



White Paper

Extending the Lower Limit of Detection in Commercial ELISA Kits Without Assay Redesign

A Streptavidin-Based Primer Approach

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Enabling Seamless BOLD Amplification in Standard Immunoassays Using Exazym® StreptaClick® Primer

Many immunoassay platforms use biotinylated detector antibodies together with streptavidin conjugated to reporter molecules or enzymes for detection. StreptaClick® Primer exploits this widely used assay format by binding directly to the biotin on the detector antibody, enabling seamless incorporation of signal amplification via Binding Oligo Ladder Detection technology (BOLD) without altering the original assay design.

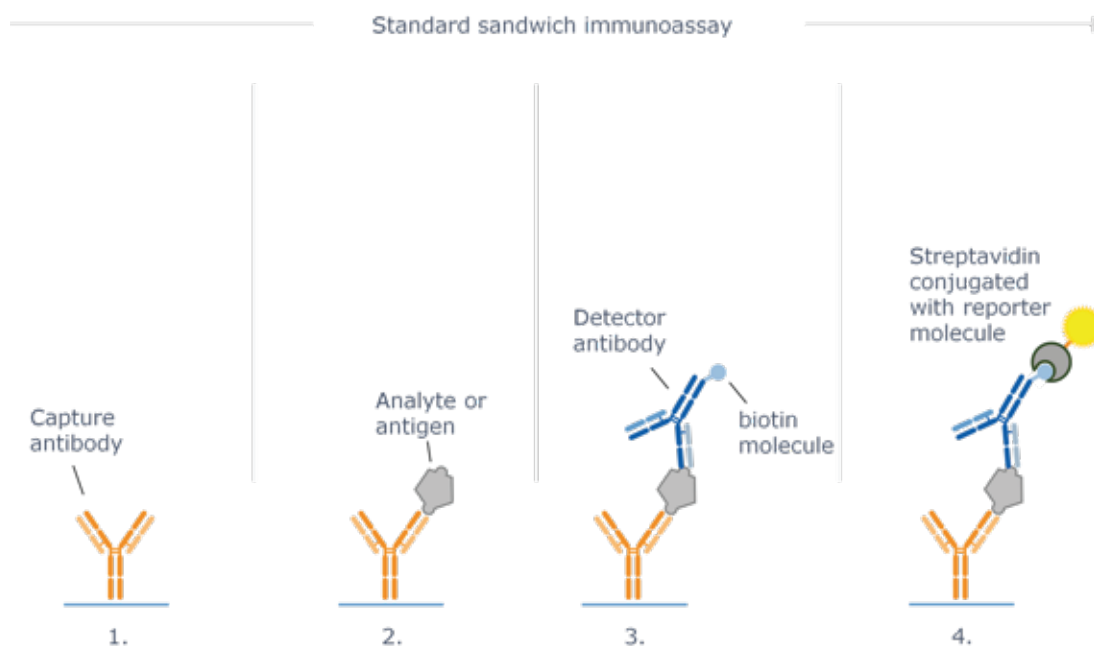


Figure 1. Overview of a conventional sandwich immunoassay. (1) The capture antibody is immobilized on a solid support. (2) The target antigen binds to the capture antibody. (3) A biotin-labeled detector antibody binds to the captured antigen. (4) Streptavidin conjugated to a reporter molecule binds to biotin, enabling detection of the antigen–antibody complex.

StreptaClick® Primer is an engineered streptavidin designed for defined 1:1 binding to biotinylated immunoassay detector antibodies. It carries an oligo-dT primer positioned distal to the biotin binding site, minimizing steric interference and preserving high affinity antibody binding. This architecture enables BOLD technology to be introduced as an orthogonal detection layer using existing assay reagents and workflows, enhancing assay sensitivity while maintaining established validation frameworks.

To date, implementation of BOLD in immunoassays has involved covalent conjugation of the Exazym® Primer to an unmodified detector antibody using click chemistry. This direct conjugation strategy remains a powerful route, particularly when developing new assays or when biotinylated detector antibodies are not available.

StreptaClick® Primer provides an alternative and complementary approach for integrating BOLD based signal amplification. By taking advantage of the widespread use of biotinylated detector antibodies in standard ELISA formats, StreptaClick® Primer enables incorporation of the oligo-dT primer without direct chemical modification of the detector antibody. This can reduce the risk of compromising antigen binding and circumvents the need to source an unmodified antibody when a validated biotinylated reagent already exists.

As such, StreptaClick® Primer offers particular advantages when enhancing an existing, up and running ELISA, or when well characterized biotinylated detector antibodies are available. Conversely, when building a BOLD amplified ELISA from the ground up, both strategies, direct Exazym® Primer conjugation and StreptaClick® Primer based incorporation, can be evaluated to determine the most suitable configuration for the specific assay and performance requirements.

Importantly, StreptaClick® Primer can be applied to the detection of virtually any biomarker for which signal generation relies on a biotinylated detector antibody. In this white paper, its utility is demonstrated through incorporation of BOLD into a model ELISA for human IL-4. Detector antibody and StreptaClick® Primer concentrations were initially optimized to achieve effective signal amplification. Following this limited optimization, the resulting BOLD enhanced immunoassay was evaluated using human IL-4 spiked into a serum based matrix, demonstrating robust performance under biologically relevant conditions.

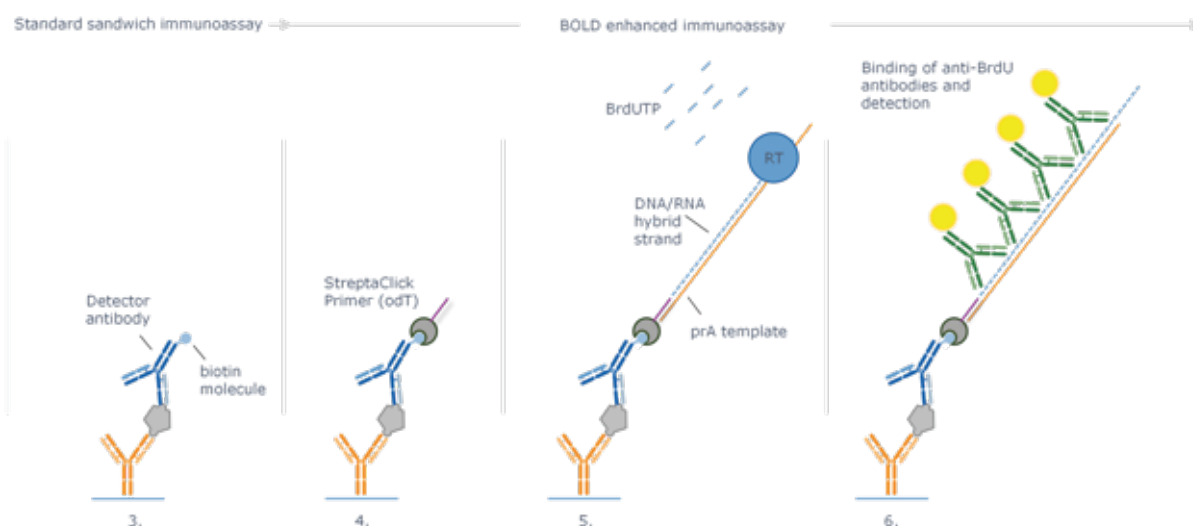


Figure 2. Overview of the signal amplification model. BOLD starts at step 3 of the standard ELISA. (3) The detector antibody labeled with biotin binds to the bound analyte. (4) StreptaClick® Primer, an engineered streptavidin molecule with a single active biotin binding site and conjugated to an oligo-dT primer, is added. (5) A reverse transcriptase (RT) and a reaction solution containing all additional key components required for reverse transcription, including the poly adenine ribose (prA) template, and BrdUTP is added. RT synthesizes a complementary DNA strand, generating a DNA/RNA hybrid. (6) Multiple anti-BrdU antibodies bind to BrdU residues within the hybrid, and their detection produces the amplified signal.

To demonstrate the practical integration of StreptaClick® Primer-mediated BOLD signal amplification into a classical sandwich ELISA format, a model assay targeting human interleukin-4 (IL-4) was established. Human IL-4 is a key cytokine in type-2 immune responses and an important biomarker in allergic and inflammatory diseases. Because IL-4 is typically present at low concentrations in biological matrices, it represents a relevant target for evaluating improvements in immunoassay sensitivity.

ELISA Assay Setup

The assay was established using monoclonal antibodies specific for human IL-4 from Mabtech. For antigen capture, the monoclonal antibody clone IL4-I was used, while clone IL4-II, supplied in biotinylated form, served as the detector antibody. Both antibodies are of the IgG1 isotype and cross-react with IL-4 from non-human primates (NHP).

These antibodies are available either individually or as part of different Mabtech ELISA kit formats that vary in the scope of supplied reagents. The most comprehensive of these is the ELISA Pro: Human IL-4 (cat. no. 3410-1HP-1), a thoroughly validated assay that includes all buffers required for reliable and reproducible measurements. The assay has a reported sensitivity of 3 pg/mL over a standard calibration range of 3.16–1000 pg/mL and is intended for quantification of human IL-4 in serum, plasma, and cell culture supernatants.

Standard and BOLD-enhanced ELISAs were established. For the BOLD-enhanced ELISA, StreptaClick® Primer was combined with the Exazym® Polymerase Reaction and Biotin Detection kits. Both assays used human IL-4 antibody clone IL4-I for capture and clone IL4-II as detector antibody, recombinant human IL-4 ELISA calibrator, ELISA diluent or phosphate buffered saline (PBS), pH 7.4 + 0.05% Tween-20® + 0.1% casein (PBS-TC) as diluent. To simulate real human IL-4 samples, human Plasma K2EDTA gender pool was used (cat. no. humanPLK2-9981-T BioIVT). For these samples the ELISA diluent was used to reduce possible interference from heterophilic antibodies and for the initial experiment for optimization of the biotinylated detector antibody and StreptaClick® Primer concentrations PBS-TC was used as diluent. Colorimetric detection was performed using streptavidin-HRP with TMB as substrate. The reaction was stopped with 2M H₂SO₄ and the product was measured by absorbance at 450 nm. All assays were performed using transparent C-type Maxisorp strip plates (Nunc). The assay is described step by step in Table 1 below, with reagents, buffers, and incubation conditions detailed for each step.

Table 1. Buffers, reagents, and assay workflow for the BOLD-amplified and standard ELISA formats, including key incubation steps and detection components.

Step	Description	Reagent / buffer	Incubation conditions	Source
1	Preparation of capture antibody (IL -4-I) surface.	Phosphate buffered saline (PBS), pH 7.4	100 µL of 0.25 µg/mL IL-4-I, incubated overnight at 2–8 °C.	Medicago 09-9400-100 and Mabtech . 3410-3-250
	Washing	PBS -T	3x400 µL	Medicago 09-9410-100
2	Blocking	Blocker™ Casein in PBS (1%)	300 µL, incubated 60 min at 20 –25 °C.	Thermo Fisher Scientific 37582
	Washing	PBS, pH 7.4 + 0.05% Tween -20® (PBS -T)	3x400 µL	Medicago 09-9410-100
3	Preparation of human IL -4 calibrators or samples.	PBS, pH 7.4 + 0.05% Tween -20® and 0.1% Casein (PBS -TC) or ELISA Diluent	100 µL of calibrators or sample, incubated 120 min at 20 –25 °C.	Medicago 09 -9410-100, Thermo Fisher Scientific 37582 and Mabtech 3410 -10, 3652-D2.
	Washing	PBS -T	3x400 µL	Medicago 09-9410-100
4	Binding of biotinylated detector antibody (IL -4-II) to human IL -4 calibrators and samples.	PBS -TC or ELISA diluent	100 µL of 62.5 -1000 ng/mL detector antibody, incubated 60 min at 20 –25 °C.	Mabtech 3410-6-250
	Washing *	PBS -T	3x400 µL	Medicago 09-9410-100
5	Binding of StreptaClick® Primer to biotinylated detector antibody.	PBS -TC	100 µL of StreptaClick ® Primer, incubated 10 min at 20–25 °C.	Cavidi 10 -0002-01
	Washing	PBS -T	3x400 µL	Medicago 09-9410-100
6	BOLD polymerization, generation of DNA/RNA hybrid strand.	Exazym® Polymerase Reaction kit	100 µl : 23 µL Reaction Solution (with Template) + 77 µL Polymerase Working Solution , incubated 30 min at 20–25 °C.	Cavidi 10-1001-01
	Washing	PBS -T	3x400 µL	Medicago 09-9410-100
7	BOLD detection – binding of biotinylated anti-BrdU antibodies to DNA/RNA hybrid strand.	Exazym® Biotin Detection kit.	100 µL Antibody Biotin Working Solution, incubated 30 min at 20–25 °C.	Cavidi 10 2001-01
	Washing	PBS -T	3x400 µL	Medicago 09-9410-100
8	Binding of streptavidin conjugate to biotinylated anti-BrdU antibodies.	SA-HRP. Dilution with PBS -TC	100 µL of SA-HRP, incubated 30 min at 20–25 °C.	R&D Systems DY998
	Washing	PBS -T	8x400 µL	Medicago 09-9410-100
9	Colorimetric detection – TMB development, stopped with 2 M H ₂ SO ₄ ; absorbance read at 450 nm.	1-Step™TMB ELISA substrate and stop solution 2M H ₂ SO ₄ .	100 µL of TMB substrate, incubated 30 min at 20 –25 °C.	Thermo Fisher Scientific 34028 and Merck 1.60315.1000

*for the standard ELISA this step is followed by colorimetric detection, step 8.

1. Optimization of Detector Antibody and Exazym® StreptaClick® Primer Concentrations

The initial experiment was designed to identify suitable concentrations of the biotinylated detector antibody and StreptaClick® Primer for use in the BOLD-amplified assay. In conventional sandwich ELISA formats, the detector antibody is typically used at approximately 1µg/mL. However, when applying BOLD signal amplification, we have observed that the detector antibody concentration often needs to be reduced to minimize nonspecific binding and background signal while maintaining antigen-specific signal intensity. To effectively integrate BOLD amplification into a standard ELISA using StreptaClick® Primer, the concentration of the Primer must be balanced with that of the detector antibody to ensure efficient signal amplification. This initial study therefore aimed to establish appropriate working concentrations for both components.

Experiments were performed using the assay format described in Table 1 with PBS-TC as diluent, with the biotinylated detector antibody and StreptaClick® Primer concentrations varied as summarized in Table 2. All other reagents, buffers, and incubation conditions were as specified in Table 1.

Table 2 summarizes the concentrations of the biotinylated detector antibody and StreptaClick® Primer evaluated in this initial study. Figure 3 illustrates the background signals obtained for the different combinations of StreptaClick® Primer and biotinylated detector antibody concentrations (B1–B5), as well as for the detector antibody used in the standard ELISA. Figure 4 presents the absorbance signals for all combinations generated from a concentration series of human IL-4 calibrators, together with the corresponding signal-to-noise ratios (SNR) calculated for each calibrator.

Table 2. Antibody and Exazym® StreptaClick® Primer concentrations evaluated in the BOLD-amplified assay and standard ELISA. Capture antibody was used at 250 ng/mL in all conditions.

Assay format	Condition ID	Biotinylated Detector Ab (ng/ mL)	StreptaClick®Primer (ng/ mL)
BOLD	B1	250	12.5
BOLD	B2	250	100
BOLD	B3	125	12.5
BOLD	B4	125	100
BOLD	B5	62.5	12.5
Standard ELISA	S1	1000	NA

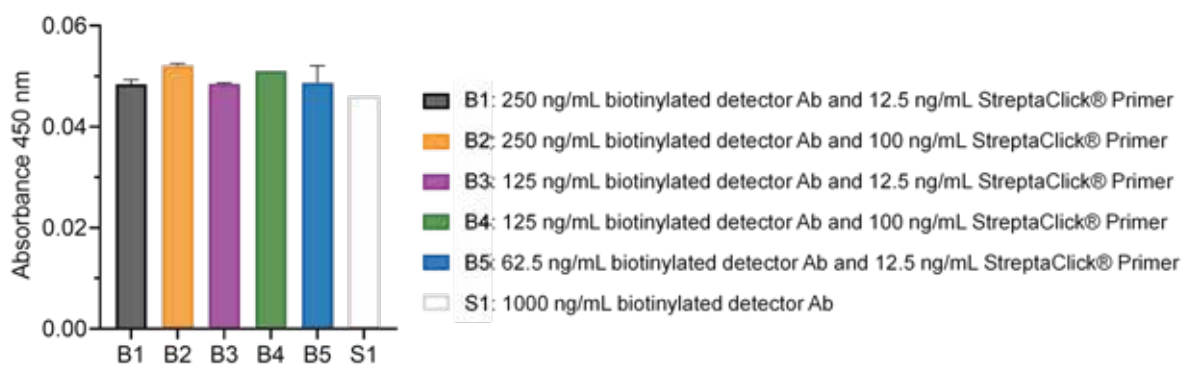


Figure 3. Background signals for detector antibody and StreptaClick® Primer combinations (B1–B5) and for the standard ELISA detector antibody. Bars are mean $\pm$ SD of duplicate wells (N=2).

The tested biotinylated detector antibody concentrations (250, 125, and 62.5 ng/mL) were well below the concentration used in the standard ELISA (S1), 1000 ng/mL. At these lower concentrations, background binding for all tested biotinylated detector antibody and StreptaClick® Primer combinations (B1–B5) (0.049–0.052) was similar to, or only slightly above, the background signal observed for the standard ELISA (0.046). These results indicate that the StreptaClick®-based detection format maintains low background levels across the evaluated detector and primer concentrations. In Figure 4, calibration curves generated using the tested human IL-4 calibrator concentrations demonstrate a clear improvement in assay signal for all StreptaClick®-based detection combinations compared with the standard ELISA (S1).

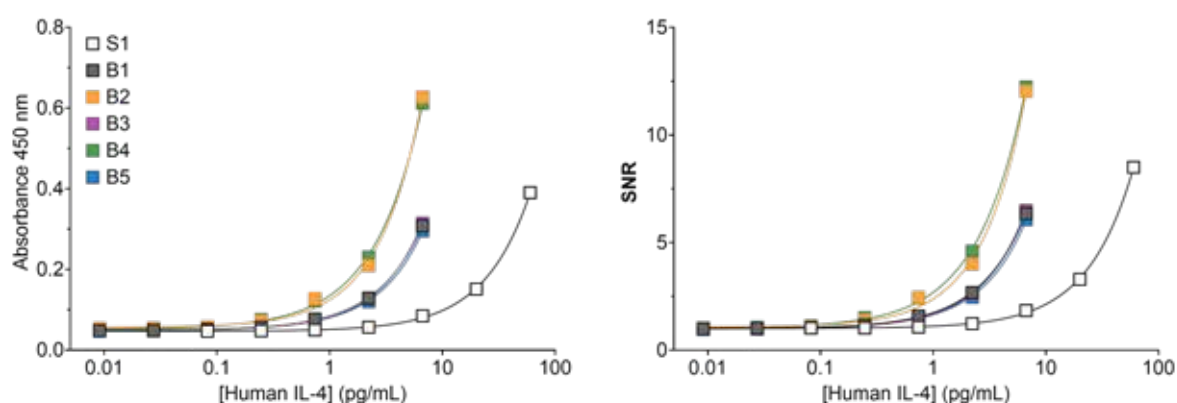


Figure 4. (Left) Calibration curve showing absorbance signal as a function of analyte concentration. Data points represent calibrator measurements and were fitted using a four parameter logistic (4 PL) model. **(Right)** SNR (Signal to noise ratios), calculated as the calibrator signal divided by the background signal, plotted against analyte concentration as an indicator of detection performance.

Table 4. Qualitative performance summary of conditions (B1–B5) and the standard ELISA (S1).

Condition	Biotinylated Detector Ab (ng/mL)	StreptaClick® Primer (ng/mL)	Background signal	Specific hIL-4 signal	SNR
B1	250	12.5	★★	★★★	★★★
B2	250	100	★★	★★★★★	★★★★★
B3	125	12.5	★★	★★★	★★★
B4	125	100	★★	★★★★★	★★★★★
B5	62.5	12.5	★★	★★★	★★★
S1 (std. ELISA)	1000	NA	★★	★★	★★

Among the evaluated combinations, B2 and B4 showed the strongest overall performance, generating the highest absorbance signals across the calibration range and resulting in high signal-to-noise ratios (SNR) compared with the standard ELISA (S1). B2 and B4 produced very similar human IL-4-specific absorbance signals and comparable background levels across the tested concentration range. These results indicate that either B2 or B4 can be used effectively, producing signal amplification relative to the standard ELISA when employing biotinylated detector antibody concentrations between 125 and 250 ng/mL in combination with a StreptaClick® Primer concentration of 100 ng/mL. Given this comparable performance, a combination of biotinylated detector antibody and StreptaClick® Primer concentrations based on the B2 and B4 conditions was selected for further testing in human plasma. Collectively, these results demonstrate that the StreptaClick® Primer-based approach provides a functional means of incorporating BOLD signal amplification into a standard sandwich ELISA format, analogous to the established implementation based on direct conjugation of the Exazym® Primer to the detector antibody, thereby offering two complementary routes for implementing BOLD in ELISA workflows.

2. Evaluation of the BOLD-amplified ELISA in Plasma Samples

In this experiment, the BOLD-amplified ELISA was evaluated in a more biologically relevant matrix using the detector antibody and StreptaClick® Primer concentrations selected in the previous section, and the same samples were run in parallel on the standard ELISA. The evaluation comprised a single experimental run intended as an initial feasibility assessment rather than a formal assay validation, and all samples were quantified against a common human IL-4 calibration curve prepared in ELISA diluent covering 0.0274–20 pg/mL across seven calibrator points, generated by 3-fold serial dilution from 20 pg/mL. Three sample types were prepared, as summarized below.

1. Dilution linearity samples: human IL-4 was spiked into human plasma (K2EDTA gender pool) at 18 pg/mL, then serially diluted 2-, 4-, and 8-fold in ELISA diluent to yield four samples containing 100%, 50%, 25%, and 12.5% plasma (with 0%, 50%, 75%, and 87.5% ELISA diluent, respectively).
2. Recovery samples in ELISA diluent: human IL-4 was prepared in ELISA diluent at 16, 8, and 2 pg/mL.
3. Recovery samples in human plasma: human IL-4 was prepared at 16, 8, and 2 pg/mL in 50% human plasma (diluted with ELISA diluent). A second set was prepared at the same concentrations in 25% human plasma (diluted with ELISA diluent).

Dilution Linearity in Human Plasma

Dilution linearity is a basic indicator of matrix compatibility: an assay that performs well in diluent should recover a spiked analyte proportionally as the plasma fraction is reduced. To assess this for the BOLD-amplified ELISA, human IL-4 spiked into 100% plasma was serially diluted in ELISA diluent and quantified against a diluent-based calibration curve run in parallel on both assay formats. Limits of detection (LOD), determined as the mean signal of the blank plus 2×SD and converted to concentration using a 4-parameter logistic (4PL) fit, were 0.025 pg/mL for the BOLD-amplified ELISA and 0.195 pg/mL for the standard ELISA, corresponding to an approximately 8-fold improvement in detection limit for the BOLD-amplified format. Back-calculated concentrations were corrected for the dilution factor and compared against the nominal spike to estimate recovery in the original 100% plasma sample.

Table 5. Human IL-4 calibration curves measured in parallel by the BOLD-amplified and standard ELISA. Values are mean absorbance at 450 nm of duplicate wells (N=2) with CV (%).

Nominal [hIL-4] (pg/mL)	BOLD ELISA		Standard ELISA	
	Mean A _{450nm} (N=2)	CV (%)	Mean A _{450 nm} (N=2)	CV (%)
20.0	1.9205	10.5	0.2246	1.1
6.67	0.7658	5.7	0.1030	1.1
2.22	0.3203	0.5	0.0663	0.7
0.741	0.1450	2.4	0.0516	0.8
0.247	0.0816	2.3	0.0475	2.9
0.082	0.0621	1.8	0.0474	0.0
0.027	0.0561	2.8	0.0473	1.1
0.0	0.0534	1.1	0.0479	0.9

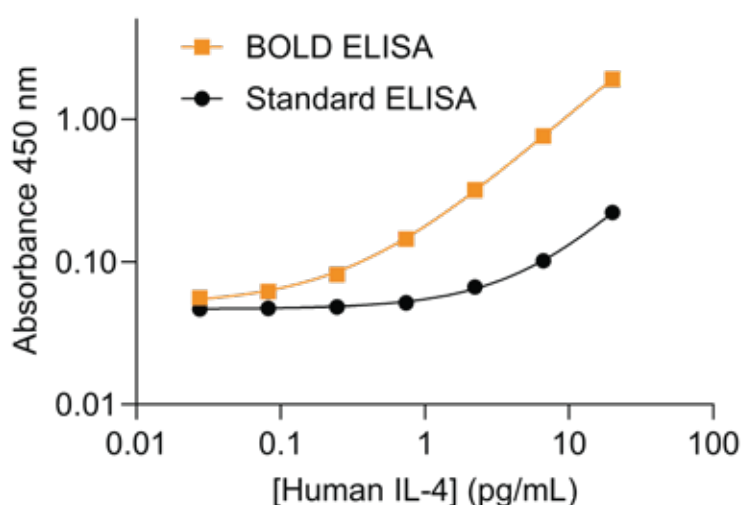


Figure 5. Human IL-4 calibration curves for the BOLD-amplified and standard ELISA (data from Table 5). Curves are 4PL fits.

Table 6. Dilution linearity of human IL-4 in human plasma measured by the BOLD-amplified ELISA. Back-calculated concentrations were multiplied by the dilution factor to give the value recovered in 100% plasma; Recovery (%) is the mean relative to the 18 pg/mL spike.

Plasma fraction	Nominal hIL-4 (pg/mL)	A ₄₅₀	Back -calculated hIL-4 (pg/mL)	Adjusted for dilution (pg/mL)	Mean recovered in 100% plasma (pg/mL)	Recovery (%)
Neat	18	1.699	17.25	17.2	16.7	93
Neat	18	1.614	16.21	16.2		
1:2	9	0.941	8.51	17.0	16.1	89
1:2	9	0.857	7.62	15.2		
1:4	4.5	0.544	4.40	17.6	17.6	98
1:4	4.5	0.544	4.40	17.6		
1:8	2.25	0.287	1.96	15.6	16.1	90
1:8	2.25	0.301	2.08	16.7		

Table 7. As Table 6, but measured by the standard ELISA.

Plasma fraction	Nominal hIL-4 (pg/mL)	A ₄₅₀	Back -calculated hIL-4 (pg/mL)	Adjusted for dilution (pg/mL)	Mean recovered in 100% plasma (pg/mL)	Recovery (%)
Neat	18	0.167	13.87	13.9	13.7	76
Neat	18	0.165	13.62	13.6		
1:2	9	0.117	8.27	16.5	16.7	93
1:2	9	0.118	8.39	16.8		
1:4	4.5	0.080	4.03	16.1	17.0	94
1:4	4.5	0.084	4.46	17.9		
1:8	2.25	0.064	2.12	16.9	19.0	106
1:8	2.25	0.068	2.64	21.1		

This dilution series was performed in a single experimental run, so the results should be interpreted as an indication of assay behavior rather than a quantitative assessment of accuracy or matrix sensitivity. Within this run, both formats produced comparable dilution linearity, with recoveries in 100% plasma in the range of 89–106% across the 2-, 4-, and 8-fold dilutions; the BOLD-amplified ELISA therefore showed no loss of linearity relative to the standard ELISA. Taken together with the higher absorbance signals and signal-to-noise ratios observed for the BOLD-amplified calibration curve (Figure 5, Table 6 and Table 7), these data are consistent with BOLD providing improved sensitivity without compromising dilution behavior in plasma.

Recovery in ELISA Diluent

Spike recovery in diluent is a basic check on assay accuracy under ideal conditions: in the absence of any biological matrix, an assay should return the nominal spike concentration across its working range. Human IL-4 was spiked into ELISA diluent at 16, 8, and 2 pg/mL and quantified against the diluent-based calibration curve on both assay formats. Recovery (%) is the mean recovered concentration relative to the nominal spike.

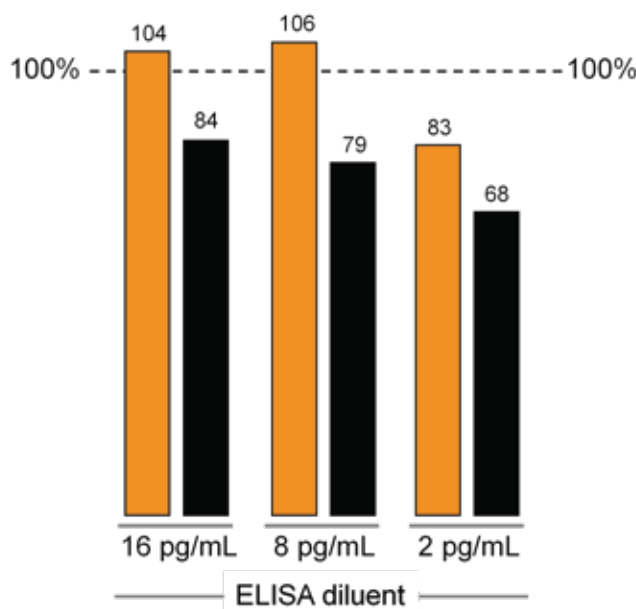


Figure 6. Recovery of human IL-4 spiked into ELISA diluent at three concentrations, measured using the BOLD-amplified ELISA (orange) and the standard ELISA (black). Each spike level was analyzed in duplicate. Recovery (%) was calculated as the mean back-calculated concentration relative to the nominal (theoretical) concentration.

The BOLD-amplified ELISA recovered the 16 and 8 pg/mL spikes at 104% and 106% respectively, and the 2 pg/mL spike at 83%, all within the conventional 80–120% acceptance interval (Figure 6). The standard ELISA under-recovered at every level (84%, 79%, 68%), with the 2 pg/mL spike falling below 80%. In the absence of any matrix, BOLD therefore tracks the nominal spike across the tested range while the standard ELISA shows a systematic negative bias that worsens at the lowest spike.

Recovery in Human Plasma

Spike recovery was then assessed in a biological matrix to determine whether the two assay formats maintain the same accuracy in the presence of plasma components. Human IL-4 was spiked into 25% and 50% human plasma (diluted in ELISA diluent) at 16, 8, and 2 pg/mL and quantified against the same diluent-based calibration curve as used for the ELISA diluent recovery experiment. Comparing recoveries across the two plasma fractions and against the matrix-free values from the previous section indicates the extent of any matrix effect on each assay.

In 25% plasma, the BOLD-amplified ELISA recovered the three spikes at 115%, 100%, and 82%, and the standard ELISA at 88%, 85%, and 114%. All values for both formats fell within the 80–120% acceptance interval (Figure 7). These results suggest that the higher sensitivity of the BOLD-amplified ELISA is not accompanied by poorer recovery than the standard ELISA in this partially diluted plasma matrix.

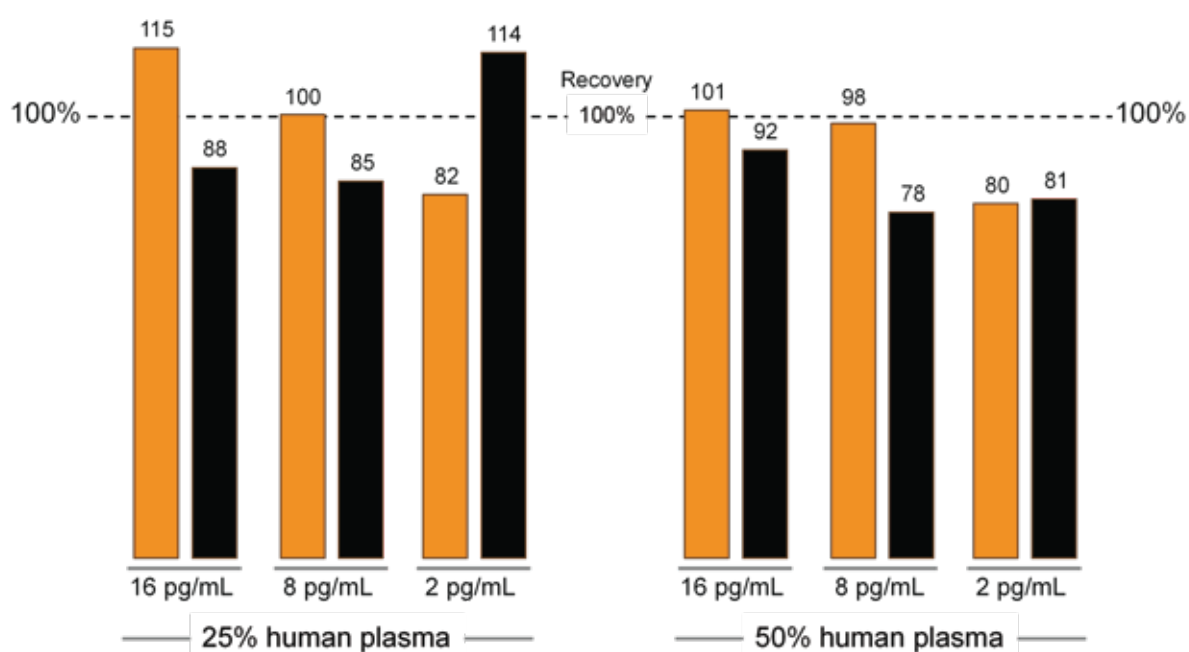


Figure 7. Recovery of human IL-4 spiked into 25% and 50% human plasma in ELISA diluent at three concentrations, measured using the BOLD-amplified ELISA (orange) and the standard ELISA (black). Each spike level was analyzed in duplicate. Recovery (%) was calculated as the mean back-calculated concentration relative to the nominal (theoretical) concentration.

In 50% plasma, the BOLD-amplified ELISA recovered the three spikes at 101%, 98%, and 80%, and the standard ELISA at 92%, 78%, and 81%. All BOLD-amplified values fell within the 80–120% acceptance interval, while the standard ELISA gave one value (78% at 8 pg/mL) just below the interval. These results suggest that the BOLD-amplified ELISA retains acceptable recovery even as the proportion of plasma in the sample is increased, whereas the standard ELISA begins to drift outside the acceptance interval at the mid concentration.

Across all three matrices and all three spike levels, the BOLD-amplified ELISA stayed within the 80–120% recovery interval, whereas the standard ELISA fell below 80% in ELISA diluent (8 and 2 pg/mL) and in 50% plasma (8 pg/mL). Within this single-run feasibility experiment, the BOLD-amplified format therefore delivered more consistent spike recovery than the standard ELISA, particularly at the lower end of the tested concentration range, which is expected for an ELISA with higher sensitivity.

Conclusion

Taken together, the work presented here illustrates that Exazym® StreptaClick® Primer offers a practical route for adding BOLD signal amplification to a standard sandwich ELISA built around a biotinylated detector antibody, without modifying the detector antibody itself. A limited optimization, varying detector antibody and StreptaClick® Primer concentrations across five combinations was sufficient to identify working conditions (125–250 ng/mL detector antibody with 100 ng/mL StreptaClick® Primer) that produced clear signal amplification relative to the standard ELISA while maintaining low background. Applied to human IL-4 spiked into human plasma, the resulting BOLD-amplified assay showed a broader linear range, a higher SNR (Figure 4), and an approximately 8-fold lower limit of detection than the matched standard ELISA, with comparable dilution linearity and spike recoveries inside the 80–120% interval across diluent, 25% plasma, and 50% plasma. As this was a single-run feasibility study rather than a formal validation, the results should not be read as a quantitative claim about accuracy, matrix tolerance, or variability of either format. Within those limits, however, Exazym® StreptaClick® Primer integrates cleanly into a standard biotinylated-detector ELISA workflow and performs at least as well as the standard ELISA in plasma, supporting it as a complementary route to direct Exazym® Primer conjugation for implementing BOLD in immunoassay development.



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 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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