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Development of ultra-sensitive immunoassay on Gyrolab microfluidic platform using Binding Oligo Ladder Detection

Enhancing Gyrolab biomarker assays using Exazym®

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Civilingenjörsprogrammet i kemiteknik

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Abstract

Immunoassays are widely used for detection of antigens in a wide range of applications including assays in pharmaceutical development. Immunoassays are continuously improved in many aspects including automatization, miniaturization and extending the dynamic range. The need to measure low abundance molecules are challenging and the need to improve the sensitivity is desired. The Gyrolab technology is a miniaturized immunoassay performed in an automated system covering a broad concentration range. In order to extend the sensitivity, the technology is combined with Binding Oligo Ladder Detection (BOLD) amplification. The technology behind BOLD or Exazym® utilizes a DNA primer, a polymerase, and a template (RNA) to generate a ladder-like modified DNA strand. Antibodies with affinity for the polymerized DNA:RNA hybrid strand (duplex) conjugated with reporter molecules are introduced to the system, resulting in an increased number of signal-generating molecules associated with each bound analyte molecule. In this thesis, the development of an ultra-sensitive immunoassay is pursued by applying Exazym® add-on reagents to the Gyrolab platform, comparing performance with the standard Gyrolab sandwich assay and other commercially available high-performing TNF-α assays.

The work includes characterization of a wide range of reaction variables involved in the BOLD signal amplification process including hybridization, polymerization, and detection of a synthetic oligonucleotide. The breakthrough involves the introduction of Allophycocyanin (APC) as a fluorescent conjugate, significantly improving sensitivity and signal-to-noise ratios. The BOLD amplified sensitivity for the TNF-α assay approaches levels seen in ultra-sensitive biomarker assays like Erenna® and Simoa®. Exazym® technology on the Gyrolab platform allows highly sensitive biomarker assays with minimal sample volume and a 1–2-hour run-time. The study marks substantial progress in achieving ultra-sensitive biomarker assays on the Gyrolab platform through BOLD signal amplification. The use of APC-conjugated detection reagents holds promise for future optimization studies.

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Populärvetenskaplig sammanfattning

Att precist kunna fastställa mängden av ett specifikt protein i ett biologiskt prov kan varav av stor vikt för gemene man. En noggrann bedömning av proteinhalten kan vara avgörande för läkare vid diagnostiska beslut, vilket i sin tur möjliggör en optimal behandling för patienten. Utöver detta så utgör det en central aspekt inom utvecklingen av nya biologiska läkemedel, särskilt de som riktas mot sjukdomar som psoriasis, ledgångsreumatism och cancer. Den traditionella industrimetoden för att kvantifiera protein, känd som ”immunoassay”, har länge använt antikroppar för att binda proteinet och generera en mätbar signal. En fångande antikropp sitter fast i botten och binder in proteinet, medan en detektionsantikropp, kopplad till en molekyl (en grupp atomer som bildar en självständig enhet) som genererar ljus, fäster på toppen av proteinet. Denna process skapar en så kallad ”macka”, där ljus produceras i proportion till mängden protein – ju högre proteinhalt, desto starkare ljus. Men vad händer när proteinhalten är så låg att ljussignalen knappt är märkbar? I dessa fall kan det vara svårt att upptäcka något protein, vilket i sin tur kan leda till felaktiga diagnoser. För att hantera detta krävs ultrakänsliga immunoassays, särskilt vid prover med extremt låga proteinhalter.

För att tackla dessa problem så har företaget Cavidi AB introducerat en banbrytande teknik kallad Exazym®, vars syfte är att förstärka signalen i olika immunoassays. Metoden baserar sig i att med hjälp av tillsatsen av ett simpelt och applicerbart additiv kunna generera mer ljus per infäst protein genom att bygga en stege bestående av organiska byggstenar som flera detektionsantikroppar kan binda in till. Metoden kommer att kallas för Binding Oligo Ladder Detection, BOLD-amplifiering eller Exazym®.

Projektets mål var att ta fram metoder som förbättrar BOLD-amplifiering på Gyrolab-plattformen, där Cavidi ABs innovativa signalförstärkning integrerades med det automatiserade, mikrofluidiska immunoassay-systemet Gyrolab®. Gyrolab, utvecklat av Gyros Protein Technologies, använder små mängder prov och antikroppar, ner till nanoliter-nivå. Där vätskor rör sig autonomt genom instrumentet med hjälp av en robotarm och en specialdesignad CD med mikrokanaler. Dessa två tekniker är möjliga att kombinera tack vare Gyrolab plattformens flexibilitet och den simpla applikationen av Exazym®-teknologin.

I projektets början konstaterades att själva Exazym® tekniken fungerar på Gyrolab-plattformen, dock utan den önskade signal-amplifieringen. Experiment utfördes då i hopp om att undersöka vilka nyckelfaktorer som sätter käppar i hjulet för själva amplifieringen. Arbetet fokuserade därefter på att detaljerat karaktärisera och analysera alla delar av BOLD-Gyrolab immunoassaysen. De flesta experimenten i detta stadie bidrog inte till någon vidare insikt och integriteten av reagensen som användes för att utföra BOLD assays började ifrågasättas. Av den anledningen utfördes ett kontrollexperiment på en standard-ELISA (en vanlig typ av immunoassay) med samma Exazym® -reagens som misstänktes vara hindret för amplifiering. ELISA-resultatet visade då på kraftig amplifiering!

Resultaten från ELISA-körningen väckte frågor om detektionsantikroppen som användes i BOLD-amplifiering på Gyrolaben. Detta eftersom de två metoderna inte använde samma antikropp men att amplifiering visades tydligt på ELISA-körningen och inte för Gyrolab-körningen. Gyrolab använder en röd laser som inte är kompatibel med den detektionsmekanism som ELISA använder, så en ny antikropp med APC (allophycocyanin) som ljusgenererande molekyl togs fram. Denna nya antikropp resulterade i kraftig amplifiering för Gyrolaben utan behövet av att gå upp i provvolym (antal molekyler), där 18 gånger så låga halter av protein nu kan mätas i jämförelse med en standard Gyrolab immunoassay.

Sammanfattningsvis har studien framgångsrikt visat att BOLD-amplifiering kan förbättra Gyrolab immunoassays. Där byte av ljusgenererande molekyl ökade känsligheten och den generade signalen, som resulterade i ultra-känslig immunoassay. Den förhöjda bakgrundssignalen som följde med den ökade känsligheten är en viktig gåta att lösa för att ytterligare känsliggöra detta assaysystem.

Contents

List of abbreviations

Abbreviation	Connotation
APC	Allophycocyanin
BOLD	Binding Oligo Ladder Detection
BrdU	5-bromo-2’deoxyuridine
CD	Compact disc
Coefficients of variation	CV
DNA	Deoxyribonucleic acid
DOL	Degree of labelling
ELISA	Enzyme-linked immunosorbent assay
fg, fM	Femtogram, femtomolar
IgG	Immunoglobulin G
iPCR	Immuno polymerase chain reaction
LOD	Limit of detection
LLOQ	Lowest limit of quantification
µg, µL, µM, mg, mL, mM	Microgram ,microlitre, micromolar milligram, millilitre, millimolar
ng, nL, nM	Nanogram, nanolitre, nanomolar
NHS	N-hydroxy succiminide
Oligo-dT	Oligo-deoxythymine
PBS	Phosphate buffered saline
PMT	Photomultiplier tube
Poly-rA	Polyriboadenylic acid
RNA	Ribonucleic acid
TNF-α	Tumor necrosis factor-alpha

1. Introduction

Biomarkers play a crucial role in the realm of biotherapeutics and diagnostics, with immunoassays reigning supreme over the past years, with applications across a broad spectrum of the food and pharmaceutical industry [1]. Immunoassays have evolved through various models, with Enzyme-linked immunosorbent assay (ELISA) emerging as the foremost choice. ELISAs unmatched selectivity, specificity, cost efficiency and time efficiency have positioned it at the pinnacle of immunoassays, especially in the biopharmaceutical sector [2].

The largest obstacle for the ELISA platform, despite its current standing, lies in evolving and enhancing sensitivity, as the biopharmaceutical industry demands heightened capabilities for the next generation of immunoassays. The industry-standard sensitivity for biomarker immunoassay kits hovers around the lower levels of the pg/mL [3]. However, achieving such levels of sensitivity on an ELISA platform has not proven as effective compared to alternatives like rolling circle amplification and immune polymerase chain reaction (iPCR) [4]. Notably, Qiu *et al.* demonstrated the exceptional sensitivity of plasmonic photothermal-iPCR (PPT-iPCR) where quantitative levels of tumour necrosis factor alpha (TNF-α) was measured for concentrations as low as 0.1 pg/mL, which is significantly lower than the sensitivity you obtain with commercially available standard ELISAs [5].

Several other techniques have been developed for sensitive TNF-α assays, including digital bead assays like Simoa® by Quanterix with a lower limit of quantification (LLOQ) on the fg/mL scale [6]. Alternatives such as Erenna® (Singulex) and Imperacer® (Chimera Biotec) offer sub pg/mL sensitivity for TNF-α application via flow cytometry and iPCR, respectively [7, 8]. A comprehensive assessment of these three methods is provided in table 1 below. Although numerous studies compare different platforms for biomarker assays, Erenna® and Simoa® stand out as the ultra-sensitive biomarker assays [3].

Table 1. Summary of high performing TNF-α assay formats. *****Calculated LLOQ was unable to be found for Imperacer® as a TNF-α assay.

Method	LOD (limit of detection)	LLOQ (lowest limit of quantification)	Sample volume	Assay model format
Simoa® (SR-X)	0.0039 pg/mL	0.0171 pg/mL	100 µL	Digital bead assay
Simoa® (HD-1/HD-X)	0.016 pg/mL	0.034 pg/mL	100 µL	Digital bead assay
Erenna®	0.080 pg/mL	0.49 pg/mL	20 µL	Flow cytometry
Imperacer®	**n/a**	*****Low pg/mL	30 µL	iPCR

Today, ELISA finds extensive applications in biomarker/metabolite quantification, determination of pharmacokinetic properties, affinity for drug candidates and more [1]. One of many advantages of the ELISA methodology is that it is very flexible, allowing it to be designed in numerous ways for various purposes. One platform that has embraced the ELISA model is the Gyrolab. The Gyrolab platform is an automated open immunoassay platform that operates with a high level of sensitivity in a microfluidic compact disc (CD) [9]. Employing a flow-through system, the Gyrolabs solid phase utilizes a 15 nL column of streptavidin beads, conveniently functionalized with biotinylated capture reagents. The short contact times between sample and the column allows for low matrix interference. The innovative system, housed within 96 to 112 micro-structured Bioaffy® CDs, enables the simultaneous automatic assessment of hundreds of samples. Generating response values for quantification as well as informative column profiles in 2D and 3D (Figure 1). The classic Gyrolab assay is typically designed as a sandwich immunoassay with a biotinylated capture antibody, the antigen, and a detection antibody conjugated with a fluorophore suitable for detection with a 635-nanometer (nm) laser, such as Alexa Fluor® 647 (Figure 1). Directly comparable to traditional ELISA, immunoassays prepared on the Gyrolab platform generally has a wider dynamic range and an improved sensitivity as compared to the standard ELISA [10].

Recent advancements within immunoassays made by Cavidi AB has highlighted that ultra-sensitive immunoassays are attainable without the need for temperature cycling nor auxiliary instruments. The method is called binding oligo ladder detection (BOLD) and is based on the Exazym® technology [12]. The technique promises great amplification whilst maintaining a low background signal along with being applicable with many different types of immunoassay platforms. The establishment of highly sensitive biomarker assays holds significant importance. Ultra-sensitive assays play a crucial role in monitoring disease progression, detecting diseases at an early stage, identifying rare biomarker, in addition to contribution to drug development studies and clinical trials [11].

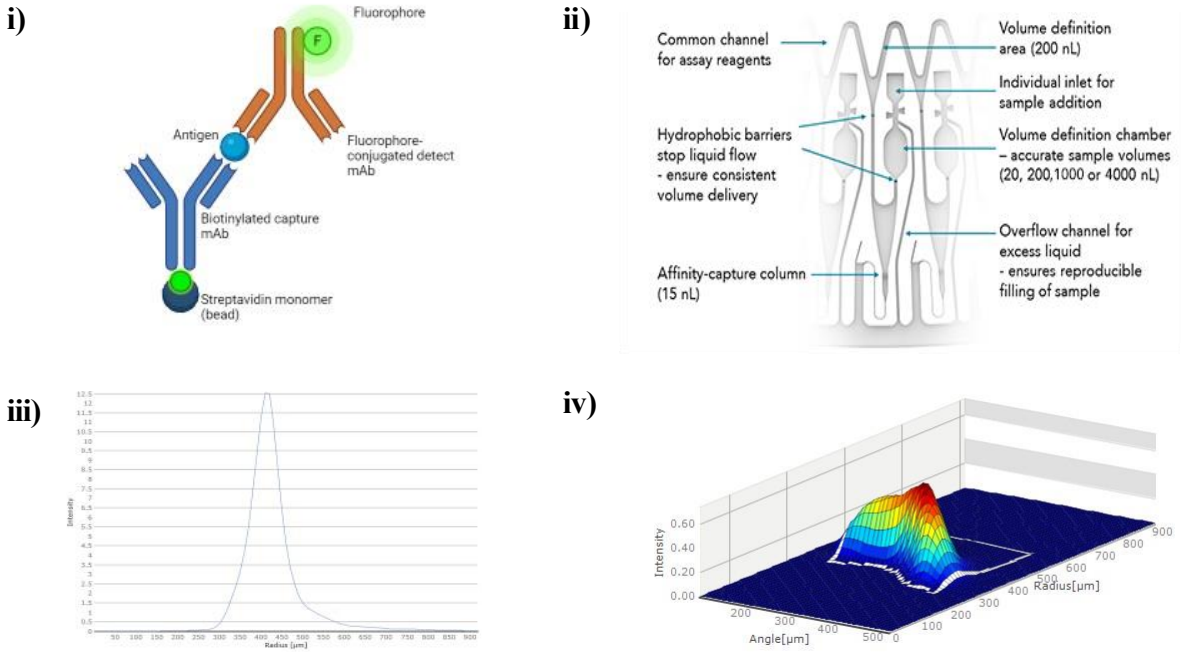


Figure 1. i) General model of a three-step sandwich immunoassay utilized on the Gyrolab platform. A biotinylated capture antibody is bound to a solid phase streptavidin bead. Thereafter, antigen binds to the capture antibodies variable domain and a fluorophore-conjugated detection antibody is lastly introduced to the system. **ii)** The microfluidic structure (one segment) for a Gyrolab Bioaffy® CD. **iii)** Two-dimensional generated data by Gyrolab, the X-axis represents the flow direction in the affinity column, and the Y-axis represents the flow direction and width of the affinity column. **iv)** Three-dimensional generated data by Gyrolab, the X- and Y-axis represents the affinity column, and the Z-axis represents the fluorescent intensity.

1.1 Aim of the study

The aim of the study is to implement the BOLD signal amplification on the Gyrolab platform to achieve an ultra-sensitive biomarker assay using small sample volumes in a fully automated instrument for a model immunoassay, such as the TNF-α assay (pg-fg/mL). The Exazym® technology and Gyrolab were previously proven to be compatible [13]. However, to sufficiently apply BOLD amplification on the Gyrolab assay further method development is needed. Optimization work will commence by analysing optimal conditions for polymerisation of the oligonucleotide and how adjusting Gyrolab methods can improve signal amplification. Two different assays are evaluated, one TNF-α assay and one simplified model system with only oligonucleotide reagents. The performance of TNF-α assay is compared with the current performance of the Gyrolab biomarker kit.

2. Theoretical background

2.1 Monoclonal antibodies and immunoassays

The immunoglobulin G (IgG)-based monoclonal antibodies (mAbs) are by far the most frequently used class of antibodies for immunoassays [14]. There are several factors for the IgG classes popularity, the fragment crystallizable (Fc) region plays a vital role in immunoassays as it is used for immobilization on solid surfaces such as membranes, plates, and beads, which opens the door for development of different immunoassays [15]. The IgG antibody also serves exceptionally well as a reagent for immunoassays due to its stability as IgGs are known to have a long half-life [16]. In order for immunoassays to perform at an ultra-sensitive capacity the interaction between antibody and antigen is critical, IgGs generally have the highest affinity and specificity for their target molecule and is therefore the first choice for many immunoassay models [17]. Furthermore, IgG classes have a wide variety of commercially available isotype-specific secondary antibodies who recognizes the constant region of the antibody which favours signal amplification in immunoassays [18]. The IgG subclasses has a way of undergoing conformational changes (flexibility) which is beneficial for assay development [18]. The inherent flexibility of IgGs contributes to heightened specificity and adaptability across various assay formats. Notably, IgGs exhibit low tendencies for nonspecific binding, minimizing background signals in applications such as microplates. This characteristic enhances the signal-to-noise ratio (S/N) ratio, ensuring the reliability of assay results.

The design of detection antibodies may vary depending on type of assay, when working with a sandwich immunoassay model it is common to conjugate a detection label to a secondary antibody [20]. The different detection labels are conjugated to the secondary antibody, which will cause a proportionate response to the amount of analyte. Examples of detection labels are enzymes such as alkaline phosphatase (ALP) and horseradish peroxidase (HRP), alternatively non-enzymatic detection labels commonly used are fluorophores, e.g., Alexa Fluor dyes such as Alexa Fluor 647. The enzymatic labels are used with a variety of signal generating substrates, including colorimetric, luminescent, or fluorescent molecules. These molecules can be detected in solution with an appropriate detector. Techniques using antibodies with fluorophores attached are more frequently applied on semi-automated systems that utilize a laser such as flow cytometry or Gyrolab miniaturized immunoassays [21].

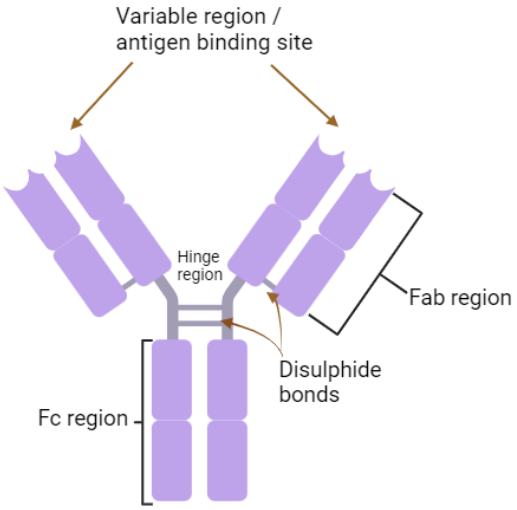


Figure 2. The structure of an IgG class antibody. The two Fc regions and four fragment antigen binding (Fab) regions are interlinked by disulphide bonds made from cysteine residues.

2.2 The Gyrolab

The Gyrolab platform is a microfluidic miniaturized immunoassay operating in the microstructures of a compact disc (CD). The instrument performs on a nanolitre scale and controls the flow of reagents and samples by utilizing the centrifugal force in a controlled pattern in addition to capillary action. There are different sizes of Bioaffy® discs, 200, 1000 and 4000, the number represents the sample volume in nanolitres. Each disc contains of structures (Figure 1, ii) which converts into one data point, the structures vary from 96 to 112 per disc, depending on the disc. All structures have one unique sample inlet that leads into a volume definition chamber followed by a hydrophobic barrier to ensure a precise sample volume. The samples are transported in the microfluidic channels and through the affinity column utilizing the centrifugal force. The reagents are all added through the common channel (Figure 1) and spun down to the affinity column in a predetermined order based on the method created in the Gyrolab software. Figure 3 below illustrates an example of the standard sandwich assay Gyrolab method sequence. Following every additive step there is a specific spin sequence based on which Bioaffy® disc is being used. The two detection steps are both executed via laser-induced fluorescence. The detection laser that is mounted to the Gyrolab is a 635 nm laser which demands a suitable fluorescent tag conjugated to the detection antibody such as Alexa Fluor 647. Detection in Gyrolab comes with the option of amplification via a photomultiplier tube (PMT), the PMT converts photons into electrical signals (electrons) by a magnitude set in the Gyrolab methods. The amplification is defined by a percentage such as 1 % or 80 % PMT and is adjusted to generate signals in a measurable range.

Figure 3. General Gyrolab method design for a three-step sandwich assay. Biotinylated capture reagent, analyte addition and detection reagent are all sample/reagent additions whilst PBS-T washes are meant to cleanse the instrument needles used to transfer samples and reagents. Background detection and detection of analyte are both measured post laser-induced fluorescence with a 635 nm laser.

The biotinylated capture antibody utilizes the extremely high affinity binding between streptavidin and biotin to functionalize the immunoassay solid phase [22]. Conjugating capture antibodies are performed using commercially available kits such as EZ-Link Sulfo-NHS-LC-Biotin (Thermo Fisher Scientific), where *N*-hydroxy succinimide (NHS) conjugation chemistry is utilized to interpolate biotin to free amine groups. The fluorophore (Alexa Fluor 647) is conjugated by the commercially available Alexa Fluor 647 labelling kits (Thermo Fisher Scientific). It is crucial that the labelling procedure contains a purification procedure to remove any unreacted coupling reagent post conjugation, as free fluorophore will increase background signals substantially and unreacted Sulfo-NHS-LC-Biotin will impede the capacity of the solid phase by competing with the primary antibody.

2.3 BOLD signal amplification

2.3.1 Exazym® platform

The Exazym® platform holds the potential to introduce ultra-sensitive detection to conventional immunoassays. The BOLD add-on reagents exhibit compatibility with nearly any immunodiagnostic platform and demonstrate remarkable signal amplification by a factor of 10-100, as illustrated in figure 4 below [12]. Unlike most assay models where the detection antibody is conjugated with a fluorescent label or an enzyme, the BOLD model utilizes a secondary antibody conjugated with a modified DNA strand (oligonucleotide). Subsequently a complementary polyriboadenylic acid template (prA template) added, hybridizing via Watson-Crick base pairing with the oligo deoxythymine primer (oligo-dT primer) alongside a polymerase solution. The enzyme uses the oligo dT-primer and poly rA-template in order to synthesize a hybrid DNA/RNA duplex of 5-bromo-2'deoxyuridylic (BrdU) and riboadenylic acid. Ultimately, secondary detection antibodies (anti-BrdU) with a conjugated fluorophore are

introduced to the polymerised hybrid duplex, resulting in multiple antibodies generating signals per bound analyte molecule.

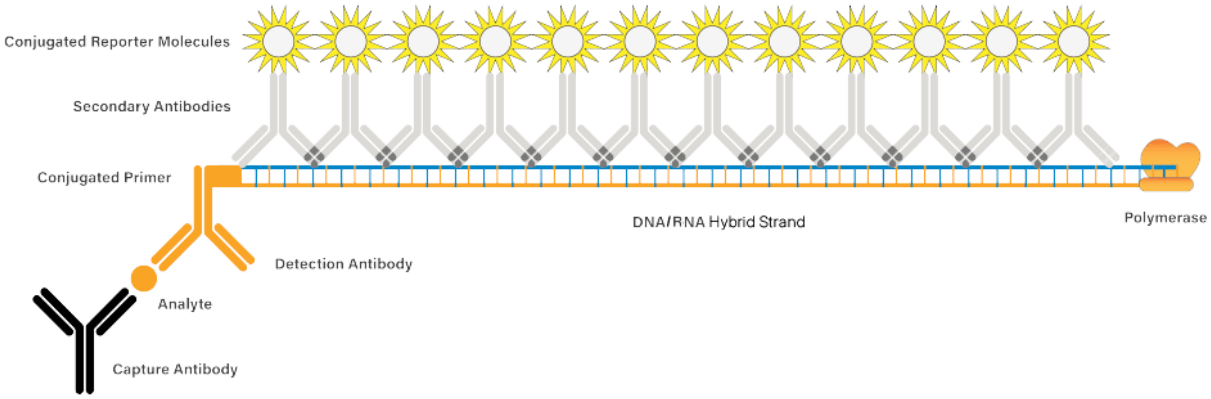


Figure 4. Illustrative sketch of how signal amplification with BOLD technology is achieved. Copyright Cavidi AB, permission to publish.

The oligo dT-primer undergoes conjugation to the secondary antibody using strain-promoted azide-alkyne cycloaddition click chemistry, abbreviated as SPAAC click chemistry. To azide-functionalize the secondary antibody, an NHS-ester is initially added, followed by the introduction of Dibenzo-bicyclo octyne (DBCO) coupled to the oligo dT-primer. The azide-alkyne reaction, widely employed in bioconjugation, is renowned for its high efficiency and compatibility with fluorophores and reactive groups [23]. The oligo dT-primer conjugation took place in a preceding project where the compatibility of Gyrolab and the Exazym® technology was evaluated [13]. Within the project, three unique configurations of the oligo dT-primer conjugated antibody were formulated. Notably, there were a total of three formulations featuring a 5, 20 and a 40-fold molar excess of NHS-PEG_4-Azide and DBCO-TEG-oligo dT-primer in relation to anti-TNF-α antibody.

2.3.2. Method development of BOLD assays on the Gyrolab platform

Assessing of parameters and the efficiency of the polymerisation carries out on a simplified BOLD model with a biotinylated oligo dT-primer, the prA template, Exazym® polymerase solution and fluorophore conjugated anti-BrdU antibodies, the two different BOLD assay models are illustrated in figure 5 below. The simplified BOLD model assay is utilized for trial runs and for developing methods for polymerisation in the Bioaffy® CD as it requires less reagents and has a shorter run time and successfully mimics polymerisation of BrdU as described in figure 4 above, only with two less steps.

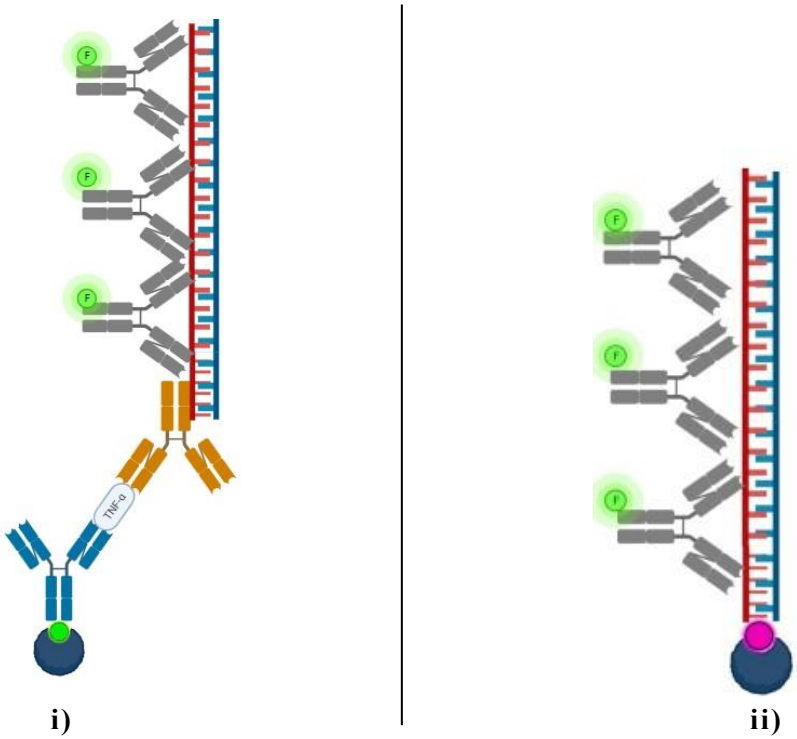
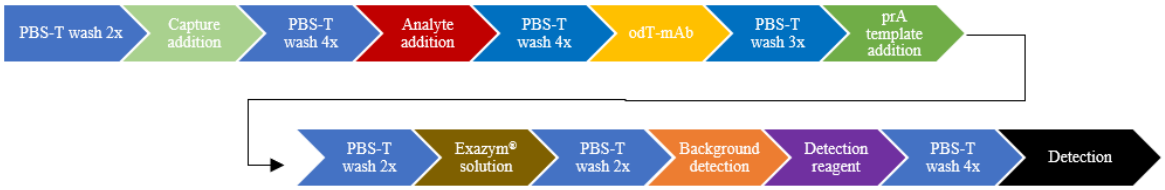


Figure 5. Illustration of the final product of the two different BOLD assays utilized in the project. **i)** End product of the BOLD amplified TNF-α assay. **ii)** End product of the simplified BOLD assay, streptavidin captured oligo dT-primer.

3. Materials and methods

3.1 Gyrolab methods

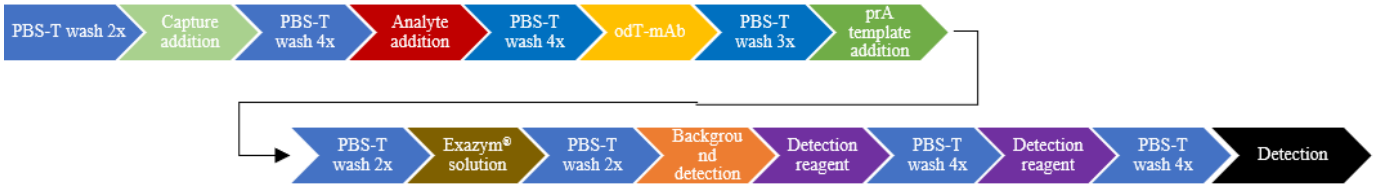
C-A-P-T-E-D Enzyme slow spin (for Bioaffy® 200, 1000 and 4000)



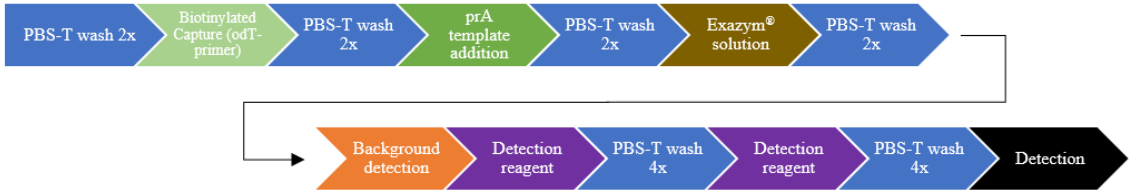
Biomarker kit method (Bioaffy® 200 and 4000)

CA-D (Bioaffy® 200)

C-A-P-T-E-D 2x detect Enzyme slow spin (Bioaffy® 1000 and 4000)



CA-T-E-2D 2x detect Enzyme slow spin (Bioaffy® 1000 and 4000)



CA-T-E-D Enzyme slow spin (Bioaffy® 200, 1000 and 4000)

Table 2. List of abbreviations used for describing Gyrolab methods. Enzyme slow spin have a 0.5 nL/s flowrate for the Exazym® polymerase sample.

Abbreviation	Connotation
C	Capture
A	Analyte
P	Primer
T	Template
E	Enzyme
D	Detect
CA	Capture + Analyte

3.2 Materials

Table 3. Consumables, reagents, and buffers used in the project.

Consumables and reagents	Details	Distributor
Gyrolab Human TNF-α Standard	1 µg/mL Human TNF-α Standard	Gyros Protein Technologies
Gyrolab Human TNF-α Kit	1 µg/mL Human TNF-α Standard, Detection reagent, Capture reagent, Wash buffer	Gyros Protein Technologies
C-Type MaxiSorp® removable ELISA strips		Thermo Fisher Scientific
Biotinylating agent	EZ-Link™ Sulfo-NHS-LC-Biotin No-Weigh™ Format	Thermo Fisher Scientific
Protein Desalting Spin Column	Pierce™ Polyacrylamide Spin Desalting Columns, 7K MWCO, 0.7 mL	Thermo Fisher Scientific
Centrifugal Spin Filter	Nanosep™ 30K, OD030C34	Pall Life Sciences
Alexa Fluor™ 647 Labelling Kit	Alexa Fluor™ 647 antibody labelling kit (A20186)	Thermo Fisher Scientific
Casein 1% in PBS	ELISA blocking buffer	Thermo Fisher Scientific
Synthetic BOLD product	Biotinylated prA-BrdU duplex (synthetic BOLD antigen)	Cavidi AB
Exazym® Template		Cavidi AB
Exazym® polymerase		Cavidi AB
Exazym® polymerase buffer		Cavidi AB
Exazym® reaction solution	BrdUTP + components for polymerisation	Cavidi AB
Exazym® working solution	18 parts Exazym® polymerase buffer, 6 parts Exazym® reaction solution and 2 parts Exazym® polymerase	Cavidi AB
Biotinylated oligo deoxythymidinic acid primer	Biotinylated oligo-dT primer	Cavidi AB
Anti-TNF-α oligo-dT primer antibody	Oligo-dT primer conjugated to anti-TNF-α antibody	Cavidi AB / Gyros Protein Technologies
Anti-BrdU-Alexa Fluor™ 647 labelled antibody	Mouse IgG, degree of labelling 0.9	N/A
Anti-BrdU antibody dilution buffer (Exazym® antibody buffer)	N/A	Cavidi AB
Anti-BrdU-APC labelled antibody	Anti-BrdU antibody conjugated with the protein APC (allophycocyanin)	N/A
Anti-BrdU antibody	Unlabelled anti-BrdU antibody	N/A
Fluorophore conjugated oligo deoxythymidylic acid primer	Alexa Fluor™ 647 labelled Exazym® oligo-dT primer	Cavidi AB

Anti-TNF-α-Alexa Fluor™ 647 labelled antibody	Alexa-labelled TNF-α detection antibody	Gyros Protein Technologies
Phosphate buffered saline (10x)		Thermo Fisher Scientific
Phosphate buffered saline (1x)	pH 7.4, 11.9 mM phosphates, 137 mM sodium chloride, 2.7 mM potassium chloride	Thermo Fisher Scientific
PBS-T	Phosphate buffered saline (1x) + 0.05% Tween20	Thermo Fisher Scientific
Rexxip A	N/A	Gyros Protein Technologies
Rexxip F	N/A	Gyros Protein Technologies
Rexxip HN	N/A	Gyros Protein Technologies

3.3 Overview of assay systems used to evaluate the use of BOLD technology on the Gyrolab platform

Initial testing was carried out on both the TNF-α (Figure 6) and the simplified BOLD assay (Figure 7), the aspiration of the experiments was to evaluate previous results regarding polymerisation of BOLD product on the Gyrolab platform.

3.3.1 BOLD applied on an immunoassay - TNF-α assay

TNF-α standard serial dilutions were freshly prepared before runs from stock solutions, containing eight points of concentration (i.e., 25600, 6400, 1600, 400, 100, 25, 6.25 and 0 pg/mL). Exazym® polymerase solution was routinely prepared as 6 parts Exazym® reaction solution, 18 parts Exazym® polymerase buffer and 2 parts Exazym® polymerase to a final enzyme concentration of 22.7 U/mL unless otherwise specified.

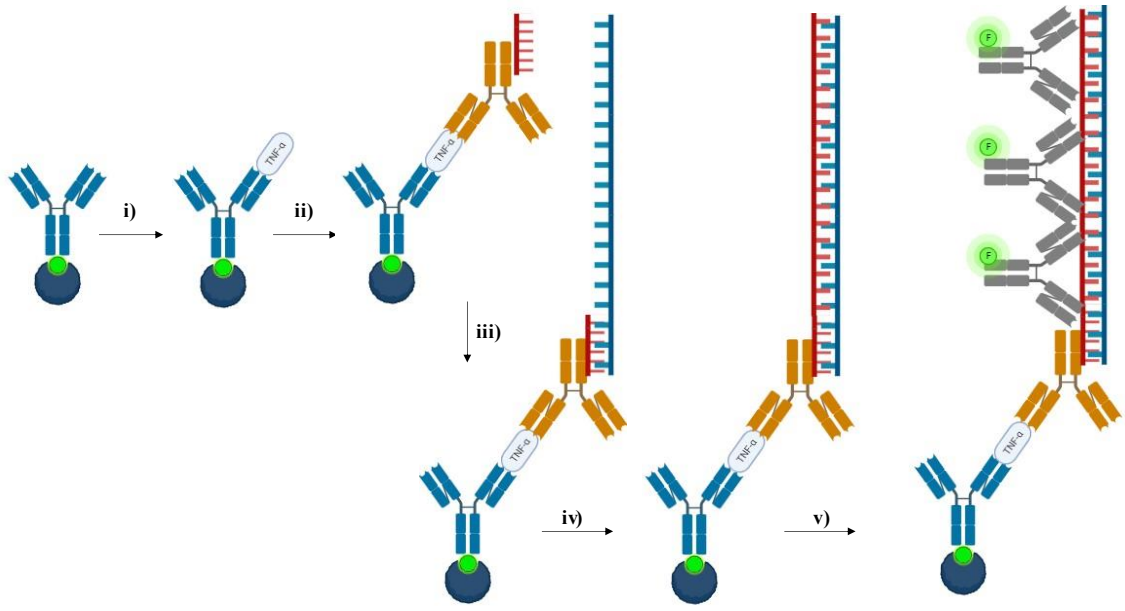


Figure 6. Illustration of the BOLD technology implemented in a TNF-α immunoassay, reagents and analyte are added (**i-v**) automatically and requires no manual labour. **i)** Addition of antigen (TNF-α) to immobilized antibody. **ii)** Addition of oligo-dT modified secondary antibody (anti-TNF-α-oligo-dT mAb). **iii)** Hybridization of oligo-dT primer to polyriboadenylic acid template (prA template). **iv)** Addition of Exazym® polymerase and polymerisation of the BrdU/prA duplex. **v)** Addition of Alexa Fluor 647 conjugated anti-BrdU detection antibodies.

3.3.2 BOLD applied on a simple streptavidin system – Capture of biotinylated oligo-dT primer to streptavidin

Evaluation of BrdU polymerisation was assessed by searching for optimal polymerization conditions by modifying the order of addition and incubation of crucial reagents such as Exazym® polymerase and Exazym® reaction solution. The typical setup used for these experiments are shown in Figure 7 below. Standard series of biotinylated oligo-dT primer were consistently prepared before runs, containing eight points of concentration (i.e., 450, 150, 50, 16.7, 5.56, 1.85, 0.617 and 0 pM), photomultiplier tube (PMT) setting 5% was used for all experiments unless otherwise specified.

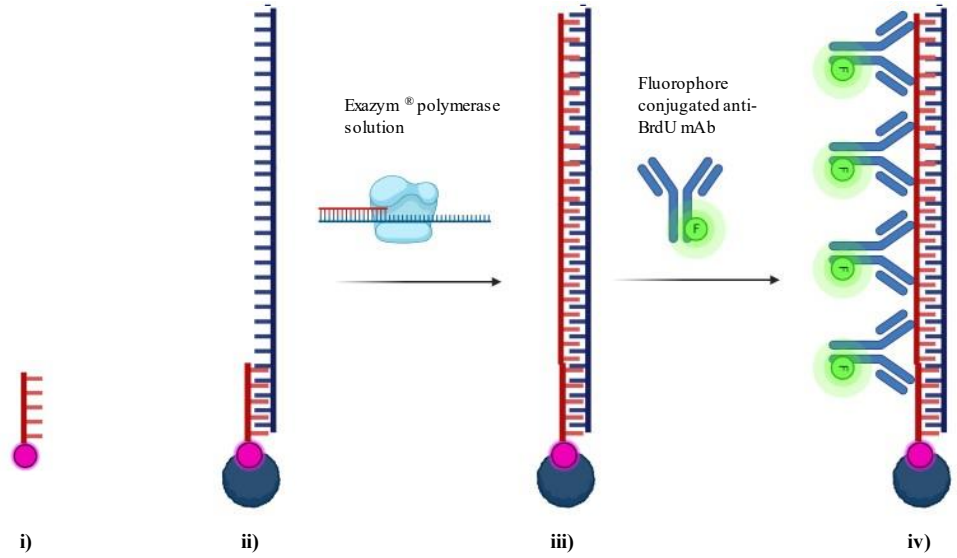


Figure 7. Illustration of the BOLD technology implemented in a simple model assay where biotinylated oligo-dT primer is used as analyte. **i)** Biotinylated oligo-dT primer. **ii)** Immobilized biotinylated oligo dT-primer post poly-rA template hybridization. **iii)** Synthesized DNA/RNA duplex. **iv)** Detected BOLD product, fluorophore conjugated anti-BrdU antibodies are introduced to the synthesized duplex.

3.4 Demonstrating conclusions from prior BOLD project

Following sections cover the methodology of Gyrolab and ELISA experiments that were conducted over the course of the project. The results of the experiments are covered in 4. Results and onward, each respective section has a corresponding section in the result segment.

3.4.1 Separating prA template from the reaction solution

A standard series from 6.25 – 25600 pg/mL of TNF-α was prepared by 4-fold serial dilution in Rexxip HN from a stock solution of 32000 pg/mL (initial dilution 1:1.25). Capture antibody (anti-TNF-α) was prepared by dilution in PBS-T to a concentration of 100 µg/mL. Anti-BrdU antibody was prepared by dilution in Exazym® antibody buffer to a concentration of 15 nM from the stock of 1000 nM. Anti-TNF-α-oligo-dT primer antibody with conjugation conditions 20x molar excess was prepared by dilution to 10 nM in Rexxip F from the stock solution. The polyriboadenylic acid template was diluted in PBS-T to a concentration of 10 µg/mL from a stock solution of 1 mg/mL. Exazym® polymerase solution was prepared by dilution of Exazym® polymerase (2 parts) in Exazym® polymerase buffer (18 parts) and Exazym® reaction solution (6 parts) from the polymerase stock solution of 295 U/mL to a total concentration of 22.7 U/mL. The Gyrolab method used for this experiment was ‘’C-A-P-T-E-D Enzyme slow spin’’ as shown below and was carried out on a Bioaffy® 200 disc.

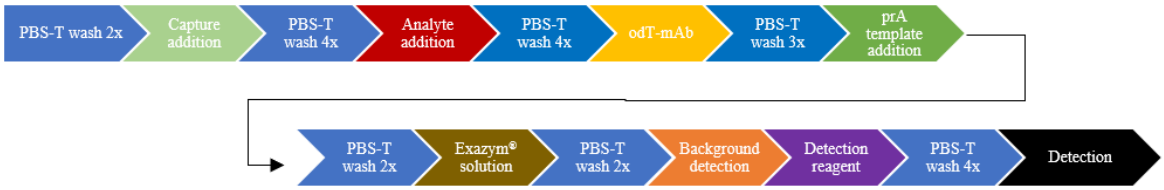


Figure 8. Gyrolab method C-A-P-T-E-D Enzyme slow spin (Bioaffy® 200, 1000 and 4000).

3.4.2 The effects of Exazym® polymerase sample preparation

A standard series from 6.17 – 450 pM of the biotinylated oligo-dT primer was prepared by 3-fold serial dilutions in PBS-T from the stock solution of 300 µg/mL. Exazym® antibody Alexa 647 was prepared by dilution of anti-BrdU-Alexa Fluor 647 to 15 nM in Exazym® antibody buffer, prA template was diluted to a concentration 10 µg/mL in PBS-T from a stock of 1 mg/mL. To assess how incubation of polymerase solution affect polymerisation/signal amplification, three Exazym® polymerase solution was prepared uniquely, one by freeze-thawing an aliquoted sample of polymerase solution, one sample was prepared and let to incubate in room temperature for one hour before run execution in addition to one polymerase sample being prepared and pipetted manually directly into the Gyrolab during the run execution. The Gyrolab method used for this experiment was ‘’CA-T-E-D Enzyme slow spin’’ as shown below and was carried out on a Bioaffy® 200 disc.

Figure 9. Gyrolab method CA-T-E-D Enzyme slow spin (Bioaffy® 200, 1000 and 4000).

3.4.3 Investigating the effects of an increased contact time of Exazym® reagents in the Bioaffy® disc

3.4.3.1 Prolonging contact time in Bioaffy® by adjusting the flowrate of Exazym®reagents

A standard series from 6.25 - 32000 pg/mL of TNF-α from a stock solution of 1 µg/mL by 4-fold serial dilution in Rexxip HN. Capture reagent was prepared by dilution of anti-TNF-α antibody to a concentration of 100 µg/mL in PBS-T, prA template solution was diluted in PBS-T to a concentration of 10 µg/mL. Oligo-dT primer conjugated anti-TNF-α antibody was prepared by dilution to 10 nM in Rexxip F. Exazym® polymerase was prepared by dilution in Exazym® polymerase buffer (18 parts) and Exazym® reaction solution (6 parts) to a final concentration of 22.7 U/mL. Anti-BrdU-Alexa Fluor 647 was diluted to 15 nM in Exazym® antibody buffer. To generate an increased contact time in the Bioaffy® CD a Gyrolab method was reprogrammed by adjusting the flowrate of the added reagents. The prA templates flowrate was reduced to 0.24 nL/s (previously 1.0 nL/s) and the flowrate of the polymerase solution was reduced to 0.16 nL/s (previously 0.5 nL/s). The Gyrolab method used for this experiment was a modified version of ‘’C-A-P-T-E-D Enzyme slow spin’’ as shown in figure 8 above and was carried out on a Bioaffy® 200 disc.

3.4.3.2 Prolonging contact time of Exazym® polymerase in Bioaffy® by multiple reagent addition

A standard series from 6.17 – 450 pM of biotinylated oligo-dT primer was prepared from a stock solution of 300 µg/mL by 3-fold serial dilution in PBS-T, prA template was diluted to a concentration of 10 µg/mL in PBS-T. Anti-BrdU-Alexa Fluor 647 was diluted was diluted to 15 nM in Exazym® antibody buffer. To achieve a longer contact time, multiple Exazym® polymerase solutions were assembled and added consecutively resulting in a longer constant flow-through of the polymerase solution. The Gyrolab method used for this experiment was a modified version of ‘’CA-T-E-D Enzyme slow spin’’ as shown in above and was carried out on a Bioaffy® 200 disc.

3.4.4 Investigation of polymerisation in a Bioaffy® disc

3.4.4.1 Enzyme titration

A standard series from 2.0 – 1500 pM of biotinylated oligo-dT primer was prepared from a stock solution of 300 µg/mL by 3-fold serial dilution in PBS-T. Anti-BrdU-Alexa Fluor 647 was diluted to 15 nM in Exazym® antibody buffer and prA template solution was diluted to 10 µg/mL in PBS-T. To examine the efficiency or potential scalability of enzyme activity dependent on concentration, a titration series of Exazym® polymerase solution was prepared ranging from concentrations 0 – 20 U/mL (0, 0.65, 2.5, 10 and 20 U/mL), by dilution in 18 parts Exazym® polymerase buffer and 6 parts Exazym® reaction solution. The Gyrolab method used for this experiment was ‘’CA-T-E-D Enzyme slow spin’’ as shown in figure 9 above, and it was carried out on a Bioaffy® 200 disc.

3.4.4.2 Titration of 5-bromo-2´-deoxyuridine 5´-triphosphate monomers (BrdUTP)

A standard series from 2.0 – 1500 pM of biotinylated oligo-dT primer was prepared from a stock solution of 300 µg/mL by 3-fold serial dilution in PBS-T. Anti-BrdU-Alexa Fluor 647 was prepared to a concentration of 15 nM by dilution in Exazym® antibody buffer, prA template was diluted to 10 µg/mL in PBS-T. To investigate polymerisation, four different samples of Exazym® polymerase was set to 22.7 U/mL. The four different Exazym® reaction solutions were prepared as a concentration titration of BrdUTP, one with no BrdUTP and three solutions with three different concentrations (0.00432 mM, 0.216 mM, and 2.16 mM). The Gyrolab method used for this experiment was ‘’CA-T-E-D Enzyme slow spin’’ as shown above and was carried out on a Bioaffy® 200 disc.

3.5 Synthetic BOLD DNA/RNA duplex

Testing on a synthetic BrdU:rA strand was carried out to assess efficiency of BOLD amplification in comparison in addition to investigating affinity of anti-BrdU antibodies in different buffers. Standard series were always freshly prepared before runs, containing eight points of concentration (i.e., 3000, 1000, 333.3, 111.1, 37.0, 12.3, 4.1 and 0 ng/mL). Photomultiplier tube (PMT) setting 5% was used for all experiments except the experiment with APC-conjugated anti-BrdU antibodies.

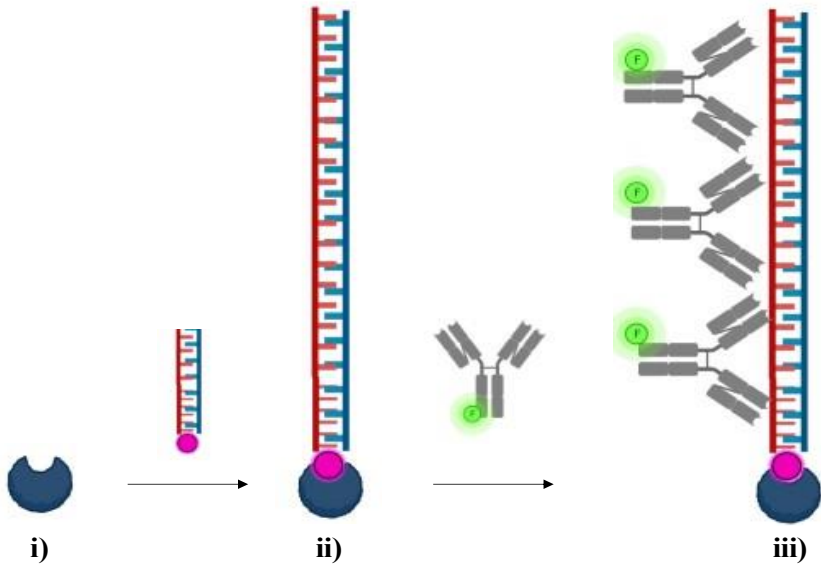


Figure 10. Illustration of an experimental set up for an assay using the biotinylated synthetic BOLD BrdU:rA duplex on the Gyrolab platform. **i)** Streptavidin bead (Gyrolab Bioaffy® affinity column). **ii)** Hybridization of biotinylated synthetic BOLD duplex of the streptavidin coated affinity column. **iii)** Addition of fluorophore conjugated anti-BrdU antibodies (detection reagent).

3.5.1 Comparison study - synthetic oligo detection vs. BOLD detection

A standard series from 0.488 – 2000 ng/mL of synthetic BOLD duplex was prepared by 4-fold serial dilution in PBS-T from a stock solution of 275 µg/mL. To evaluate the efficiency of enzymatic activity, the comparison study was carried out versus the simplified BOLD assay (Figure 7). Therefore, a standard series 0.617 – 450 pM of biotinylated oligo-dT primer was prepared by 3-fold serial dilution in PBS-T. Anti-BrdU Alexa Fluor 647 was prepared by dilution to 15 nM in Exazym® antibody buffer, and prA template was diluted to 10 µg/mL in PBS-T. Exazym® polymerase was prepared by dilution to a final concentration of 22.7 U/mL. The Gyrolab methods used for this experiment was ‘’CA-D’’ (synthetic BrdU:rA duplex assay) as shown below and ‘’CA-T-E-D Enzyme slow spin’’ (BOLD biotinylated oligo-dT primer assay (Figure 9), both experiments were carried out on Bioaffy® 200 discs.

Figure 11. Gyrolab method CA-D (Bioaffy® 200)

3.5.2 Synthetic BOLD product – Limit of Detection

3.5.2.1. Limit of Detection – anti-BrdU-Alexa Fluor 647

A standard series from 4.12 – 3000 ng/mL of synthetic BOLD product was prepared by 3-fold serial dilution in Rexxip A from a stock solution of 275 µg/mL. Anti-BrdU-Alexa Fluor 647 was prepared by dilution to 15 nM in Exazym® antibody buffer. The Gyrolab method used for this experiment was ‘’CA-D’’ as shown above and was carried out on a Bioaffy® 200 disc.

3.5.2.2. Limit of Detection – anti-BrdU-APC

A standard series from 0.00764 – 31.3 ng/mL of synthetic BOLD product was prepared by 4-fold serial dilution in Rexxip A from a stock solution of 275 µg/mL. Detection reagent was prepared by dilution of anti-BrdU conjugated with allophycocyanin (APC) to a concentration of 15 nM in Exazym® antibody buffer from a stock solution of 173.3 nM. The Gyrolab method used for this experiment was ‘’CA-D’’ as shown above and was carried out on a Bioaffy® 200 disc (PMT1%).

3.5.3 Buffer testing – Rexxip F vs. BOLD antibody buffer

A standard series of 0.0244 – 100 ng/mL of synthetic BOLD product was prepared by 4-fold serial dilution in Rexxip A from a stock solution of 275 µg/mL. Detection reagents were prepared by dilution of anti-BrdU-APC to a concentration of 15 nM in both Rexxip F in addition to Exazym® antibody buffer from a stock solution of 173.3 nM. The Gyrolab method used for this experiment was ‘’CA-D’’ as shown above and was carried out on a Bioaffy® 200 disc (PMT1%).

3.6 BOLD amplification on the ELISA platform

A working solution of capture anti-TNF-α antibody was prepared by dilution (1:250) to a concentration of 2.0 µg/mL in PBS from a stock solution of 500 µg/mL, 12 transparent C-Type MaxiSorp® strips were coated with 100 µL of capture anti-TNF-α antibody solution. The microplate (figure 12) was sealed with sealing tape and incubated at 4-6 °C overnight.

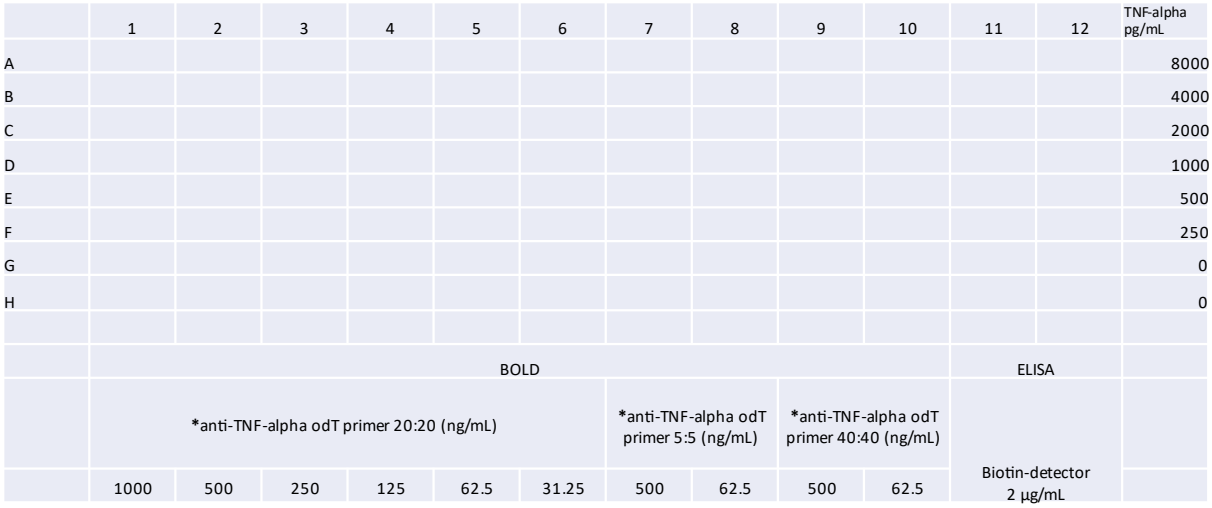


Figure 12. Schematic illustration of ELISA microplate, C-Type MaxiSorp® strips are numbered 1 to 12 and represents each column of the microplate. The bottom two rows represent the oligo-dT primer conjugated antibody used for respective column of wells, therefore, each well in column 1 uses 1000 ng/mL of oligo-dT primer conjugated TNF-α antibody and so forth. Rows A – H represent the rows of the microplate which represents the standard series, the concentrations are seen in the furthest right column. *Oligo-dT primer-conjugated antibodies where 40:40, 20:20 and 5:5 represent the molar excess of oligonucleotide in respect to anti-TNF-α antibody during conjugation (NHS-PEG$_4$-azide: DBCO-oligo-dT primer).

The ELISA dilution buffer was prepared and consisted of 44.75 mL 1x PBS, 5 mL of 1% casein solution and 250 µL of 10% Tween 20. The microplate was washed with PBS-T, 300 µL of casein ELISA blocking buffer, 1% casein (w/v) in PBS was added in each well, the microplate was covered and incubated in room temperature for 60 minutes. A standard series of TNF-α between 0.25 – 8.0 ng/mL was prepared by 2-fold serial dilution in ELISA dilution buffer from a 1 µg/mL stock solution of TNF-α as shown in table 4 below. The microplate was then washed with PBS-T and loaded with 100 µL of TNF-α dilution series as described in figure 12 above, the plate was covered and incubated for an additional 60 minutes.

Table 4. Schematic overview of serial dilution of TNF-α for ELISA.

Test tube	Concentration TNF-α (ng/mL)	Transfer TNF-α (stock solution)	Transfer	Buffer	Total volume
1	8	22 µL	-	2778 µL	2800 µL
2	4	-	1400 µL	1400 µL	2800 µL
3	2	-	1400 µL	1400 µL	2800 µL
4	1	-	1400 µL	1400 µL	2800 µL
5	0.5	-	1400 µL	1400 µL	2800 µL
6	0.25	-	1400 µL	1400 µL	2800 µL

Table 5. Illustration of primary antibody dilution scheme, stock solution concentration for 20:20 oligo-dT mAb (872 µg/mL), for 40:40 oligo-dT mAb (890 µg/mL) and for 5:5 oligo-dT mAb (957 µg/mL).

Tube	Concentration (ng/mL)	Oligo-dT mAb (20:20) stock solution	Transfer	ELISA Dilution Buffer	Total
1	1000	2.3 µL		1998 µL	2000 µL
2	500	-	1000 µL	1000 µL	2000 µL
3	250	-	1000 µL	1000 µL	2000 µL
4	125	-	1000 µL	1000 µL	2000 µL
5	62.5	-	1000 µL	1000 µL	2000 µL
6	31.25	-	1000 µL	1000 µL	2000 µL

Tube		Oligo-dT mAb (5:5) stock solution	Transfer	ELISA dilution buffer	Total
1	500	2.0 µL		3 826 µL	3 828 µL
2	62.5	-	250 µL	1 750 µL	2 000 µL
Tube		Oligo-dT mAb (40:40) stock solution	Transfer	ELISA dilution buffer	Total
1	500	2.0 µL		3 558 µL	3 560 µL
2	62.5	-	250 µL	1 750 µL	2 000 µL

Different concentrations of oligo-dT primer conjugated anti-TNF-α was prepared by dilution to working concentrations in ELISA dilution buffer according to table 5 from respective stock solution. Exazym® antibody biotin (biotinylated anti-BrdU) was prepared, 9.4 µL of antibody was added to 2100 µL of ELISA dilution buffer to a concentration of 2 µg/mL from the stock solution of 447 µg/mL. Subsequently the microplate was washed with PBS-T and 100 µL of oligo-dT antibody solution was added to each well according to the schematic in figure 12 above. The microplate was covered and incubated 60 minutes in room temperature.

Exazym® polymerase was diluted to a concentration of 0.65 U/mL in Exazym® polymerase dilution buffer and the plate was washed with PBS-T. The ELISA strips (column 11 and 12, figure 12) were sealed with sealing tape and stored in the fridge. 73 µL of Exazym® polymerase solution and 23 µL of Exazym® reaction solution was added to each BOLD well and put to incubate for 30 minutes in room temperature. 0.3 µg/mL of biotinylated anti-BrdU antibody was prepared by dilution in Exazym® antibody buffer from the stock solution. The BOLD strips were washed with PBS-T and 100 µL of 0.3 µg/mL biotinylated anti-BrdU was added, the microplate was covered and incubated for 30 minutes in room temperature. SA-HRP (streptavidin-horseradish peroxidase) solution was prepared and diluted to 62.5 ng/mL in ELISA dilution buffer from a stock solution of 1.25 mg/mL. Subsequently, the BOLD strips were washed with PBS-T and the ELISA strips were mounted back on the microplate frame. 100 µL of SA-HRP was added to each well and let to incubate for 30 minutes in room temperature, after which the microplate was washed with PBS-T. 100 µL of TMB (3-3´-5-5´-tetramethylbenzidine) substrate was added to each well, the microplate was put to incubate for 12 minutes in room temperature, protected from light. Subsequently 100 µL of 2M H_2SO_4 (stop solution) was added to each well, absorbance measurements were carried out at 450 nm.

3.7 TNF-α – Standard Gyrolab assay and BOLD amplified assay

These experiments were intended to compare the robustness and sensitivity of standard Gyrolab TNF-α assay (Figure 13) with the BOLD TNF-α assay (Figure 6). The experiments were not exclusive to biomarker assays and therefore also include data from the simplified BOLD assay (Figure 7) for method development studies and limit of detection screening. Bioaffy® 4000, 1000 and 200 were used for these experiments, however, the vast majority of experiments were carried out on the Bioaffy® 200 due to availability. Exazym® polymerase solution was consistently prepared by adding 18 parts Exazym® polymerase buffer, 6 parts Exazym® reaction solution and Exazym® polymerase to a final concentration of 22.7 U/mL unless specified otherwise. Template solution (prA) was consistently prepared by diluting it to a working concentration of 10 µg/mL in PBS-T from its stock solution of 1 mg/mL unless specified otherwise. Detection reagent, fluorophore labelled anti-BrdU antibodies were always prepared in Exazym® antibody buffer to a final concentration of 15 nM unless specified otherwise. Standard series were continuously prepared before runs and contained eight points of concentrations (e.g., 25600, 6400, 1600, 400, 100, 6.25 and 0 pg/mL), photomultiplier tube (PMT) setting 5% was used for all experiments unless specified.

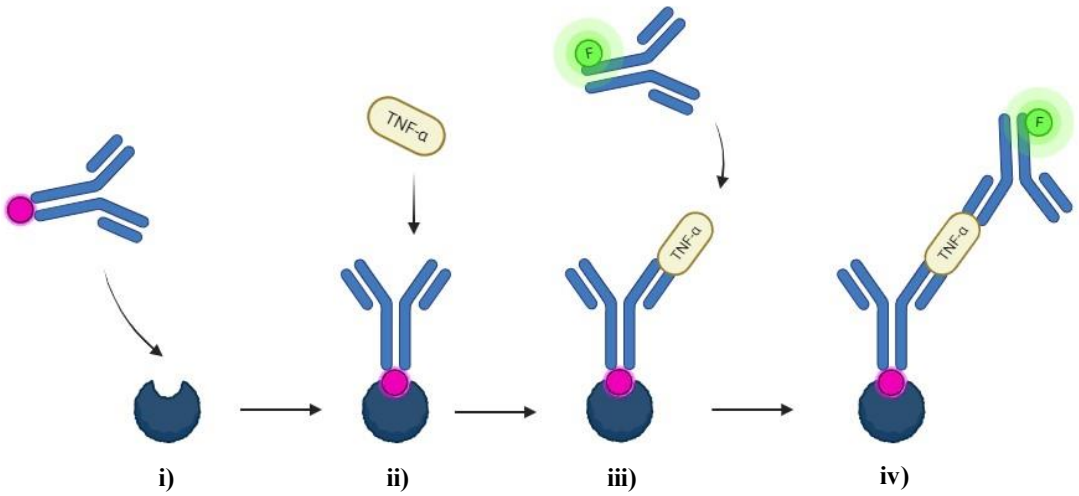


Figure 13. Standard Gyrolab sandwich assay and fitting model for biomarker assays, preferably using Gyrolab method ''Biomarker kit method'' on a Bioaffy® 4000 disc. **i)** Biotinylated capture anti-TNF-α is introduced to streptavidin bead (Gyrolab affinity column). **ii)** TNF-α is added to the immobilized capture anti-TNF-α. **iii)** Fluorophore conjugated anti-TNF-α antibody is introduced to the system (detection reagent). **iv)** Detected product, laser-induced fluorescence with a 635 nm laser.

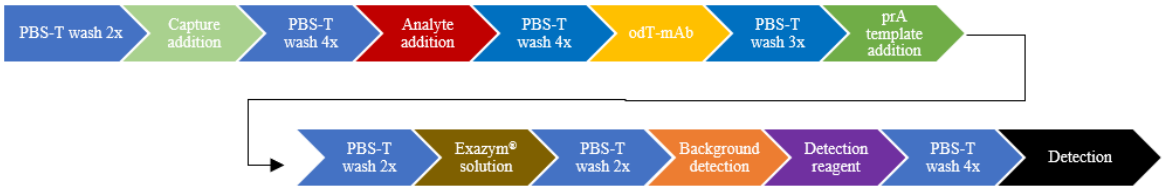


Figure 14. Gyrolab method C-A-P-T-E-D Enzyme slow spin (Bioaffy® 200, 1000 and 4000).

Figure 15. Gyrolab method Biomarker kit method (Bioaffy® 200 and 4000)

3.7.1 Initial testing

3.7.1.1 BOLD TNF-α assay vs. Gyrolab sandwich assay

A standard series of 6.25 – 25600 pg/mL of TNF-α was prepared by 4-fold serial dilution in Rexxip HN from a stock solution of 1 µg/mL. Detection reagent was prepared by dilution of Alexa Fluor-labelled anti-BrdU in Exazym® antibody buffer to a concentration of 15 nM (BOLD assay), Alexa Fluor-labelled anti-TNF-α was also diluted to a concentration of 10 nM (normal assay). Exazym® polymerase solution was prepared by dilution to 22.7 U/mL in 6 parts Exazym® reaction solution and 18 parts Exazym® polymerase buffer. Oligo-dT-labelled anti-TNF-α (20:20) was diluted to a working concentration of 10 nM in Rexxip F, prA template was diluted to a concentration of 10 µg/mL. The Gyrolab methods used for this experiment was ''C-A-P-T-E-D Enzyme slow spin'' (BOLD TNF-α assay) and ''Biomarker kit method'' (sandwich TNF-α assay), both conducted in Bioaffy® 200 discs, the two different Gyrolab methods are shown above.

3.7.1.2 Titration of secondary Exazym® antibody (oligo-dT-anti-TNF-α mAb)

A standard series of 7.81 – 32000 pg/mL of TNF-α was prepared by 4-fold serial dilution in Rexxip HN from a stock solution of 1 µg/mL. Exazym® polymerase solution was prepared by dilution to a working concentration of 22.7 U/mL in Exazym® polymerase buffer and reaction solution. Secondary detection

reagent was prepared by dilution of anti-BrdU conjugated with Alexa Fluor 647 to a concentration of 15 nM in Exazym® antibody buffer from a stock solution of 1000 nM. Primary detection reagent was prepared by dilution of oligo-dT primer conjugated anti-TNF-α (20:20) antibody to concentrations 10, 25, 50 and 100 nM in Rexxip F from the stock solution of 872 µg/mL. The Gyrolab method used for this experiment was ‘’C-A-P-T-E-D Enzyme slow spin’’ shown above and was conducted in a Bioaffy® 200 disc.

3.7.1.3 Enzyme titration – higher concentrations of Exazym® polymerase

A standard series of 0.617 – 450 pM of biotinylated oligo-dT primer was prepared by 3-fold serial dilution in PBS-T from a stock solution of 300 µg/mL. Detection reagent was prepared by dilution of anti-BrdU-Alexa Fluor 647 in Exazym® antibody buffer to a final concentration of 15 nM from a stock solution of 1000 nM, prA template was prepared by dilution in PBS-T to 10 µg/mL from a stock solution of 1 mg/mL. Exazym® polymerase solution was prepared in six different concentrations 0.65, 2.5, 10, 22.7, 35 and 50 U/mL, the polymerase solutions were prepared as 18 parts dilution buffer, 6 parts reaction solution and 2 parts polymerase. The Gyrolab method used for this experiment was ‘’CA-T-E-D Enzyme slow spin’’ shown below and was conducted in a Bioaffy® 200 disc.

Figure 16. Gyrolab method CA-T-E-D (Bioaffy® 200, 1000, and 4000)

3.7.1.4 Addition of Exazym® reaction solution after polymerase

A standard series of 0.617 – 450 pM of biotinylated oligo-dT primer was prepared by 3-fold serial dilution in PBS-T from a stock solution of 300 µg/mL. Alexa Fluor 647 conjugated anti-BrdU was prepared as detection reagent by dilution to 15 nM in Exazym® antibody buffer from a stock solution of 1000 nM, prA template solution was prepared by dilution to 10 µg/mL in PBS-T. Exazym® polymerase was diluted to a concentration of 22.7 U/mL in Exazym® polymerase dilution buffer and Exazym®, reaction solution was prepared by dilution in polymerase dilution buffer (6 parts reaction solution and 20 parts dilution buffer) to replicate the concentration of the reaction solution for a traditional BOLD assay. A standard BOLD assay was prepared and executed aligned with this experiment as reference data using the same standard series, the reagents were materialised as described in section 3.7. The Gyrolab methods used for this experiment was ‘’CA-T-E-D Enzyme slow spin’’ shown above in addition to a modified version of the method and was conducted in Bioaffy® 200 discs.

3.7.2 Screening of a new anti-BrdU conjugate

3.7.2.1 Biotinylated oligo-dT primer - anti-BrdU-APC vs. anti-BrdU-Alexa Fluor 647

A standard series of 0.617 – 450 pM of biotinylated primer was prepared by 3-fold serial dilution in Rexxip A from a stock of 300 µg/mL. Exazym® polymerase solution was prepared to a final concentration of 22.7 U/mL by dilution in Exazym® polymerase buffer (18 parts) and reaction solution (6 parts), prA template was diluted to a concentration of 10 µg/mL. Two detection reagent solutions were prepared, one Alexa-labelled anti-BrdU antibody solution diluted to 15 nM in Exazym® antibody buffer in addition to an APC-labelled anti-BrdU antibody solution diluted to 15 nM in Exazym® antibody buffer. The Gyrolab methods used for this experiment was ‘’CA-T-E-D Enzyme slow spin’’ shown above and was conducted in a Bioaffy® 200 disc (PMT1%).

3.7.2.2 BOLD vs. sandwich TNF-α assay – anti-BrdU-APC vs. anti-BrdU-Alexa Fluor 647

A standard series of 6.25 – 25600 pg/mL of TNF-α was prepared by 4-fold serial dilution in Rexxip HN from at stock of 1 µg/mL. Exazym® polymerase solution, oligo-dT-labelled anti-TNF-α was prepared by dilution to 10 nM in Rexxip F, and prA template was materialised by dilution to a concentration of 10 µg/mL in PBS-T. Detection reagents were prepared by diluting anti-BrdU-Alexa and anti-BrdU-APC in Exazym® antibody buffer to a concentration of 15 nM (BOLD assay), Alexa-labelled anti-TNF-α was diluted to 10 nM in Rexxip F (normal assay). The Gyrolab methods used for this experiment was

‘’C-A-P-T-E-D Enzyme slow spin’’ (BOLD TNF-α assays, Figure 14) and ‘’Biomarker kit method’’ (normal sandwich assay, Figure 15), both conducted in Bioaffy® 200 discs.

3.7.3 Screening the limit of detection

3.7.3.1 Limit of detection – oligo-dT primer

A standard series of 0.0032 – 50 pM of biotinylated oligo-dT primer was prepared by 5-fold serial dilution in Rexxip A. Detection reagent was prepared by dilution of anti-BrdU-APC in Exazym® antibody buffer to a final concentration of 15 nM, prA template solution was prepared by dilution to 10 µg/mL in PBS-T. Exazym® polymerase was prepared to a working concentration of 22.7 U/mL. The Gyrolab method used for this experiment was ‘’CA-T-E-D Enzyme slow spin’’ (Figure 16), the experiments were conducted on Bioaffy® 200 and 1000 (PMT1%).

3.7.3.1 Limit of detection – BOLD TNF-α assay

A standard series of 0.0977 – 400 pg/mL of TNF-α was prepared by 4-fold serial dilution in Rexxip HN from at stock of 1 µg/mL. Exazym® polymerase was diluted to a final concentration of 22.7 U/mL in 6 parts Exazym® reaction solution and 18 parts Exazym® polymerase buffer, and prA template solution was prepared by dilution to 10 µg/mL in PBS-T. Oligo-dT-labelled anti- TNF-α was diluted to 10 nM in Rexxip F and detection reagent was prepared by diluting anti-BrdU-APC in Exazym® antibody buffer to a concentration of 15 nM. The Gyrolab method used for this experiment was ‘’C-A-P-T-E-D Enzyme slow spin’’ (Figure 14), the experiments were carried out on Bioaffy® 200 and 4000 (PMT1%).

3.7.4 Final testing

3.7.4.1 Enzyme titration

A standard series of 0.320 – 5000 pg/mL of TNF-α was prepared by 5-fold serial dilution in Rexxip HN from a stock solution of 1 µg/mL. Detection reagent was prepared by dilution of anti-BrdU-APC in Exazym® antibody buffer to a final concentration of 15 nM, prA template solution was prepared by dilution in PBS-T to a working concentration of 10 µg/mL from a stock of 1 mg/mL. Four different concentrations of Exazym® polymerase were prepared by dilution in 18 parts Exazym® polymerase buffer, 6 parts Exazym® reaction solution and 2 parts Exazym® polymerase to concentrations 0.65, 2.5, 10 and 22.7 U/mL. The Gyrolab method used for this experiment was ‘’C-A-P-T-E-D Enzyme slow spin’’ (Figure 14), and experiment was carried out on a Bioaffy® 200 (PMT1%).

3.7.4.2 Incubation test

A standard series of 0.0640 – 1000 pg/mL of TNF-α was prepared by 5-fold serial dilution in Rexxip HN from a stock solution of 1 µg/mL. Anti-BrdU-APC was diluted to 15 nM in Exazym® antibody buffer, and prA template was prepared to a concentration of 10 µg/mL. Two Exazym® polymerase solutions were prepared by dilution to a working concentration of 22.7 U/mL in 6 parts Exazym® reaction solution and 18 parts Exazym® polymerase buffer. One Exazym® polymerase solution was prepared one hour in advance. The Gyrolab method used for this experiment was ‘’C-A-P-T-E-D Enzyme slow spin’’ (Figure 14), the experiment was carried out on a Bioaffy® 200 (PMT1%).

3.7.4.3 Double anti-BrdU antibody addition

A standard series of 0.0032 – 50 pM of biotinylated oligo-dT primer was prepared by 5-fold serial dilution in Rexxip A. Exazym® polymerase solution was prepared by dilution to a final concentration of 22.7 U/mL in Exazym® polymerase buffer (18 parts) and Exazym® reaction solution (6 parts), prA template solution was prepared by dilution in PBS-T to a concentration of 10 µg/mL. Detection reagent was prepared by dilution of anti-BrdU-APC antibodies to a working concentration of 15 nM in Exazym® antibody buffer. The Gyrolab method used for this experiment was ‘’CA-T-E-2D 2x detect Enzyme slow spin’’, as shown below the experiments were carried out on Bioaffy® 1000 and 4000 (PMT1%).

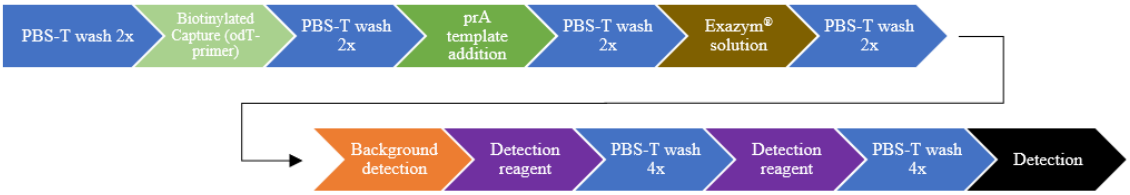


Figure 17. Gyrolab method CA-T-E-2D 2x detect Enzyme slow spin (Bioaffy® 1000 and 4000)

3.8 Conjugation of capture and detection reagent

3.8.1 Biotinylation of anti-TNF-α antibody

Biotinylation reagent (EZ-Link Sulfo-NHS-LC-Biotin, Thermo Fisher Scientific) was dissolved in ice-cold distilled water to a final concentration of 1 mg/mL and vortexed. 4.5 µg of dissolved biotinylation agent was added to 200 µL of 0.5 mg/mL anti-TNF-α antibody solution (12-time molar excess), the mixture was vortexed and incubated in room temperature for an hour while gently shaken. A protein desalting spin column (Thermo Scientific) was prepared by suspension of slurry and centrifuged at 1500 x g for one minute for removal of excess liquid. The biotinylation mixture was then added and the sample was let to flow around the resin bed. The mixture was then centrifuged at 1500 x g for two minutes where the biotinylated antibody solution was collected in the collection tube.

3.8.2 Alexa Fluor™ 647 labelling of anti-BrdU antibody

A Nanosep™ 30K filtration unit was pre-rinsed with 500 µL of deionized water. The membrane was then washed with azide-free 1x PBS and centrifuged at 11000 x g three times. 220 µL of anti-BrdU antibody and 400 µL of 1x PBS was added to the column and the filter column was centrifuged at 11000 x g for 3 minutes, 1x PBS washing of the filter membrane was repeated twice. 1 M of sodium bicarbonate buffer was added (1:10) to the anti-BrdU antibody solution,100 µL of the detection reagent was added to the reactive dye and vortexed gently. The reaction mixture was incubated at room temperature for one hour while gently shaken, protected from light. Alexa Fluor™ 647 purification column was prepared by filtration with PBS, 1 mL of the purification resin was then added to the moistened purification column. Additional purification resin was added until the resin bed reached approximately 1.5 mL, the column was placed in a collection tube and centrifuged at 1100 x g for three minutes. The column was loaded with 100 µL of the labelled reagent and centrifuged at 1100 x g for five minutes, the content of the collection tube was transferred to a dark vial.

4. Results

4.1 Demonstration of prior conclusions regarding BOLD amplification on the Gyrolab platform

These experiments were carried out to confirm conclusions made from earlier experiments and application of the BOLD technology on the Gyrolab platform [13]. These experiments were performed in the beginning of the project and were executed using the same reagent preparations used in the preceding BOLD-Gyrolab project.

4.1.1 Separation of prA template from the reaction solution

The result of separating the prA-template from the Exazym® reaction solution was assessed using the BOLD TNF-α assay. Signals from the Exazym® polymerase solution without prA template shows a higher response along with S/N-ratio, whereas the Exazym® polymerase solution indicates better coefficients of variations (CV). However, since the background signal was greater for the assay where prA is separated from the reaction solution this assay protocol was used as the standard method for the rest of the project (Figure 18).

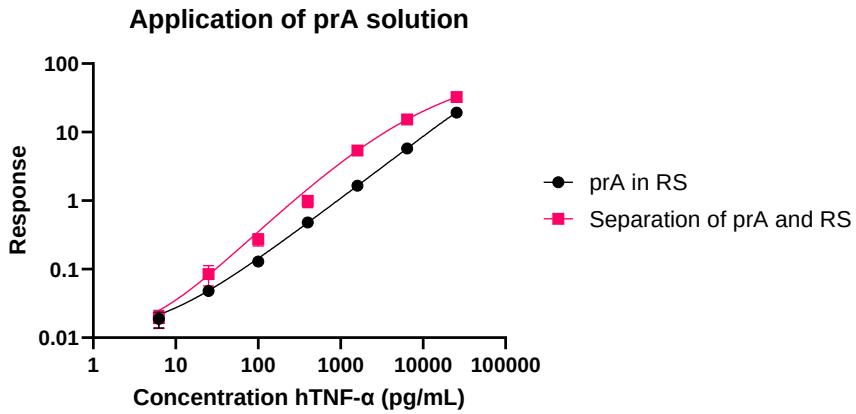


Figure 18. Standard series of TNF-α (6.25 – 25600 pg/mL) using a Bioaffy® 200. Two different preparations of Exazym® polymerase solutions were prepared for this experiment, one with the prA template in the reaction solution and one where the prA template is added prior to introducing the polymerase solution.

4.1.2 Exazym® polymerase sample preparation

The simplified BOLD assay using the biotinylated oligo-dT primer was used to assess differences in response signal based on how the polymerase solution is prepared. There were no significant differences in the results of the different Exazym® polymerase sample preparations, although, the directly injected sample had poor CV values in the lower concentration area (figure 19). However, poor CVs are not to be judged too harshly for this experiment as the result contains standard deviations (SD) for two datapoints.

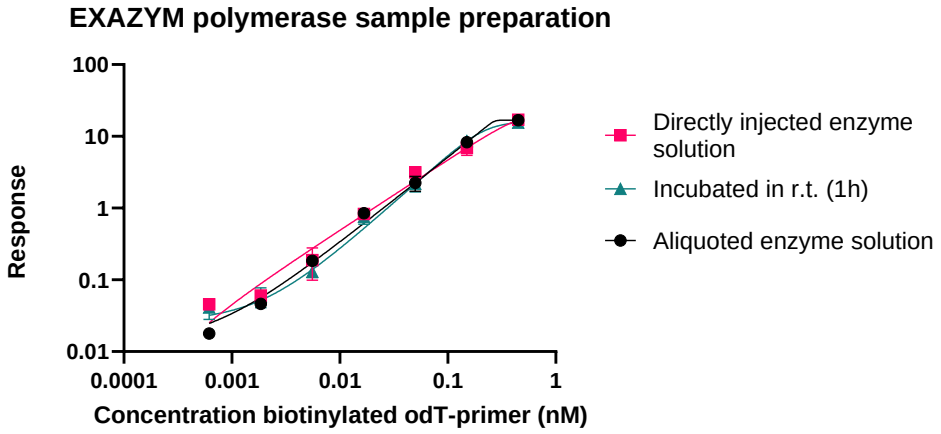


Figure 19. A standard series of (0.617 – 450 pM) biotinylated oligo-dT primer using a Bioaffy® 200. The three different Exazym® polymerase solutions were prepared uniquely. One polymerase solution was let to incubated in room temperature for an hour, one sample was an aliquot from the freezer and the final sample was manually pipetted into the Gyrolab during the run execution.

4.1.3 Reagent contact time in the Bioaffy® column

Both the BOLD TNF-α assay and BOLD biotinylated oligo-dT primer assay were used when investigating the effects of increased contact time between reagents in the Bioaffy® column. An increased contact time did not yield a significantly increase response signal nor was it beneficial with respect to background signal. The background signal measured for experiment with repeated enzyme addition was of ~8x higher than of the original method, where polymerase solution is added just once (figure 20, i). Experiment where spin sequences for the Bioaffy® was prolonged was able to generate a slightly higher signal especially for the lower concentration range of the analyte (figure 20, ii). However, the increased signal was not significant in addition to experimental run times increasing and was therefore not adopted as standard method.

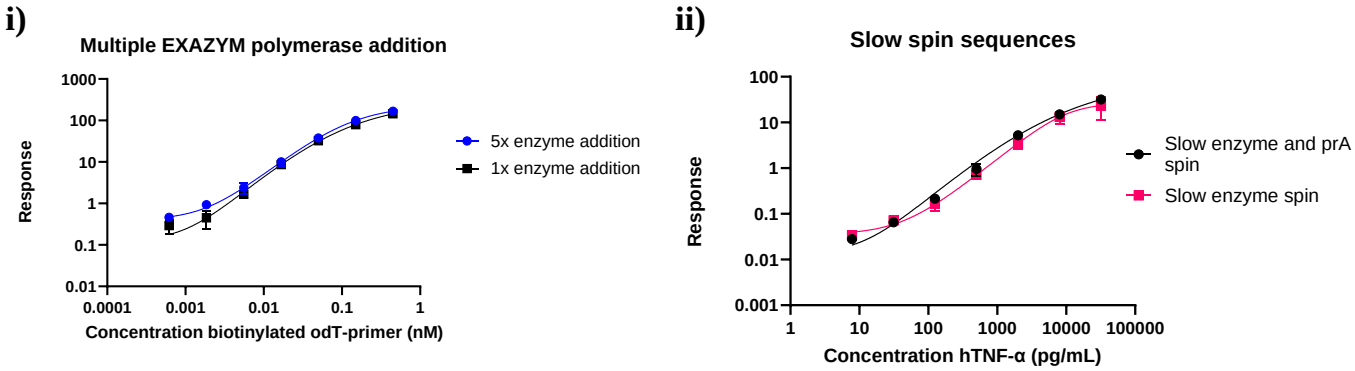


Figure 20. Two different methods to assess potential optimisation as a result of an increased contact time in the Bioaffy® CD, these experiments were carried out on both the TNF-α BOLD assay and simplified BOLD model system. **i)** A standard series of 0.617 – 450 pM of biotinylated oligo-dT primer. The Gyrolab method ''CA-T-E-D Enzyme slow spin'' was modified by looping the Exazym® solution sequence five times (PMT5%). **ii)** A standard series of 7.81 – 32000 pg/mL of TNF-α. The Gyrolab method ''C-A-P-T-E-D Enzyme slow spin'' was modified to reduce the flowrates for ''prA template addition'' (0.24 nL/s) and ''Exazym® solution addition'' (0.16 nL/s), creating a longer contact time for the reagents in the column.

4.1.4 Enzymatic activity in the Bioaffy® column

The efficiency of the on-column polymerase activity was evaluated using the simplified oligo-dT primer assay. Cross reaction testing was conducted by titrating concentrations enzyme and BrdUTP

concentrations. The titration of BrdUTP did not yield any information regarding concentration optimum for the nucleotide. However, the results show that the BrdUTP is not limiting the reaction and omitting the nucleotide generate blank response levels (figure 21, ii). However, the data suffices the conclusion that there is more than enough BrdUTP in the reaction solution and that an excess of BrdUTP does not impede the results in any way. The polymerase titration generated information regarding optimal concentration ranges for the enzyme. Data from both **i)** and **iii)** (figure 21, below) concluded that the best signal-to-noise ratio was obtained at the polymerase concentration of 22.7 U/mL, this concentration was later chosen as the standard for future assays. The effect of different concentrations was assessed with a new detection antibody later in the project, however, the data was not able to incentivize any new conclusions regarding the concentration of Exazym® polymerase.

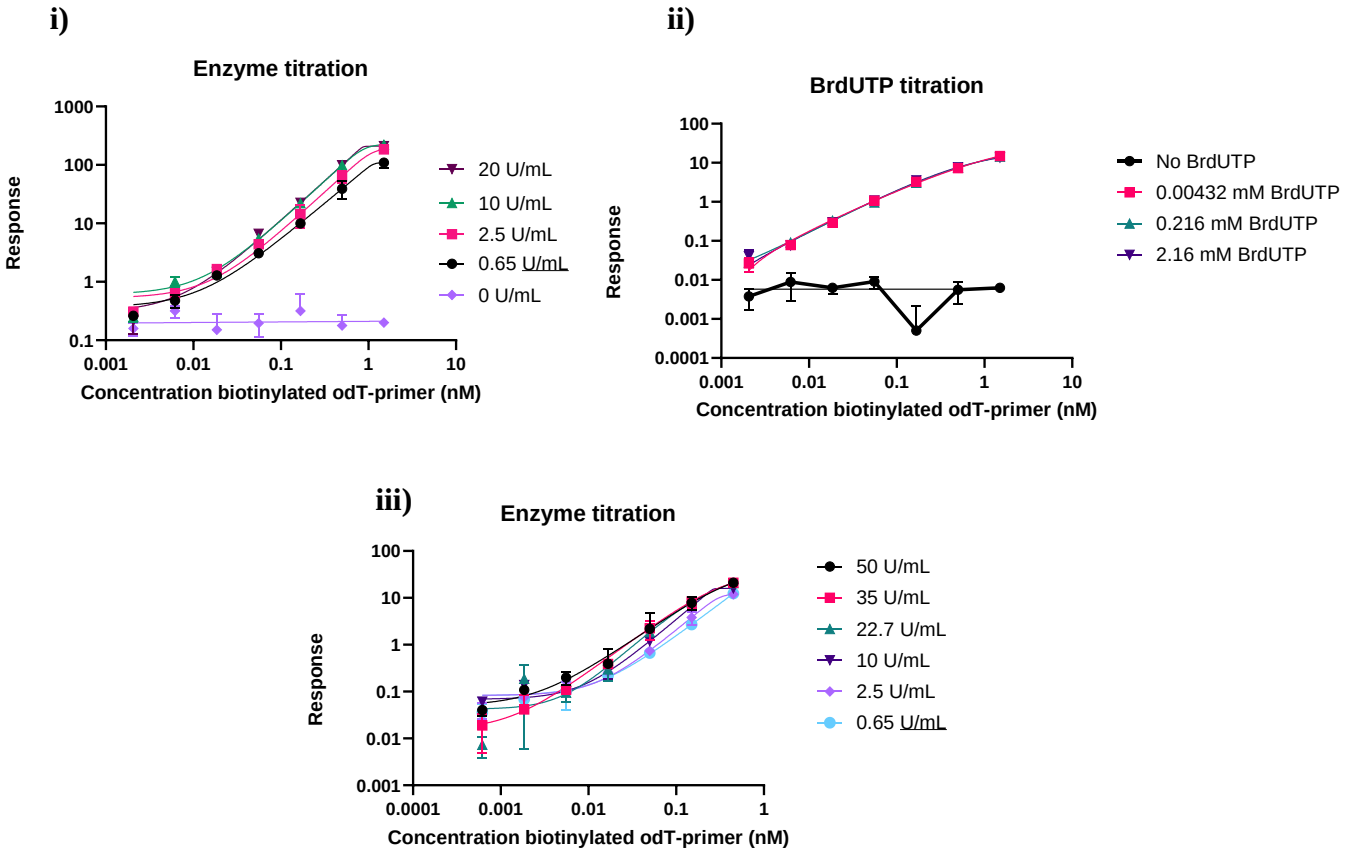


Figure 21. A standard series of 2.06 – 1500 pM biotinylated oligo-dT primer using a Bioaffy® 200. The design of the assay models was both directed toward understanding enzymatic activity based on concentrations of the crucial reagents for synthesizing BrdU. The concentration BrdUTP was constant for the enzyme titrations and the enzyme concentration was set to 22.7 U/mL for the BrdUTP titration**.** The Gyrolab method used for all runs were ‘’CA-T-E-D Enzyme slow spin’’. **i)** Five different enzyme concentrations were studied to compare how enzymatic activity scales along the polymerase concentration (PMT25%). **ii)** Titration curve of the modified nucleotide BrdUTP, different concentrations were studied to get an understand on how concentration BrdUTP affects the response in a BOLD assay (PMT1%). **iii)** An additional enzyme titration with higher polymerase concentrations to study how higher concentrations of enzyme simulated response (PMT5%).

4.2 New Exazym® reagents

The experiments in section 4.1 were carried out with older reagents that had been stored at ~4 ºC for multiple months. The stability of the reagents was unknown and therefore new batches of Exazym® polymerase solution, prA template, polymerase dilution buffer, synthetic BOLD duplex, biotinylated oligo-dT primer, and the different constituents of the reaction solution was sent by Cavidi.

4.2.1 BOLD TNF-α assay vs. Gyrolab sandwich assay

Signals from measurement was higher for the standard assay compared to the BOLD assay although the BOLD assay had a lower blank signal (figure 22, below). The two different assay designs visualized promising sensitivity with detectable signals in the lower range of the pg/mL scale, however, the BOLD assay model is expected to surpass the sensitivity of the standard sandwich assay.

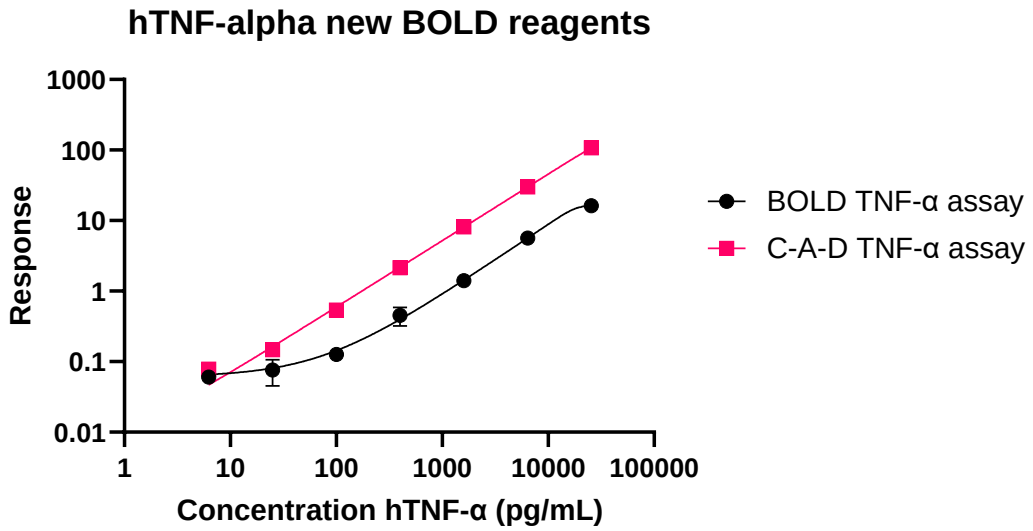


Figure 22. One standard series 6.25 – 25600 pg/mL of TNF-α the experiment was carried out in Bioaffy® 200 discs, comparison study between a BOLD TNF-α assay and a standard Gyrolab sandwich assay. The reagents used for the C-A-D assay are the Gyrolab TNF-α kit reagents (capture anti-TNF-α-mAb and Alexa-labelled anti-TNF-α antibody). The reagents used for the BOLD assay are the 20:20 oligo-dT primer-labelled anti-TNF-α antibody, the same capture antibody, Exazym® polymerase reagents and an Alexa-labelled anti-BrdU antibody (PMT5%).

4.2.2 Titration of oligo-dT-labelled anti-TNF-α antibody

When comparing various concentrations of oligo-dT primer-labelled antibodies, it becomes clear that one factor affecting unwanted background noise is the concentration of the antibody itself. Higher concentrations of this antibody seem to directly increase the background signal in a somewhat linear fashion. The experiment did not yield any suggestion for any benefit when it comes to using a higher concentration of oligo-dT primer-labelled mAb. Therefore, another oligo-dT primer conjugated antibody titration experiment was conducted using the same setup. However, with oligo-dT primer antibody concentrations of 15, 7.5 and 1.0 nM. The signals from the two lower concentrations were feeble in comparison to the one of 10 and 15 nM.

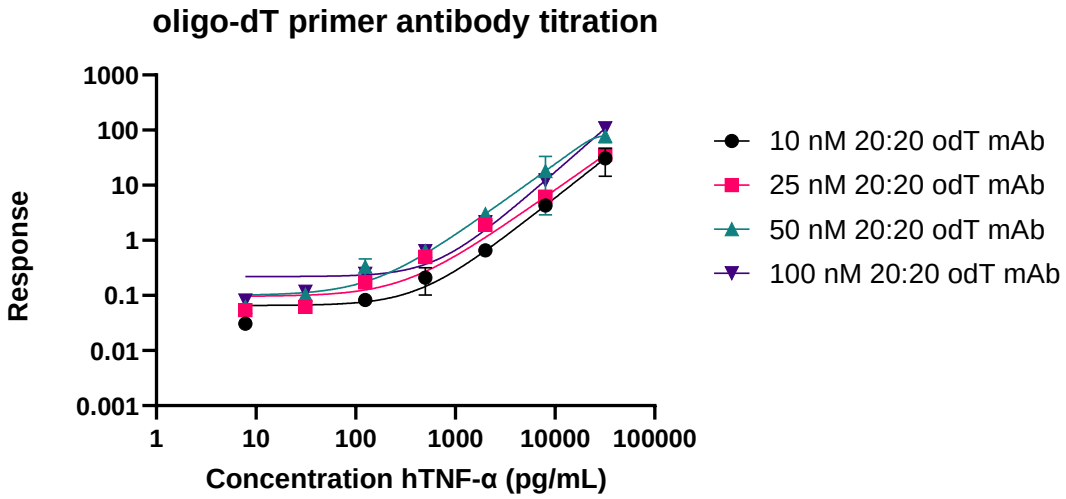


Figure 23. A standard series of 7.81 – 32000 pg/mL of TNF-α, the experiment was carried out on a Bioaffy® 200 disc. The standard concentration of oligo-dT primer-labelled antibody is 10 nM in respect to what concentration is used as standard for other detection reagents (PMT5%).

4.2.3 Addition of Exazym® reaction solution post hybridization of polymerase

In order to further improve the amplification, polymerase was added to the column prior to the Exazym® reaction solution with the hypothesis that the polymerase interaction with the oligo-dT primed poly-rA DNA/RNA duplex was the limiting step. Judging from the data of the experiment (see figure 24), introducing the polymerase to the oligo-dT primer before adding the reaction solution was proven inefficient.

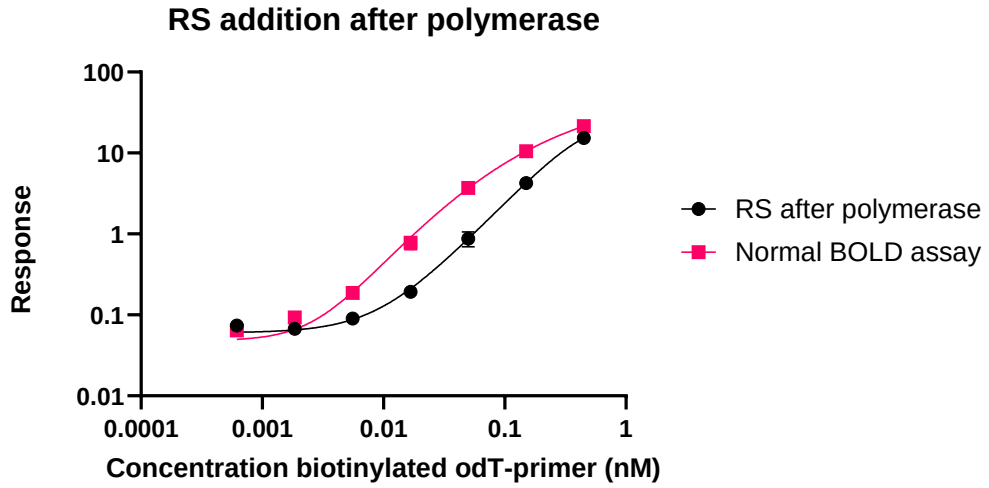


Figure 24. Standard curve of 0.617 – 450 pM of biotinylated oligo-dT primer, the experiment was carried out on a Bioaffy® 200 disc. There were no further tests conducted with separation of reaction solution and polymerase (PMT1%).

4.2.4 Biotinylated oligo-dT primer vs. synthetic BOLD product

The data from the experiment showcases the capability of the Exazym® technique (figure 26, below), the BOLD amplification model of the biotinylated oligo-dT primer is able to display a clear signal for concentrations as low as 1.85 pM (>4 S/N). The synthetic BOLD product is not capable of reaching the same level of sensitivity, which is expected for a much shorter DNA/RNA hybrid ladder. However, the difference in between the two sensitivities is roughly ~195x where the synthetic BOLD showed a LOD

of around 8.8 ng/mL, which translates into 360.5 pM. The limit of detection was calculated by adding the average blank to two standard deviations of the blank.

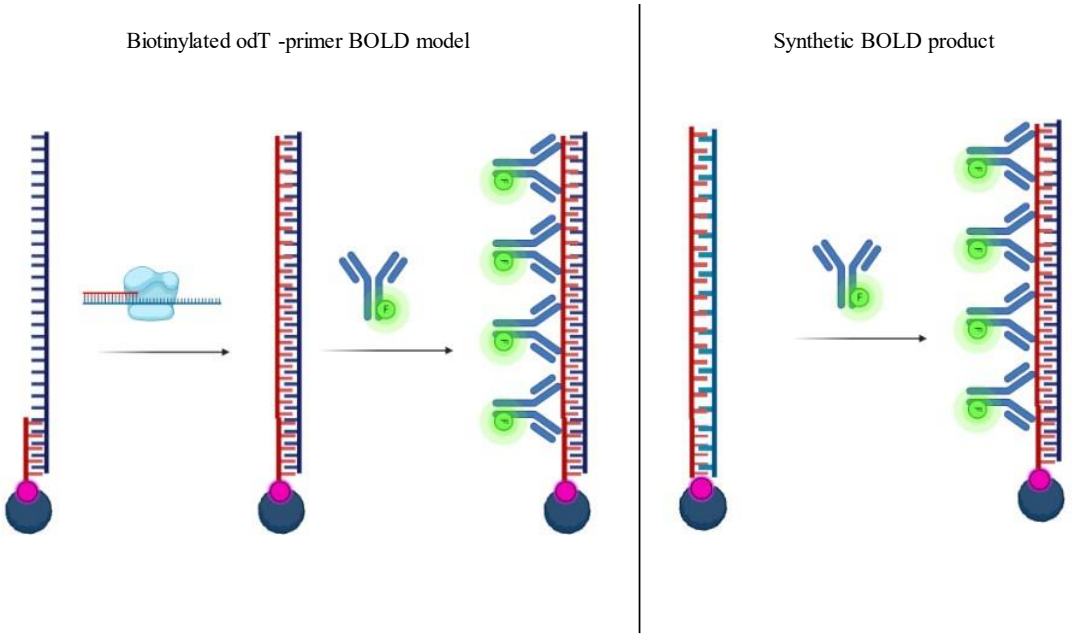


Figure 25. Visual representation of the two BOLD models compared in this experiment, the two model systems are notably alike with the only difference being enzymatic activity (biotinylated oligo-dT primer). Therefore, the two BOLD models can be seen as comparable and as a satisfactory approach of measuring the efficiency of the Exazym® polymerase.

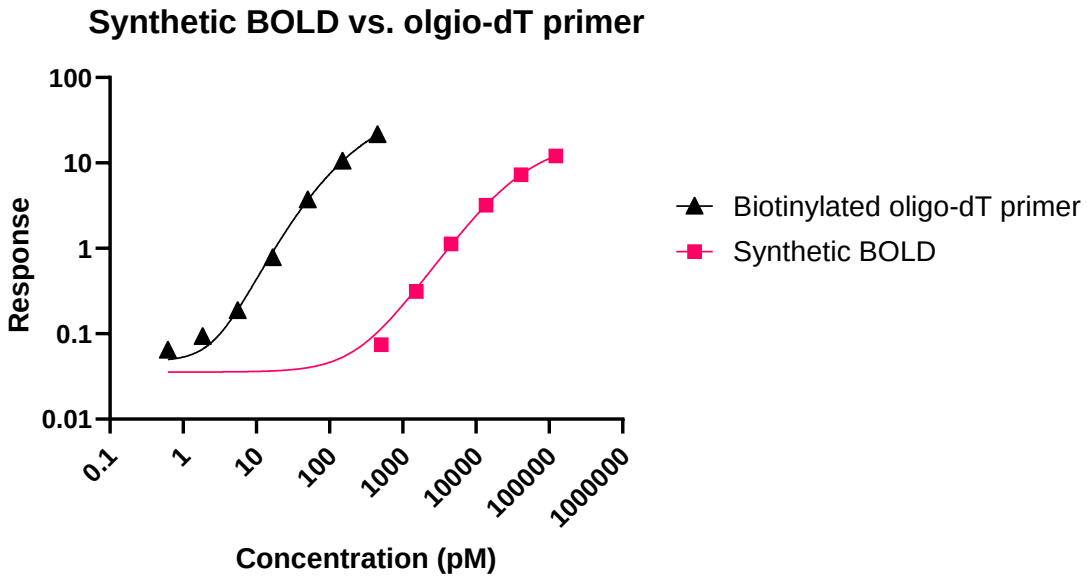


Figure 26. Two standard series were prepared, 0.617 – 450 pM of biotinylated oligo-dT primer and 0.500 – 123 nM of synthetic BOLD product, the experiment was carried out on Bioaffy® 200 discs. The graph displays the great order of magnitude that the Exazym® technique is able to boost signal amplification in the Gyrolab (PMT5%).

4.3 BOLD amplification on the ELISA platform

In order to investigate the reason why BOLD amplification was not performing up to expectations in Gyrolab, the TNF-α assay was arranged in the ELISA format using the same reagents. The assay was

compared with a standard TNF-α ELISA assay without amplification, see figure 27. The results showcased the potency of the oligo-dT primer-conjugated antibody, where the only difference is the detection reagent that is unable to be assessed in comparison to the anti-BrdU-Alexa Fluor647 used on the Gyrolab. Therefore, it is crucial to note that the ELISA uses another type of detection mechanism with streptavidin-horseradish peroxidase (chemiluminescence) rather than fluorescent Alexa Fluor 647 used on the BOLD assays on Gyrolab. The ELISA experiment yielded evidence that the Exazym® TNF-α reagents were potent, where the only difference is the detection reagent that is unable to be assessed in comparison to the anti-BrdU-Alexa Fluor647 used on the Gyrolab.

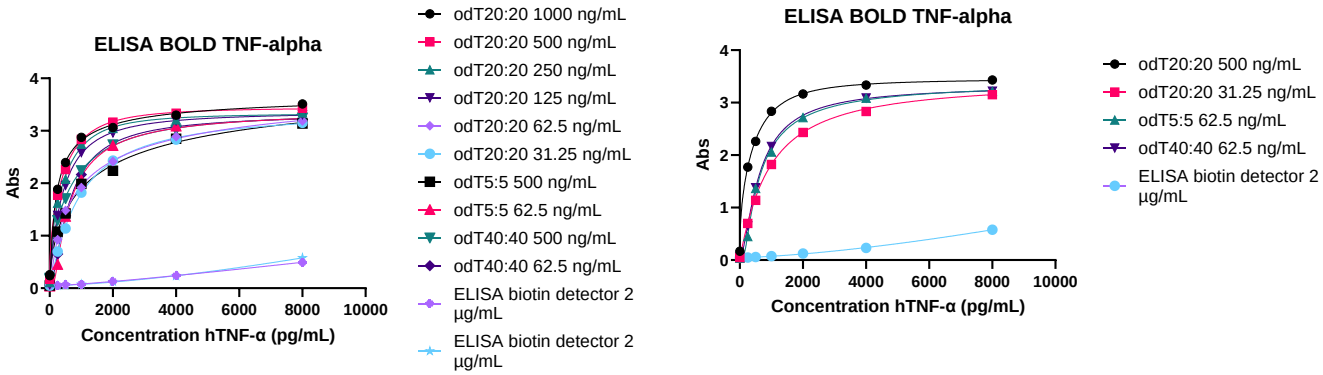


Figure 27. Data from a BOLD TNF-α assay on the ELISA platform where amplification is apparent, a standard curve from 0.25 – 8000 pg/mL, a schematic view of the microplate can be seen in section 3.6 (**Figure 12**). Different conjugations and concentrations of the oligo-dT primer-labelled were produced to isolate the best performing primary detect antibody. Notably, all conjugates resulted in a polymerase reaction (amplification), and roughly a 50-time increase in sensitivity is achieved. **i)** Results from all the ELISA strips of the microplate. **ii)** ELISA BOLD data from the same experiment, however, here the most prevalent of data was chosen for each respective oligo-dT primer-conjugated antibody concentration (including an extra for the 20:20 mAb). Note that data marked with ''ELISA biotin detector 2 µg/mL'' is a standard ELISA sandwich TNF-α assay (Figure 12).

4.4 Screening of a new fluorophore – allophycocyanin (APC)

Evaluation of ELISA data suggested that the limiting step for BOLD amplification is the detection reagent, as it was the sole differentiating factor. Instead of using a fluorophore based on a small organic molecule (Alexa 647), a fluorescent conjugate based on the fluorescent protein allophycocyanin (APC) was evaluated. The results in figure 28 showcase the significant increase in response using the APC conjugate in both the biotinylated oligo-dT primer assay and BOLD TNF-α assay. It is noteworthy that the appearance of the plotted values in the figure below may occur as parallelly moved to a certain order of magnitude. However, that is not the case as the signal for the APC-conjugated detection model increase in great proportion than the blank signal, where response values increased up to 100 times in the upper concentration area of the curve. The average blank signal for the data was 0.0463 (BOLD biotinylated oligo-dT primer assay) and 0.0339 (BOLD TNF-α assay) which is 4.3 and 10.7 times larger in respectively in comparison to Alexa-labelled anti-BrdU. The results obtained from demonstrating a new detection antibody gave reason to assess the improvement of sensitivity.

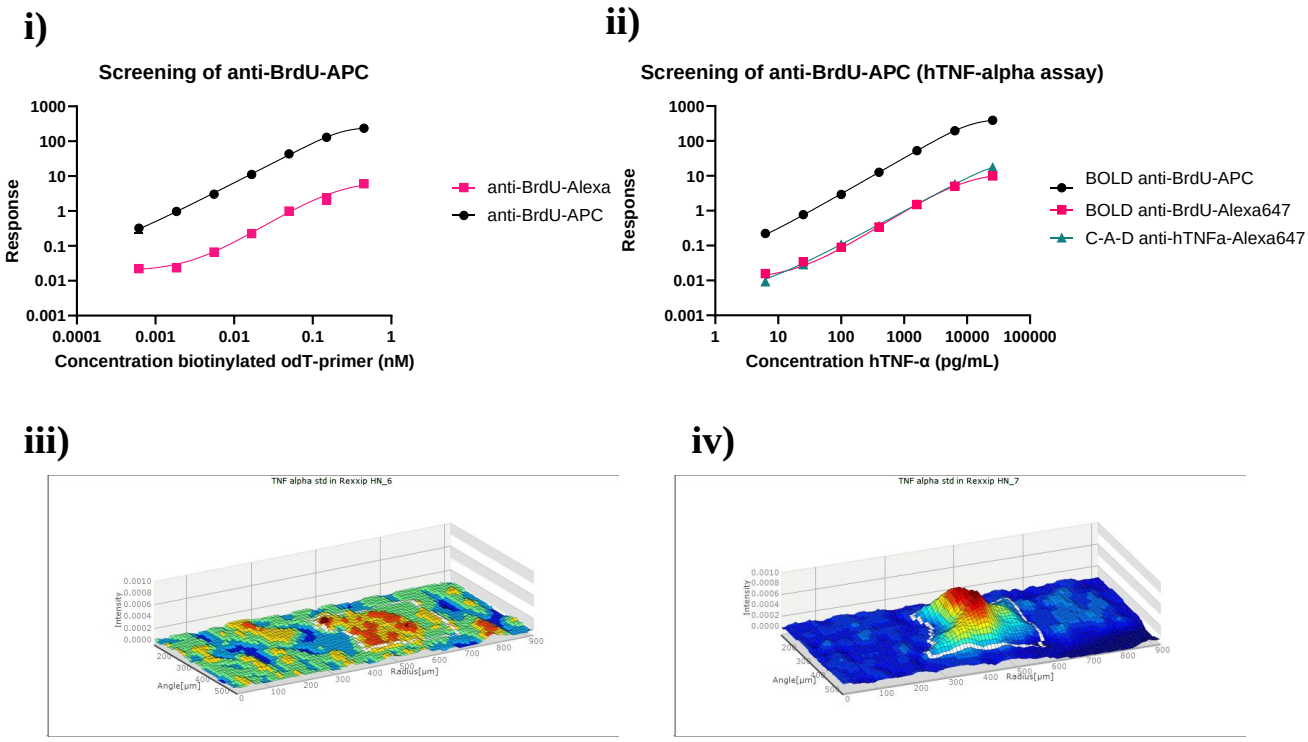


Figure 28. Screening of anti-BrdU-APC for both BOLD assay model systems carried out on the Bioaffy® 200 (PMT1%). The results showed a massively improved amplification in comparison to the old BOLD setup with an Alexa-labelled anti-BrdU antibody for the simplified BOLD model system in addition to the BOLD TNF-α assay. The results also showcased the expected difference in signal between the three-step sandwich assay model in comparison to the amplification. **i)** Screening of anti-BrdU-APC as detection reagent in the simplified BOLD model. **ii)** Screening of anti-BrdU-APC as detection reagent in the BOLD TNF-α assay, the new fluorophore is compared to the Alexa-labelled BOLD setup in addition to the standard Gyrolab sandwich assay. **iii)** 3D column profile for 25 pg/mL TNF-α (Alexa-labelled anti-BrdU) which shows a signal with a magnitude of 3x S/N. However, it is extremely difficult to separate the signal from the blank. **iv)** 3D column profile for 6.25 pg/mL TNF-α (APC-labelled anti-BrdU), the column profile displays a clear signal (~7x S/N).

4.5 Results using the APC conjugate

The revelations of the APC-conjugated anti-BrdU antibody led to further experiments carried out later in the project, where limit of detection and multiple older concepts that were trialled earlier in the project was tested.

4.5.1 Stability of the Exazym® polymerase

The stability of the polymerase after incubation in room temperature was tested earlier in the project (section 4.1.2, above) and did not indicate any loss of enzymatic activity. However, when repeating the experiment with the APC conjugated detection antibody, it is apparent that there are differences in signal in addition to background signal depending on how the polymerase sample is prepared. Figure 29 below shows that background signal is reduced by letting the polymerase solution incubate in room temperature for one hour before run execution, although the loss of raw signal is also apparent for the incubated polymerase sample. The column profiles in figure 30 (i and ii) showcase the loss of signal post incubation of polymerase solution in comparison to the more intense column profile of iii.

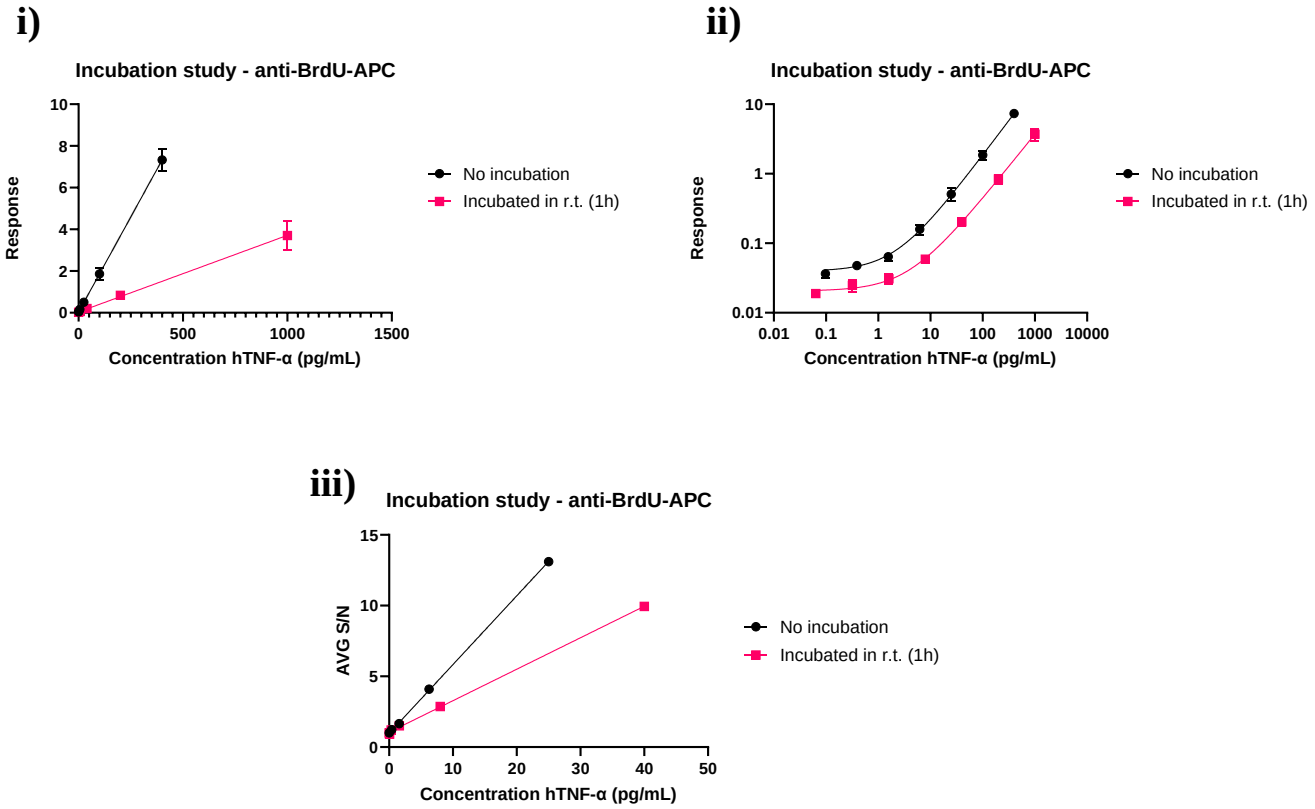


Figure 29. A standard series of 0.064 – 1000 pg TNF-α (datapoints for incubated polymerase solution), the data is plotted against the LOD screening for the BOLD TNF-α assay on the Bioaffy® 200, which is the reason for different concentrations used as datapoints for the two datasets. Exazym® polymerase incubation study on the TNF-α BOLD assay, the experiment was carried out on the Bioaffy® 200 (PMT1%). The three different graphs are from the same dataset and represent the differences in preparation of the Exazym® polymerase solution. **i)** Linear regression of assay data. **ii)** Non-linear regression of assay data (standard). **iii)** Linear regression of average signal-to-noise ratio between the two different sample preparations. The results exhibits that when incubating the polymerase solution, the amplitude of the blank and raw signal is reduced in comparison to the sample preparation where no incubation takes place. However, it is noteworthy that there is a total loss of S/N-ratio in favour of the method without incubation.

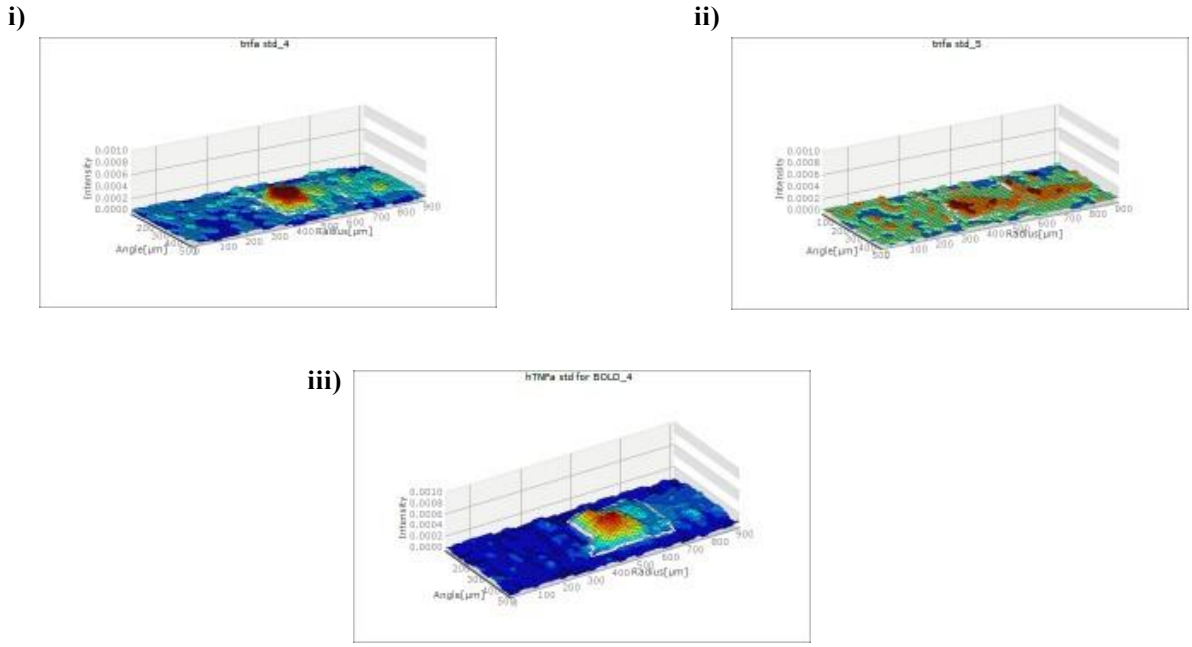


Figure 30. Incubation test of Exazym® polymerase solution in addition to TNF-α LOD screening for Bioaffy® 200. **i)** The column profile represents a datapoint for 8 pg/mL of TNF-α ~3 S/N. **ii)** Column profile representing incubation test of polymerase solution at TNF-α concentration 1.6 pg/mL (~1 S/N). **iii)** The column profile representing the non-incubated polymerase sample, the column profile represents a datapoint for 3.91 pg/mL of TNF-α (~5 S/N).

4.5.2 Double addition of anti-BrdU-APC antibody

The new APC-conjugated detection antibody that was used in the later stages of the project yielded a higher background signal. Notably, the average blank signal for the APC-conjugated antibody is ~3-10 times higher than that of the Alexa-labelled detection antibody. While the signal amplification surpasses the elevation in the blank signal, it is imperative to explore measures for its reduction, as a heightened blank signal poses a more significant challenge at lower concentrations of analyte. The experiment showed an increase in S/N ratio for the newly tested method, although the increase did not align with the anticipated outcomes. The blank signal for two-time anti-BrdU addition was measured to 0.0141, and the one for single addition of detection reagent was measure to 0.0371.

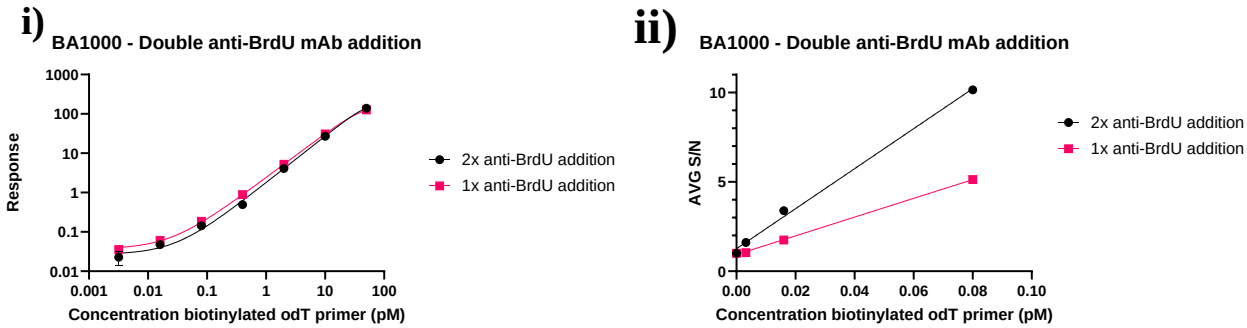


Figure 31. A standard curve of 0.0032 – 50 pM biotinylated oligo-dT primer. The reasoning for the experiment was to investigate the feasibility to decrease the blank signal or to increase the analyte signal. **i)** Double anti-BrdU-APC mAb addition in a Bioaffy® 1000 disc. **ii)** Linear regression of dataset **i** (three lowest concentrations)**,** however, the S/N ratio is plotted as the Y-axis.

4.5.3 APC conjugate dilution buffer screening – Rexxip F vs. Exazym® antibody buffer

With the knowledge that nucleotides are charged molecules and interactions between them may be influenced by ionic strength, two different detection dilution buffers were evaluated. A quick detection reagent buffer screening where Rexxip F is compared to the low-salt Exazym® antibody buffer that showed a significant increase in response values and improvement in sensitivity, see figure 32. To investigate the importance of using low-salt buffers such as the Exazym® antibody buffer for the detection reagent, an experiment was set up using the synthetic duplex assay. By using an assay model without the presence of polymerase, the experiment would therefore yield information regarding a correlation between high salt concentrations and poor response values.

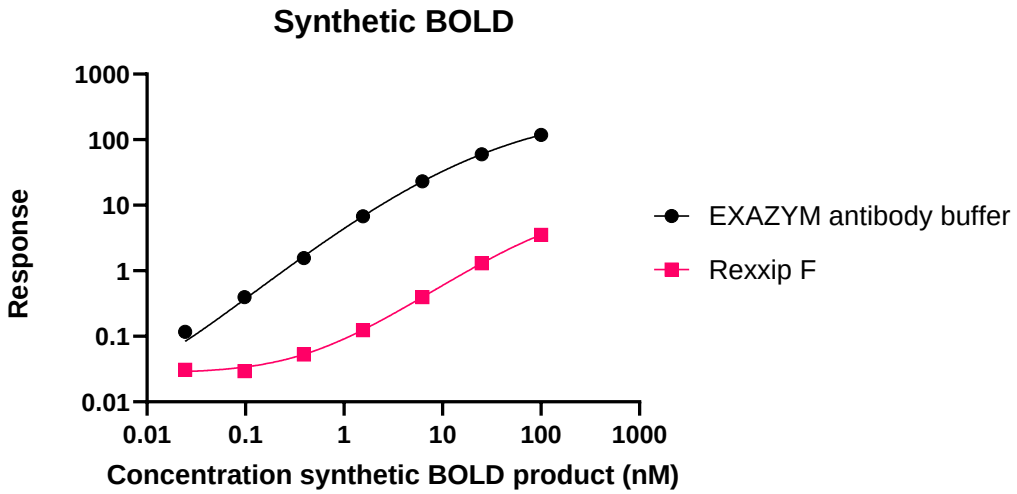


Figure 32. A two-step assay model with a standard series from 0.0244 – 100 nM of synthetic BOLD product, the experiment was carried out in a Bioaffy® 200 disc. The data provides the deduction that high-salt buffers like Rexxip F are crucial to avoid for the sake of hybridization between the anti-BrdU antibody and synthesized BrdU (PMT1%).

4.5.4 LOD screening – BOLD assays

The limit of detection was calculated for the two different BOLD assays. The limit of detection for the BOLD TNF-α assay was evaluated for Bioaffy® 200 and 4000, and the limit of detection for the biotinylated oligo-dT primer assay was assessed for Bioaffy® 200 and 1000. The limit of detection of the synthetic BOLD product (BrdU:rA duplex) was also evaluated for the Bioaffy® 200. The limit of detection is calculated by taking the average blank added to two standard deviations of the blank ($\mu_{blank} + 2\sigma_{blank}$) and is calculated by the Gyrolab instrument software. Every LOD screening are made with a total of 10 blank samples to generate a considerate pool of a blank signals.

4.5.4.1 Limit of detection – synthetic BOLD product

The limit of detection is calculated to 0.017 ng/mL (figure 33), which translates into ~0.70 pM. A more conservative estimate of LOD can also be established by using 3x the blank signal, by that principle the LOD for the synthetic BOLD would be < 0.48 ng/mL or < 20 pM.

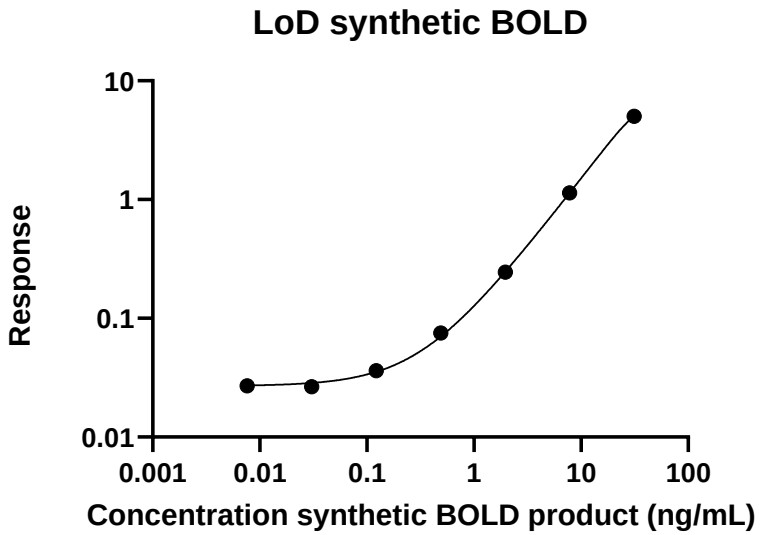


Figure 33. LOD for synthetic BOLD product measured on Bioaffy® 200. Limit of detection is calculated to 0.017 ng/mL which is a great improvement from previous the measurement of ~8.8 ng/mL (PMT1%).

4.5.4.2 Limit of detection – biotinylated oligo-dT primer

The limit of detection for the biotinylated oligo-dT primer BOLD assay was calculated to 42.7 fM for Bioaffy® 200 and 12.3 fM for Bioaffy® 1000 (figure 34, i). Using the conservative estimate method (3x blank), the LOD can be approximated to ~16 fM for Bioaffy® 1000 and ~80 fM for Bioaffy® 200.

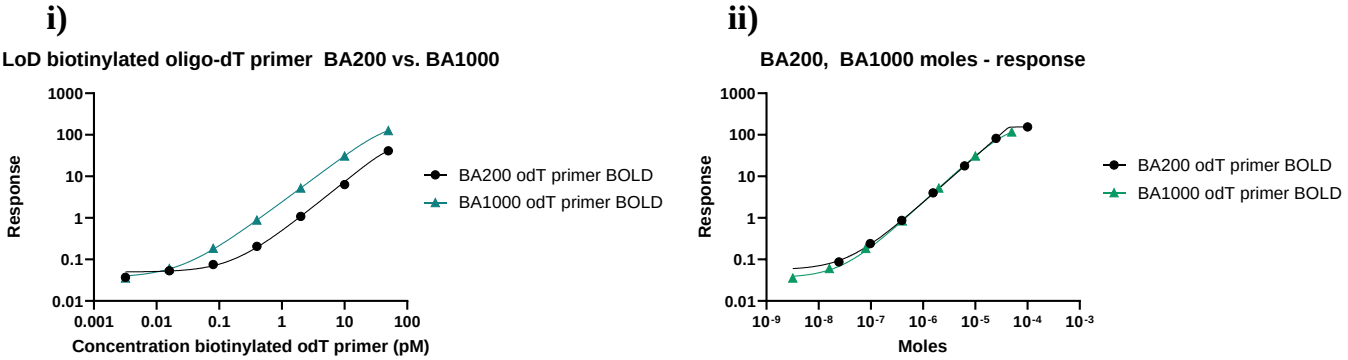


Figure 34. **i)** Comparison between the LOD screenings for biotinylated oligo-dT primer on the Bioaffy® 200 and 1000. Calculated limit of detection was set to 0.0427 pM for Bioaffy® 200 and 0.0123 pM for Bioaffy® 1000 (PMT1%). **ii)** Data from LOD screening of biotinylated oligo-dT primer where response is fitted to moles instead of concentration oligo-dT primer. The graph suggests that sensitivity in the Bioaffy® scales linearly with volume, meaning that the sensitivities are equal for both discs with respect to total amount of biotinylated oligo-dT primer passing the affinity column.

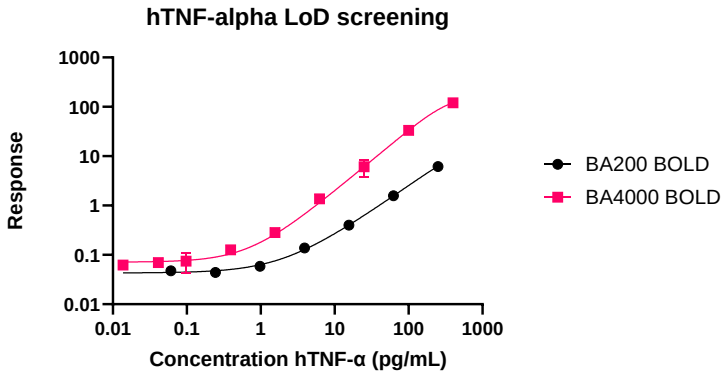
4.5.4.3 Limit of detection – TNF-α assay

The limit of detection calculated for the TNF-α assay in the Bioaffy® 200 was 0.97 pg/mL and a conservative estimate of the LOD is approximated to < 3.91 pg/mL (figure 35, i). A massive improvement from earlier screenings where the TNF-α assay for Bioaffy® 200 would not issue fluorescent signals at concentrations as high as 50 pg/mL. For Bioaffy® 4000, the limit of detection was calculated to 0.039 pg/mL and the conservative estimate is deducted to < 0.39 pg/mL (figure 35, i). A comparison between BOLD amplified TNF-α assays on Gyrolab and other ultra-sensitive TNF-α assays is shown in the table below.

Table 6. Assessment of high performing TNF-α assays in comparison to the Gyrolab sandwich assays and Gyrolab BOLD assays for Bioaffy® 200 and 4000. The data showcases a major increase in sensitivity when comparing the standard sandwich assay model to the BOLD amplification model without the need for an increased sample volume, calculated values for LOD are featured.

Method	LOD (limit of detection)	LLOQ (lowest limit of quantification)	Sample volume	Assay model format
Simoa® (SR-X)	0.0039 pg/mL	0.0171 pg/mL	100 µL	Digital bead assay
Simoa® (HD-1/HD-X)	0.016 pg/mL	0.034 pg/mL	100 µL	Digital bead assay
Erenna®	0.080 pg/mL	0.49 pg/mL	20 µL	Flow cytometry
Imperacer®	**N/A**	Low pg/mL	30 µL	iPCR
Gyrolab sandwich assay (Bioaffy® 200)	< 5.6 pg/mL	**N/A**	0.2 µL	Gyrolab
Gyrolab sandwich assay (Bioaffy® 4000)	< 0.70 pg/mL	1.0 pg/mL	4 µL	Gyrolab
BOLD Gyrolab assay (Bioaffy® 200)	< 0.97 pg/mL	**N/A**	0.2 µL	Gyrolab + Exazym®
BOLD Gyrolab assay (Bioaffy® 4000)	< 0.039 pg/mL	**N/A**	4 µL	Gyrolab + Exazym®

i)



ii) **iii)**

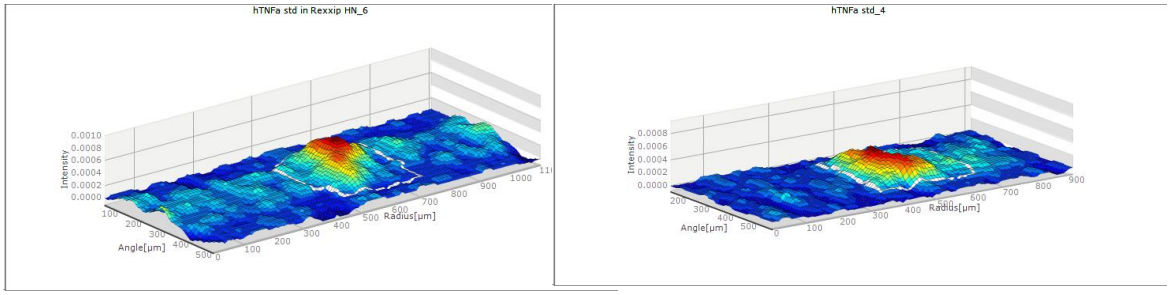


Figure 35. Limit of detection screening for a BOLD TNF-α assay carried out on Bioaffy® 200 and 4000. Calculated limit of detection for Bioaffy® 4000 was measured to 0.039 pg/mL and for the 200 CD it was 0.98 pg/mL. **i)** Data for LOD screenings for the BOLD TNF-α assays, for Bioaffy® 200 and 4000. **ii)** 3D column profile for the conservative LOD estimate (Bioaffy® 4000), a clear peak is illustrated at concentration 0.391 pg/mL. **iii)** 3D column profile for the conservative LOD estimate (Bioaffy® 200) where a clear peak is illustrated at concentration 3.91 pg/mL.

5. Discussion

5.1 Evaluation of initial BOLD experiments

The project endeavoured to successfully incorporate and implement the BOLD technology on the Gyrolab platform with the objective of establishing an ultra-sensitive assay platform for biomarkers. In order to assess the efficacy of the amplification method, initial testing involved the examination of conclusions formulated in a preceding project [13]. Cross-reaction tests were subsequently executed (Figure 21, above) to validate enzymatic activity, entailing the titration of concentration of Exazym® polymerase and BrdUTP (in reaction solution). An optimization strategy was implemented by isolating the Exazym® prA-template from the reaction solution through hybridization to the oligo-dT primer-labelled antibody before introduction of the polymerase. The assessment of how prolonged contact time in the affinity column would affect the BOLD signal amplification was conducted through a series of tests - repeated addition of Exazym® polymerase solution and extended Bioaffy® CD spin sequences, facilitating a slower flowrate. Lastly, the extent of response was measured and compared by uniquely preparing three different aliquots of the Exazym® polymerase solution.

The results of the experiments (section 4.1, above) did not reveal any significant distinctions between the data from the experiments, except for the instance where the prA template was separated from the reaction solution. However, it is crucial to note that the other experiments did not hinder the overall response.

5.2 Developing a method for BOLD amplification on the Gyrolab platform

Along the new Exazym® reagents, new BOLD tests were carried out in order to assess if there was any difference in potency for the old vs. new reagents. Generally, the data for experiments concluded with new Exazym® reagents barely yielded a higher signal, however, the CVs were improved. The new reagents were used to compare performances for the BOLD TNF-α assay in comparison to a standard sandwich TNF-α assay in a Bioaffy® 200 disc (Figure 22). The results for the BOLD TNF-α assay still demonstrated disappointing results, as the non-amplified sandwich model was able to generate a greater signal. The results created a concern for the BOLD assay model on the Gyrolab, even though the enzyme had been proven to synthesize the detectable polymer in the cross-reaction testing (Figure 21,above), the BOLD assay was therefore expected to at least reach the performance-level of the sandwich assay. The main suspicion was that reagents for one or multiple steps of the BOLD assay model were had an inadequate affinity. This led to alternative hybridizing strategies being tested, one where the oligo dT-labelled anti-TNF-α antibody (secondary antibody) was titrated in concentrations ranging from 1.0 nM to 100 nM. In addition to designing the BOLD assay around the polymerase hybridizing to the oligo-dT primer before introducing the prA template (Figure 24), which led to a loss of signal. Since the data was inconclusive as to what was the weakest link affinity-wise, the efficiency of the polymerase was demonstrated by comparing the performance and level of sensitivity to the synthesized BOLD product (Figure 26), detection of the BOLD assay showed itself as more than 100 times sensitive than the synthesized product.

Issues with affinity are a frequent and crucial complication amidst microfluidic flow-through assay models such as the Gyrolab. Barbosa *et al.* studied the change of the affinity constant (K_{eq}) with respect to changes in the association constant (k_{on}) in addition to the dissociation constant (k_{off}) based on flowrate in microfluidic heterogenous immunoassays [24]. The study was made using microfluidic strips and heterogenous immunoassays with reagent flowrates ranging from 10 to 1000 μL/min, which is significantly higher than what is used in the Gyrolab (usually 0.3 – 0.12 μL/min). The study yielded conclusions that low antigen concentrations in a reagent flow system (compared to stagnant flow) caused a 2-fold increase of the limit of detection which implies that fluid flow may interfere with molecular binding and non-specific binding (background).

5.3 BOLD amplification on the ELISA platform

At the project's midpoint, a BOLD ELISA TNF-α assay was conducted to evaluate the oligo-dT-labelled anti- TNF-α antibodies. This assessment included a comparison with a standard ELISA. The same set of reagents were employed for both the BOLD ELISA assay and he BOLD Gyrolab assay, with the only difference being the detection reagent. In the BOLD ELISA assay, the detection reagent consisted of anti-BrdU-biotin-SA-HRP in combination with TMB, as opposed to the Alexa-labelled anti-BrdU antibodies. The results of the ELISA showcased that the reagents were in fact potent together with the BOLD signal amplification working as intended. It is of importance to understand that the data (Figure 27) are from a non-optimized ELISA with a TMB substrate incubation time of 12 minutes. This is showcased by the huge amplification for the BOLD ELISA as the signals exceeded the dynamic range of the ELISA reader. The illustrated potency of BOLD amplification caused suggestions towards the anti-BrdU detection antibody, as it was the only differing factor between the Gyrolab and ELISA setup.

5.4 Signal amplification on the Gyrolab platform – a new fluorophore

The BOLD amplification was demonstrated by replacing the Alexa-labelled anti-BrdU antibody with an APC-conjugated anti-BrdU antibody, which thereby served as the standard detection reagent. This modification of fluorophore highlighted the synergistic potential for biomarker assays utilizing BOLD amplification on the Gyrolab platform. As the obtained results began to exhibit anticipated signals indicative of effective amplification in addition to producing improved CVs, even for lower concentrations. The sensitivity for the different BOLD assays and the synthetic BOLD product one-step assay were massively improved, however, this also indicates that the first tests showcasing the efficiency of the Exazym® polymerase was exaggerated (section 4.2.4, above). Where the biotinylated oligo-dT primer was estimated to be approximately 195 times more sensitive than the synthetic BOLD product. Repeating the same experiment, however, this time with APC as the fluorescent conjugate (section 4.5.4, above). The difference in sensitivity between the two are now about 16 times in favour of the biotinylated oligo-dT primer model assay, which is significantly less than before. Although, a number aligning with the claims of Cavidi regarding Exazym® [25]. The BOLD TNF-α assay was also exceedingly improved, where concentrations ~25 times lower than before were able to be distinguished in comparison with the old fluorescent conjugate (Alexa Fluor 647) on the Bioaffy® 200. Direct correlations between the standard Gyrolab sandwich assay and the new BOLD assay were not assessed as there was no TNF-α detection reagent conjugated with APC available, which constitutes an interesting experiment for future works. It is therefore difficult to pinpoint the exact reason for the increase in response without an additional APC-conjugated detection antibody for another model assay. Both Alexa Fluor 647 and APC are proclaimed as very bright fluorophores. However, APC being a large molecule (105 kDa) carries multiple chromophore units and notoriously exhibits a high ratio of quantum yield (70%) [26], APC therefore has more than two times the quantum yield of Alexa Fluor 647 (33%) [27]. The quantum yield is the measurement of which a fluorophore converts absorbed photons into emitted photons, usually generating a higher signal than most fluorophores [28].

Another simpler potential reasoning for the newly generated amplified signal may be because of the APC-conjugated anti-BrdU antibody might obtain a higher affinity for the synthesized BrdU duplex. The possibility of a higher-affinity antibody does not seem improbable as the background signal is considerably higher with the APC-conjugated variant, an increased affinity for BrdU could further potentially inflate the background signal by binding non-specifically to over-labelled oligo-dT primers on the primary detection antibody. One reason for believing that the APC-conjugated antibodies have a better binding frequency is because the Alexa Fluor conjugates might possibly be poorly conjugated, it is therefore feasible that multiple Alexa Fluor conjugates are conjugated to the binding site of the detection antibody due to its small molecular weight (~1 kDa). In addition to this, the Alexa-labelled antibody was announced to have a degree of labelling of 0.9, which may give more room for a decreased signal. One possible way of combating the effect of poor conjugations is by testing click chemistry conjugation methods such as GlyClick (Genovis, Sweden) [29]. The method promises highly specific

incorporation of conjugates (Fc-region specific) with a low degree of labelling, resulting in a stable and homogenous antibody conjugate which are suitable for sensitive applications. By utilizing glycan-mediated conjugation chemistry for the oligo-dT primer and Alexa Fluor647, the risk of conjugating the DNA primer or the fluorophore to the Fab-region of the antibody will diminish.

The successful application of BOLD signal amplification on the Gyrolab led to a small number of former experiments being revisited. The possibility of further signal amplification was examined by utilizing multiple addition of the detection reagent (section 4.5.2, above) in order to combat the newly gained blank signal. The result for the experiment yielded better CVs and a higher S/N ratio, however, not in the fashion that was expected as an approximately 3 times lower blank signal was generated for the new method in comparison to the single addition of detection reagent. Through multiple addition of detection reagent, the suggestion was believed to increase the raw signal in addition to the blank by binding into the synthesized BrdU duplex in a higher quantity. In company of the successfully lowered blank signal the method yielded a lower calculated limit of detection (3.3 fM) for Bioaffy® 1000 which is about four times lower than what previously was calculated (12.3 fM). It is crucial to understand that this experiment was only conducted once, and that there is limited research available on multiple reagent flow addition of detection agent in microfluidic assay systems. However, it is a possibility that the BOLD assay model in the Gyrolab can utilize optimization strategies via multiple reagent addition.

Incubation of the Exazym® polymerase solution was assessed once more, where the polymerase was prepared according to section 3.3.1 (above) and let to incubate for 1 hour in room temperature before run execution. The outcome of the experiment is telling of a decline in enzymatic activity, thus resulting in a generally lower signal generated in addition to a lower S/N ratio, which aligns accordingly to Cavidi's findings. However, it is apparent that the incubation strategy generates a much lower background signal that may suggest a higher sensitivity when calculating the limit of detection. Although, judging from generated 3D column profiles for the different methods (Figure 30), the lower calculated limit of detection may therefore be interpreted incorrectly. This experiment was not thoroughly tested with APC as the fluorescent conjugate and would be interesting to further investigate, in addition to a retrial of experimental setup 3.4.2 (above). Moreover, there is a possibility that incubation of the Exazym® polymerase solution may be beneficial for optimizing sensitivity in a BOLD assay on the Gyrolab platform, as reducing background signal is implied to be one of the greatest challenges moving forward.

APC as a fluorescent conjugate has contributed to revealing the potential for signal amplification for BOLD biomarker assays on the Gyrolab platform, as the BOLD TNF-α assay has ~18 times better sensitivity in comparison to the standard Gyrolab TNF-α kit for the Bioaffy® 4000. Application of BOLD technology with APC-conjugated detection antibodies to the Gyrolab has resulted in improvement for all assays in comparison to the BOLD assay model with Alexa-labelled detection agents. However, moving forward in the project, a further understanding regarding how amplification scales with each fluorescent conjugate is necessary. Further studies should involve utilizing an APC-conjugated anti-TNF-α antibody in a Gyrolab sandwich assay. This approach aims to exemplify the impact of APC and provide insight into its potential efficacy, along with a comprehensive assessment of the anticipated rise in background signal. The uncertainty surrounding the substantial increase in background noise when substituting Alexa Fluor 647 with APC necessitates closer examination. There is a concern that the background escalates disproportionately due to potential nonspecific binding of the primary detection agent. The oligo-dT primer-conjugated anti-TNF-α antibody was found to be over-labelled with oligo-dT primer due to the harsh conjugation conditions [13]. Therefore, an anti-BrdU antibody that employs a higher affinity might result in an increased unwanted binding frequency (unspecific binding), contributing to an elevated background signal.

Conclusion

In summary, the journey to enhance the BOLD assay on the Gyrolab platform unfolded with an evaluation of initial experiments, addressing concerns about reagent stability and replication challenges. The pivotal change to APC-conjugated antibodies as detection reagent was inspired by the BOLD ELISA assay. As the ELISA TNF-α assay showcased reagent potency and successful BOLD signal amplification, sparking the idea to substitute Alexa-labelled anti-BrdU antibody with an APC-conjugated counterpart for the Gyrolab platform.

The introduction of APC as a fluorescent conjugate marked a transformative breakthrough, significantly enhancing sensitivity and signal-to-noise ratios. The new conjugate also served as a beacon of light, showcasing that BOLD amplification is applicable on a miniaturized flow-through Gyrolab assay. In addition to proving that every molecular process that BOLD amplification relies on are fully operational on a nanolitre-scale. The observed improvement of sensitivity with the new fluorescent conjugate for the BOLD assays were considerable. The BOLD TNF-α Gyrolab assay demonstrated LOD values clearly below the pg/mL-scale and an improvement of 18 times in comparison to the sandwich Gyrolab assay for Bioaffy® 4000 (see Table 6). In comparison to the Alexa-labelled detection antibody, concentrations 25 times lower were able to be determined for the BOLD amplified TNF-α assay with APC-labelled detection reagent for Bioaffy® 200.

Moreover, the BOLD-enhanced sensitivity of the TNF-α assay on the Gyrolab marked substantial progress, the measured limit of detection is nearing the levels of sensitivity of ultra-sensitive biomarker assays such as Simoa® HD-X and SR-X. Notably, application of Exazym® technology on the Gyrolab platform allowed for automated and sensitive biomarker assays with a run-time of approximately 1.5 hours without the need for auxiliary instrumentation.

To reach lower sensitivity levels, studies where APC-conjugated detection reagents are used in Gyrolab sandwich assays are critical for understanding the efficacy and addressing potential challenges with background signal. Further studies should also contain newly conjugated oligo-dT primer antibodies, preferably with glycan-mediated conjugation technology to potentially achieve lower nonspecific binding resulting in lower background signals for the assay. Future work should also consider how to decrease the background signal by utilizing new dilution buffers, blocking buffers and wash solutions for the Gyrolab. Efforts should also be made to determine the lower limit of quantification (LLOQ) along with performing quantification studies in different sample matrices going forward. Gaining an understanding and investigating how the background signal is affected based on assay environment is crucial for decreasing the background signal.

The Gyrolab and BOLD technologies complement each other well, resulting in a significant improvement in sensitivity without increasing the sample volumes nor the addition of auxiliary instruments, which is a requirement for other ultra-sensitive assay models. As we delve deeper into the dynamics of amplification with different fluorescent conjugates, the journey continues on the cutting edge of bioanalytical innovation. Striving for precision that matches industry benchmarks, the ongoing efforts promise to reshape the landscape of advanced biomarker detection on the Gyrolab platform.

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Appendix

$$\frac{k_{on}}{k_{off}} = K_{eq}$$

Figure A. Equation for the association constant.

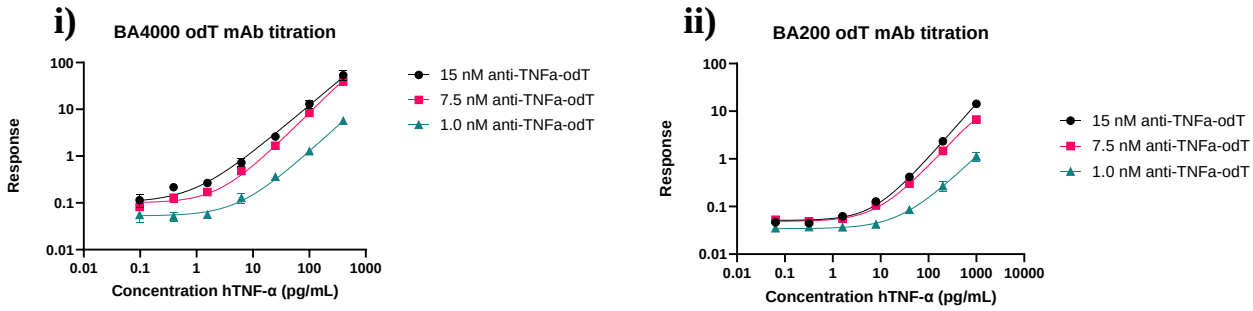

Figure B. Data from **4.2.2 Titration of oligo-dT-labelled anti-TNF-α antibody**. The two graphs display the same exact experiment, however, **i)** is performed on a Bioaffy® 4000 disc and **ii)** is carried out on a Bioaffy® 200 disc.

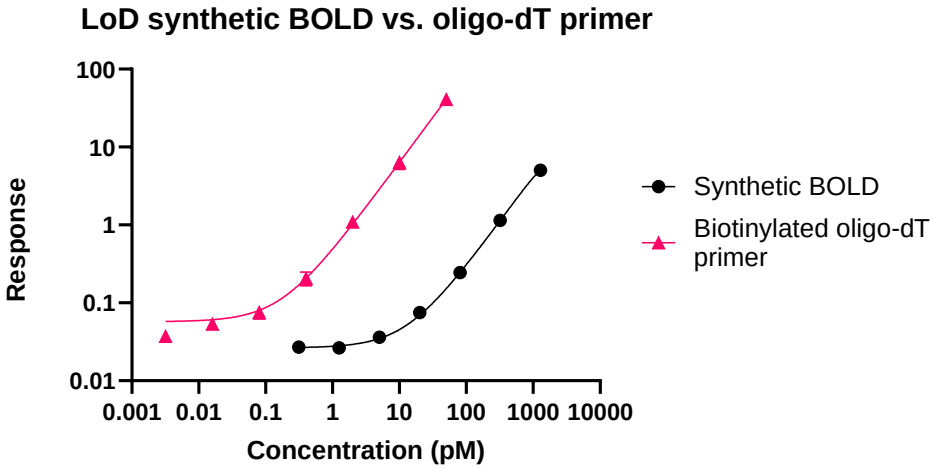

Figure C. LOD screening data from biotinylated oligo-dT primer (**Figure 34**) vs. LOD data for the synthetic BOLD duplex (**Figure 33**). The data from Bioaffy® 1000 is excluded from this graph as no testing for the synthetic BrdU:rA duplex was made for the disc.

i) **ii)**

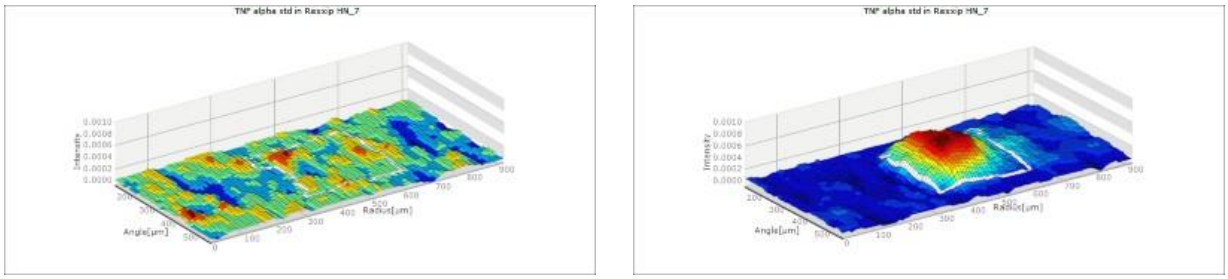

Figure D. 3D column profile data for BOLD TNF-α assay (Bioaffy® 200), the column profiles are both for 6.25 pg/mL TNF-α and visualize the great difference of intensity between APC and Alexa Fluor647 conjugated anti-BrdU. **i)** BOLD TNF-α assay with anti-BrdU-Alexa Fluor647 as detection reagent. **ii)** BOLD TNF-α assay with anti-BrdU-APC as detection reagent.