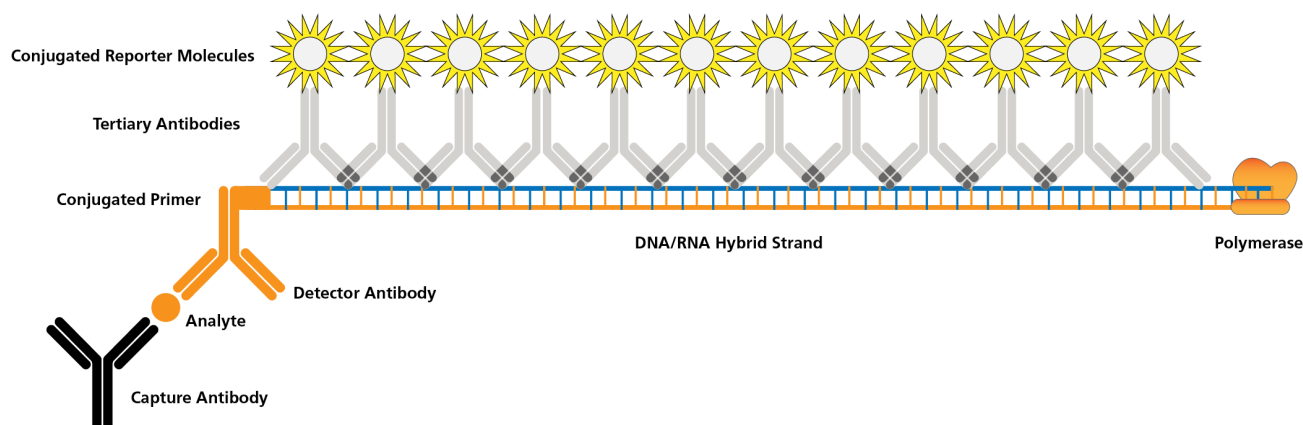


Figure 1. Schematic illustration of BOLD technology and the principle of BOLD amplification.

Application Examples

To illustrate the ability of the BOLD technology to enhance the performance of a standard ELISA, we measured human anti-spike IgG antibodies, a biomarker for post SARS-CoV-2 infection or for immunization with one of the current vaccines, in a patient serum sample. For these experiments (figure 2 and 3), the SARS-CoV-2 spike protein (S-antigen) was attached to the bottom of the plate (indirect ELISA), followed by the addition of human samples containing anti-SARS-CoV-2 spike antibodies in a serial dilution, with and without applying BOLD technology. As seen in Figure 2, by applying BOLD amplification to the ELISA, the positive signal was higher at all dilutions and still positive when the standard ELISA signal was at background level.

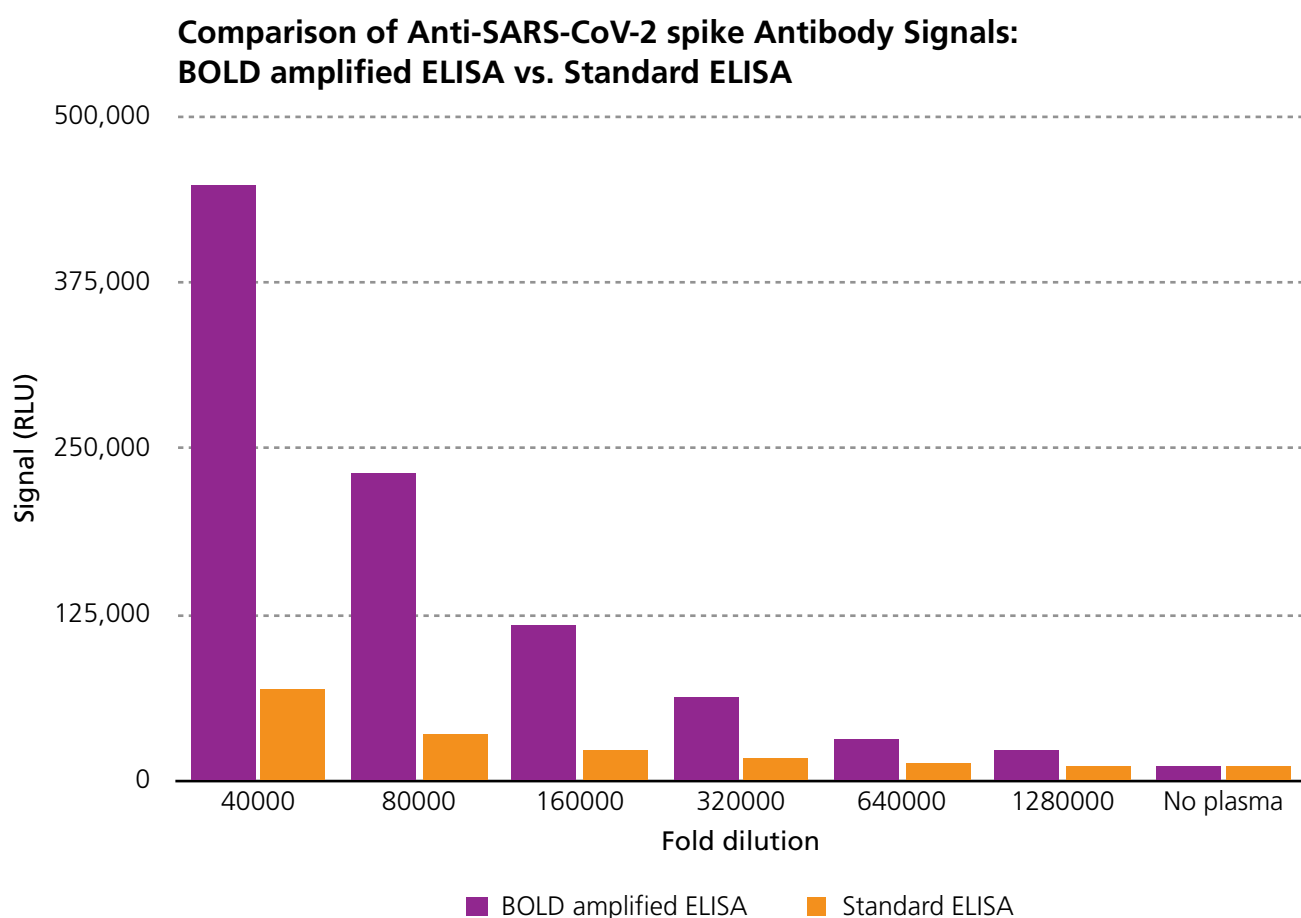


Figure 2. BOLD amplified and standard ELISA detection of anti-SARS-CoV-2 spike protein antibodies in patient sera. Orange bars represent standard ELISA, and purple bars represent BOLD amplified ELISA. RLU denotes Relative Light Units.

To further assess the applicability of BOLD amplification, we prepared an amplified ELISA where the signals were calibrated against the first WHO International standard for anti-SARS-CoV-2 spike immunoglobulin. The antibody titers can then be expressed as binding antibody units (BAU)/ml instead of a dilution factor. see Figure 3. Again, the signal from a positive sample was clearly distinct from that of a negative sample, even when diluted down to a few μ BAU/ml.

Comparison of Anti-SARS-CoV-2 Spike Antibodies in Positive and Negative Serum Samples Using BOLD Amplified ELISA

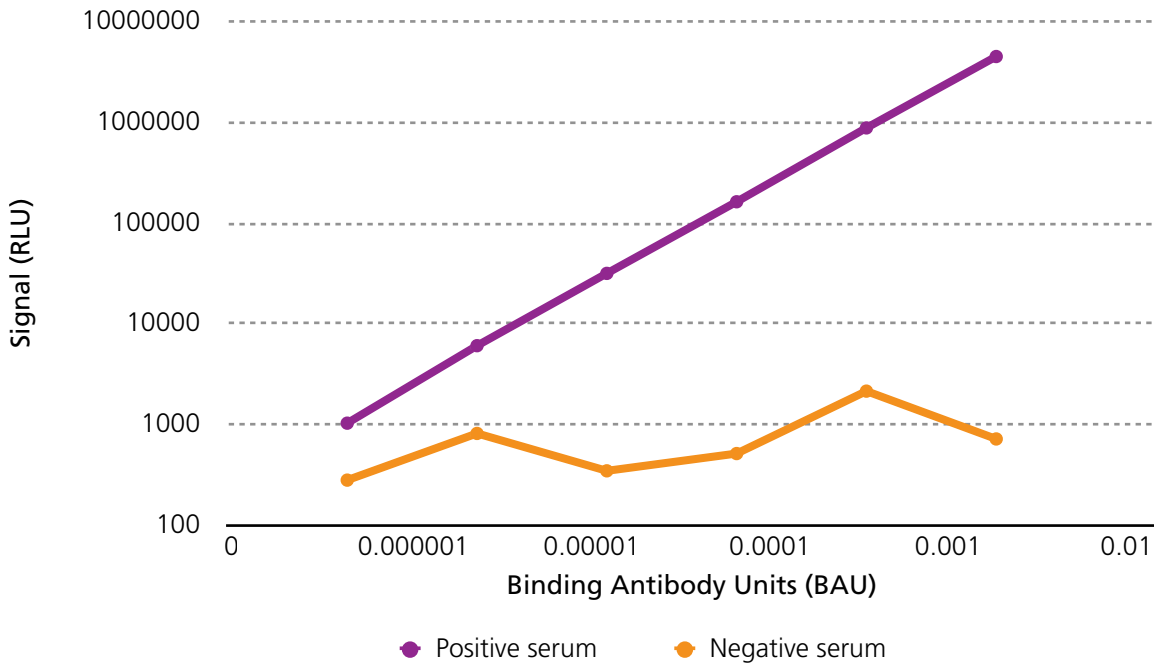


Figure 3. Quantification of anti-SARS-CoV-2 spike protein antibody titers assayed with BOLD amplified ELISA. Purple line: Anti- SARS-CoV-2 spike protein positive serum, Orange line: Anti- SARS-CoV-2 spike protein negative serum. RLU denotes Relative Light Units.

To further explore the ability of the BOLD technology to amplify the signals for different ELISA types, we next established a sandwich ELISA for the measurement of the SARS-CoV-2 spike protein, S-antigen. In the first experimental setup, we diluted the S-antigen in six fourfold steps from 100 to 0 ng/ml. As illustrated in Figure 4, using the sandwich ELISA approach, a linear increase in chemiluminescence was achieved, yielding a maximum of about $1,5 \cdot 10^6$ relative light units (RLU) at 100 ng/ml antigen concentration. However, when employing BOLD amplification to the same sandwich ELISA, a significant increase in signal led to saturation of the luminescence reader already at 25 ng/ml, the saturation level illustrated by the dotted line in Figure 4.

S-antigen Signal Comparison: BOLD Amplified vs. Standard Sandwich ELISA

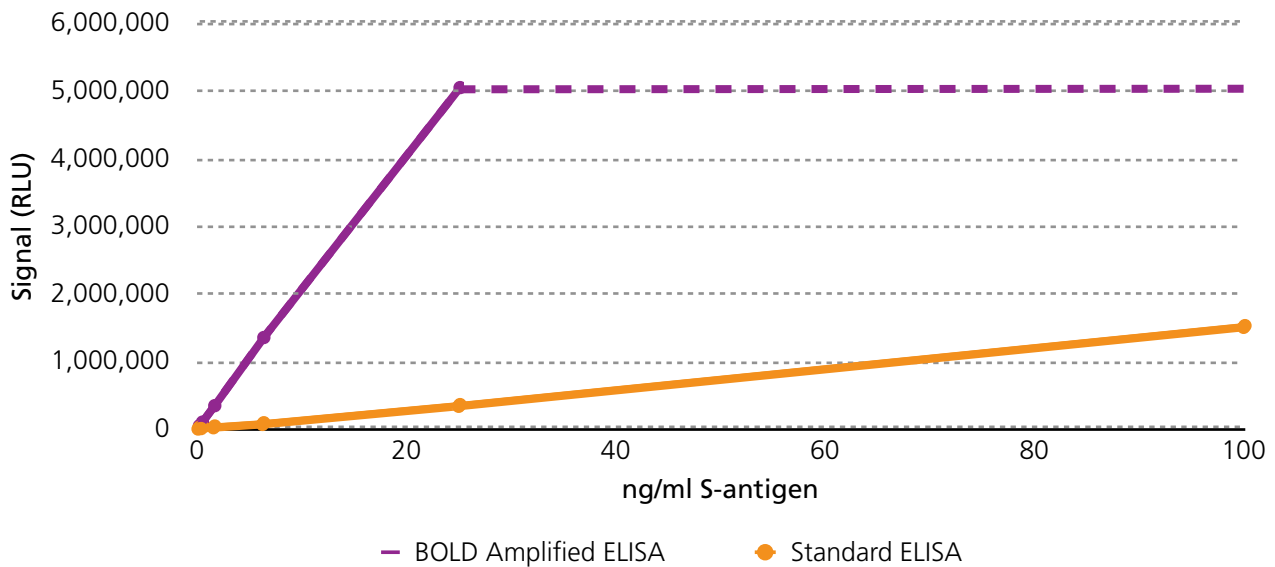


Figure 4. Comparison of chemiluminescence signal generated in a sandwich ELISA with and without BOLD amplification. Orange line: Standard ELISA. Purple line: BOLD amplified ELISA. The purple dotted line indicates the level at which the luminescence reader has been saturated.

To explore the level of signal amplification at lower concentrations of the S-antigen, i.e., within the dynamic range of the reader, a separate experiment was performed using dilutions of the spike protein from 0 to 10 ng/ml. The signals in relation to the Limit of Blank (LoB) at these lower concentrations of the spike protein are illustrated in Figure 5.

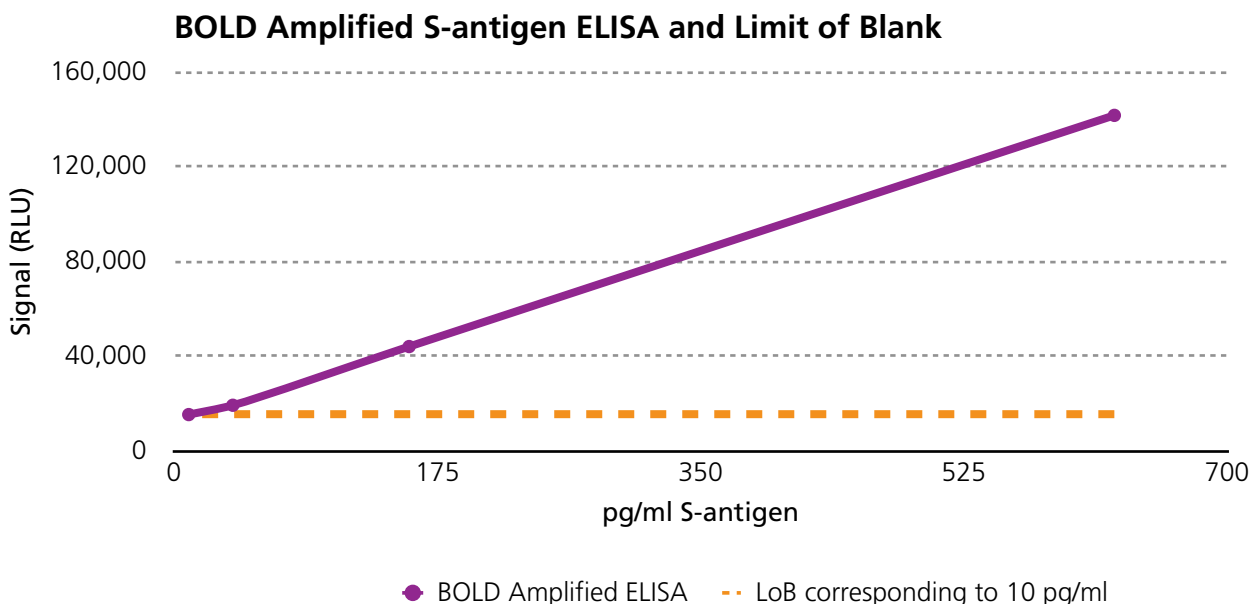


Figure 5. Purple line: Chemiluminescence signals for a BOLD amplified S-antigen ELISA tested at concentrations from 0 to 10 ng/ml (data shown for 10-625 pg/ml).

To compare the standard ELISA with the BOLD amplified ELISA, we evaluated the data where both ELISA tests gave signals in the linear range. By normalizing the standard ELISA signal to 1, we found that using BOLD amplification resulted in an increase in signal by approximately 15 times (see Figure 6).

Signal Amplification with BOLD

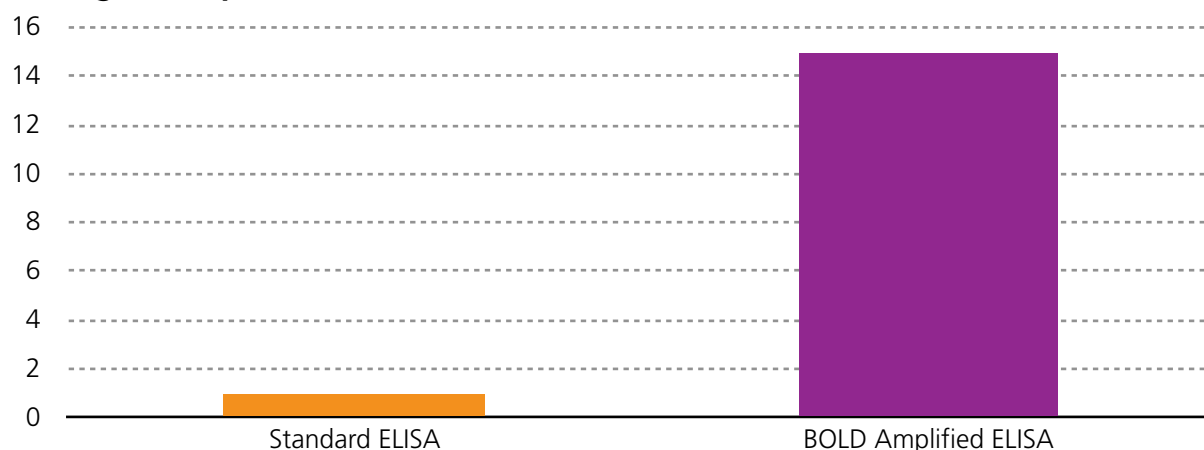


Figure 6. Illustration of signal amplification achieved using BOLD technology. The comparison was made by setting the signal level from the standard S-antigen ELISA to 1. Employing BOLD amplification resulted in an approximately 15-fold increase in chemiluminescence signal.

While the true signal was significantly increased by approximately 15 times, the determination of the amount of antigen is highly dependent on the level of background signal. To assess resolution at low levels of antigen (< 10ng/ml), we calculated the Level of Blank (LoB) defined as average background + 1,645 * SD. As seen in Figure 5, the BOLD signal is higher than the LoB down to an antigen concentration corresponding to approximately 10 pg/ml.

Comparison of ELISA Resolution: Standard vs. BOLD Amplified

	LoB (RLU)	LoB (ng/ml)
BOLD	12626	0.027
ELISA	5400	0.29
ELISA/BOLD		10.7

Table 1. Comparison of the level of resolution in a standard ELISA vs. ELISA with BOLD amplification was done by assessing the limit of blank (LoB). LoB was calculated as background average +1,645*SD and demonstrates that BOLD amplification in these sets of experiments resulted in an approximately 10-fold increase in assay sensitivity.

Discussion

The data shows that by applying BOLD technology, a significant increase in assay performance can be achieved. It also indicates the opportunity to upgrade an existing standard ELISA to get a highly meaningful improvement in sensitivity without the high cost of investing in new dedicated instrumentation. Alternatively, the BOLD technology could be adapted to fit in an existing immunoassay IVD instrument. The illustrations in the present examples are all based on ELISA setups, however, Exazym® would be equally applicable to any immunoassay-based technology, as for example, applications in immunohistochemistry or any approach where the readout is linked to interactions with antibodies. It should, however, also be noted that the BOLD technology does not come in a box with fixed performance properties. Rather, it can be adapted to the properties of a pre-existing assay and its unique operational constraints. With this flexibility, the BOLD technology should be suited for applications in a broad range of therapeutic areas, including oncology, neurology, cardiology, inflammation, and infectious disease.

We are presently investigating various aspects of the BOLD technology components and procedure, exploring ways to further improve signal generation during BOLD amplification, while simultaneously maintaining a low background signal. To further amplify the signal generated by BOLD, either increasing the time of the polymerase incubation or increasing the concentration of the polymerase may be used. Further, there is a choice of polymerases that will allow BOLD amplification at room temperature instead of the standard 37°C enzyme reaction temperature. In the present setup, applying BOLD to a given capture-detector ELISA assay is done by conjugating a DNA primer (oligo-dT Primer) to the unmodified detector of the standard assay. This conjugation serves as a crucial bridge for the addition of the BOLD amplifications steps to the standard ELISA.

Another aspect of the amplified signal-generating capacity is that it can be used to work with more diluted samples, thus decreasing matrix effects. Back-calculated to the undiluted samples, sensitivity may be the same as in the original standard assay, but the value will be more reliable. It may also be possible to reduce the amount of capture and detector monoclonals used in the assay, retaining the sensitivity while reducing reagent costs.

Cavidi is seeking strategic partners for Exazym® in in vitro diagnostics. If you are interested in further discussions, please contact us at <https://cavidi.se/contact>



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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