Signal Amplification of a Human Cardiac Troponin I Sandwich ELISA

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The BOLD Immunoassay Signal Amplifier

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INRODUCTION

Cardiac troponin I (cTnI) is a 24 kDa protein in the troponin complex that regulates cardiac muscle contraction by inhibiting actin–myosin interaction in the absence of calcium [1]. It contains a unique N-terminal extension that distinguishes it from skeletal isoforms [2], see Figure 3.

cTnI is exclusively expressed in cardiac muscle, making it a highly specific biomarker for myocardial infarction (MI). It is released into the bloodstream upon cardiac injury and can be detected within hours [3]. Normal circulating levels are typically below 20 pg/mL in men and 15 pg/mL in women [4], though baseline levels may vary with age, sex, and renal function [5]. A two- to three-fold increase above these baseline levels may indicate early myocardial injury and should be considered for further clinical evaluation.

While skeletal troponin I (skTnI) can cause cross-reactivity, modern assays use highly specific antibodies to minimize this issue [6]. Ultra-sensitive cTnI assays are essential for early MI diagnosis and may also enable detection of subclinical cardiac conditions in healthy individuals [7].

OBJECTIVE

To develop a standard ELISA and a high-sensitivity ELISA using BOLD signal amplification for cardiac troponin I (cTnI). The assays were optimized through systematic evaluation of key parameters and final performance was assessed by evaluating the limit of detection (LoD) in serum.

The following steps summarize the key stages and critical steps of this development process:

1. **Selection of Capture and Detector Antibodies** – Evaluated various capture antibodies and dual-capture setups for optimal binding. Identified detector antibodies with strong signal and low background.
2. **Downstream Assay Optimization** – Fine-tuned polymerase activity, buffer composition, and incubation conditions to maximize amplification.
3. **LoD Evaluation in Serum** – Assessed assay sensitivity in serum using the fully-optimized system.

BOLD TECHNOLOGY OVERVIEW

Cavidi has developed BOLD (Binding Oligo Ladder Detection), a molecular biology-based signal amplification technology that enables ultra-sensitive detection in virtually any immunodiagnostic assay. BOLD can be integrated into existing immunoassays to improve sensitivity and lower the limit of detection by over 100-fold.

To implement BOLD, an oligo-dT primer is conjugated to an unmodified detector antibody of the standard assay using click chemistry, see Figure 1. This conjugate acts as a bridge, enabling the integration of BOLD amplification steps into standard immunoassays, see Figure 2 for steps 1–6.

Illustration of Two Different Processes (A and B) for Conjugating an Oligo-dT primer to a Detector Antibody Using Click Chemistry

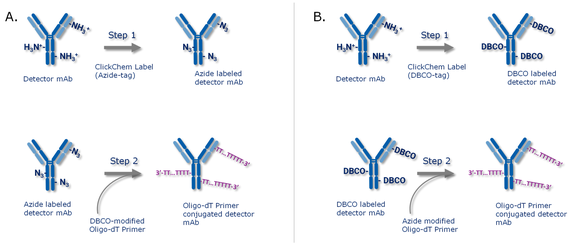


Figure 1. *Two-step processes (A and B) for conjugating an oligo-dT primer to a detector antibody using click chemistry. A: Standard chemistry (Exazym®): The detector monoclonal antibody (mAb) is labeled with an azide tag and then conjugated with a DBCO-modified oligo-dT primer. B: Alternative chemistry: Similar to A, but the mAb is labeled with a DBCO tag and then conjugated with an azide-modified oligo-dT primer.*

Steps of a BOLD Amplified Sandwich Immunoassay

1. **Capture Antibody Coating** – Immobilize capture antibody on the assay surface.
2. **Antigen Binding** – Add sample; antigen binds to capture antibody.
3. **Detector Binding** – Apply oligo-dT primer-conjugated detector antibody.
4. **Hybrid Strand Generation** – Add poly-rA template, polymerase with reversed transcriptase activity, and BrdUTP to synthesize DNA/RNA hybrid strand.
5. **Hybrid Detection** – Bind biotinylated anti-DNA/RNA antibody to the hybrid strand.
6. **Signal Generation** – Add streptavidin conjugated to a reporter molecule or enzyme (ELISA) that enables the desired detection modality (e.g., colorimetric, fluorescence, or luminescence).

BOLD TECHNOLOGY OVERVIEW

Illustration of BOLD Implementation in a Sandwich Immunoassay

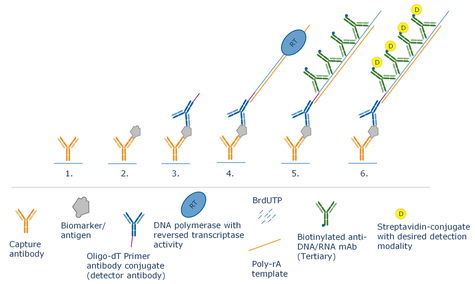


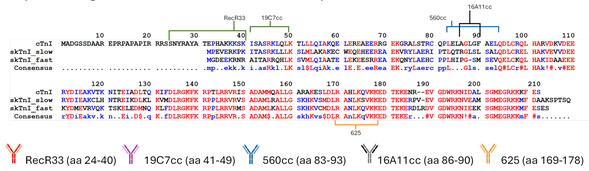
Figure 2. *Schematic drawing of the steps in an amplified sandwich immunoassay.*

METHODS AND RESULTS

1. Selection of Capture and Detector Antibodies

The first step in setting up both the standard and amplified ELISA was to identify suitable antibody pairs for cTnI. Figure 3 presents the amino acid sequence alignment of cardiac Troponin I (cTnI) with the slow and fast skeletal variants (skTnI). The mAbs directed against regions with low sequence similarity between cTnI and the skeletal variants are highlighted in blue, see Figure 3. The mAbs (Hytest), clones noted in Figure 3, were selected to minimize cross-reactivity with the skeletal Troponin I sequences, ensuring specificity for cTnI.

Sequence Alignment of Cardiac and Skeletal Troponin I



RecR33 (aa 24-40) 19C7cc (aa 41-49) 560cc (aa 83-93) 16A11cc (aa 86-90) 625 (aa 169-178)

Figure 3. *Sequence alignment of cardiac Troponin I (cTnI) with the slow and fast skeletal variants (skTnI). The alignment highlights regions with low sequence similarities. Monoclonal cTnI specific antibodies (625, 19C7cc, 560cc, and 16A11cc) are indicated at these regions, demonstrating their binding sites on cTnI.*

Evaluation of Capture antibodies for Standard and Amplified ELISAs

These anti-cTnI capture antibodies were evaluated for use in both standard and amplified ELISA. The mAbs tested included clones 19C7cc, 625, and RecR33. These were assessed individually and in pairs in both ELISA formats.

In the studies, a biotinylated detector antibody (clone 16A11cc) at a concentration of 2.5 µg/mL was used for all standard ELISA experiments. For the amplified ELISA, an oligo-dT primer-conjugated detector antibody (clone 560cc) at a concentration of 1 µg/mL was used. The oligo-dT primer-conjugated detector antibody was prepared according to process B (Figure 1), using a molar excess of the DBCO-tag (20:1) relative to the antibody, and a molar excess of 5:1 for the azide-modified oligo-dT primer relative to the DBCO-labelled antibody (20:5).

Evaluation of Capture Antibodies for Standard and Amplified ELISAs

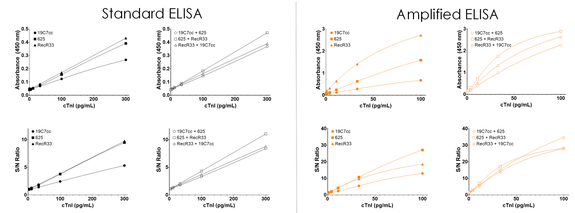


Figure 4. Top row: *calibration curves derived using difference capture mAbs for cTnI concentrations ranging from 0 to 300 pg/mL for the standard ELISA (left Panel) and from 0 to 100 pg/mL for the amplified ELISA (right panel). Experiments performed at room temperature, (22-25 °C).* ***Bottom row:*** *corresponding Signal to noise (S/N) ratios determined for cTnI concentrations of the respective calibration curves. Streptavidin-HRP and TMB substrate were used for signal generation and absorbance was measured at 450 nm.*

METHODS AND RESULTS

Evaluation of Capture Antibodies for Standard and Amplified ELISAs

As seen in Figure 4, all antibodies were able to capture cTnI concentration dependently and in general the use of two capture antibodies resulted in higher signals as well as S/N ratios. In the standard and amplified ELISA RecR33 showed good performance, both individually and in combination with the other two capture antibodies. However, RecR33 also contributed to high background levels after optimizing downstream layers of standard and amplified ELISA assays. Consequently, the combination of 19C7cc and 625 was selected as capture mAbs, as they provided signal levels and overall performance comparable to those of RecR33 and 625, while minimizing background interference. The combination of 19C7cc and 625 at a concentration of 0.5 µg/mL was chosen for both standard and amplified ELISAs, which enhanced the performance by reducing background and improving signal, especially after downstream layers and processes of the assay were fine-tuned.

Evaluation of Background Signals for Standard and Amplified ELISA

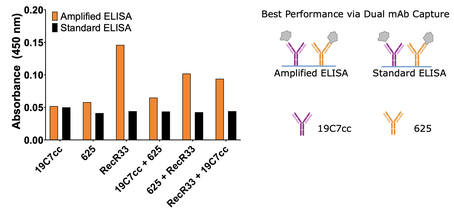


Figure 5. *Background signals obtained for standard and amplified ELISA using capture antibodies 19C7cc, 625, and RecR33.*

Evaluation of Detector Antibodies for Standard and Amplified ELISAs

The next step involved identifying detector mAbs with the highest specificity and lowest background signal within both standard and amplified ELISAs. For the standard ELISA biotinylated detector antibodies were tested, see Figure 6. To integrate BOLD technology into a standard ELISA, an oligo-dT primer was conjugated to an unmodified anti-cTnI detector antibody using click chemistry as described Figure 1. Both processes were tested, as well as different amino reactive tags for the modification of the antibody were evaluated. Best performing oligo-dT Primer conjugates were obtained when using process B in Figure 1 using a molar excess of the DBCO-tag (20:1) relative to the antibody, and a molar excess of 5:1 for the azide-modified oligo-dT primer relative to the DBCO-labelled antibody (20:5), see Figure 6.

Evaluation of Signal-to-Noise Ratios for Standard and Amplified ELISA

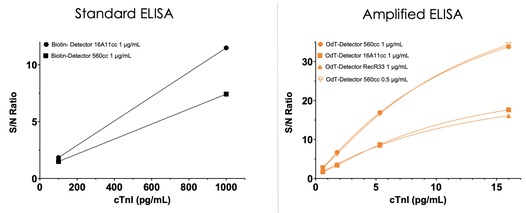


Figure 6. *Relationship between cTnI concentrations and Signal-to-Noise ratio using different detector antibody conjugates for standard and amplified ELISA. Experiments performed at room temperature. Detector antibodies were evaluated using cTnI antigens captured on a surface prepared with both 19C7cc and 625 capture antibodies.*

Best performing detector antibody for the standard ELISA was 16A11cc at a concentration of 1 µg/mL and for the amplified ELISA, best performing detector was 560cc at a concentration of 0.5 µg/mL.

2. Downstream Assay Optimization

Additional parameters critical to the amplified ELISA were evaluated, including the oligo-dT primer-conjugated detector antibody in combination with polymerase parameters (concentration and time) and the concentration of the biotinylated anti-DNA/RNA antibody. Using the optimal conditions identified for both standard and amplified ELISA, we compared absorbance signals from their cTnI calibration curves.

Figure 7 shows calibration curves for cTnI antigen detection using the standard ELISA (black trace) and the amplified ELISA (orange trace). For both standard and amplified ELISA, 0.5 µg/mL each of capture mAbs 19C7cc and 625 were used. As detectors, 0.5 µg/mL oligo-dT primer conjugate (560cc) was used for the amplified ELISA and 2.5 µg/mL of biotinylated 16A11cc were used for the standard ELISA. For the amplified ELISA 1.3 U/mL polymerase, and 0.3 µg/mL biotinylated anti-DNA/RNA antibody (tertiary antibody) were used. Detection in both ELISAs was achieved via Streptavidin-HRP and TMB substrate, with absorbance measured at 450 nm.

Antigen Calibration Curves for the Standard and Amplified ELISAs in Buffer

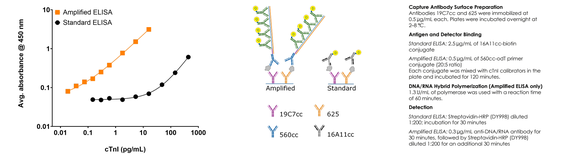


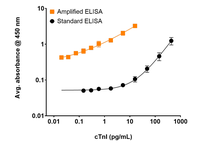
Figure 7. Left panel: *Calibration curves for cTnI antigen in a PBS-T buffer, ranging from 0 to 432 pg/mL for the standard ELISA and 0 to 16 pg/mL for the amplified ELISA. Experiments were conducted at room temperature. Curves represent the best fit using a four-parameter logistic (4PL) model. Data are shown as the mean of four replicates (n = 4).* ***Middle panel:*** *Schematic overview of the ELISA setups.* ***Right panel:*** *Summary of key assay parameters used in the ELISA protocols.*

3. LoD Evaluation in Serum

Using the optimized parameters identified during downstream development, we compared absorbance signals obtained from the standard and amplified ELISAs using cTnI antigen prepared in 25% cTnI-free serum. Figure 8 presents the resulting calibration curves: the standard ELISA is shown in black, and the amplified ELISA in orange. Experiments were conducted at room temperature.

To assess compatibility with automated systems, the amplified cTnI ELISA was tested at 37° C using a polymerase optimized for that temperature, see Figure 9.

Antigen Calibration Curves for the Standard and Amplified ELISAs in Serum



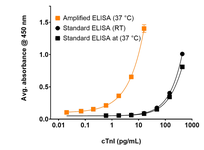
LoD when Signal/background ratio = 2

LoD in standard ELISA = 12.5 pg/mL
LoD in amplified ELISA = 0.07 pg/mL

=> improvement ≈ 180-fold

Figure 8. *Calibration curves for cTnI antigen in 25 % cTnI free serum at concentrations ranging from 0 to 432 pg/mL (standard ELISA) and from 0 to 16 pg/mL (amplified ELISA). Experiments performed at room temperature. Data represent the mean calibrator signals from three independent analytical runs.*

Antigen Calibration Curves for the Standard and Amplified ELISAs at 37°C



LoD when Signal/background ratio = 2

LoD in amplified ELISA = 0.46 pg/mL
LoD in standard ELISA = 22.7 pg/mL
LoD in standard ELISA (RT) = 17.9 pg/mL

=> improvement (37 °C) ≈ 50-fold

Figure 9. Left panel: *Calibration curves for cTnI antigen in PBS-T buffer (n=3) at concentrations ranging from 0 to 432 pg/mL (standard ELISA) and from 0 to 16 pg/mL (amplified ELISA). Experiments performed at 37 °C.* ***Right panel:*** *sensitivity comparison between the standard and amplified ELISA.*

CONCLUSIONS

- At room temperature (22-25 °C) in serum, the BOLD-amplified cTnI ELISA showed up to a 180-fold increase in sensitivity and a broad dynamic range compared to standard ELISA.
- Initial tests at 37° C, typical for some automated systems, suggested that a substantial signal amplification can be expected, with sensitivity improved up to 50-fold.
- Testing different antibody combinations for both capture and detection is a critical step in developing high-performance ELISAs.
- Systematic optimization of assay parameters, such as reagent concentrations, incubation times, and enzyme activity, was essential for maximizing performance.
- Simultaneous use of two different capture antibodies significantly enhanced assay sensitivity and overall performance. This dual-capture strategy may be applicable to the detection of other biomarkers, not just cTnI.

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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® product range.