UPTEC X 06 009
JAN 2006
ISSN 1401-2138

MARIA THURFJELL

Development of an assay for quantification of anti-dsDNA autoantibodies using CD microlaboratories

Master’s degree project

UPPSALA UNIVERSITET

Molecular Biotechnology Programme

Uppsala University School of Engineering

UPTEC X 06 009	**Date of issue 2006-01**

Author

Maria Thurfjell

Title (English)

Development of an assay for quantification of anti-dsDNA autoantibodies using CD microlaboratories

Title (Swedish)

Abstract

The presence in serum of anti-dsDNA autoantibodies is one of the diagnostic criteria for systemic lupus erythematosus. A microfluidic method for detection of these antibodies was developed using the Gyrolab Bioaffy™ platform. Immobilized synthetic dsDNA was utilized for capture of sample antibodies, which were then detected by fluorescence-labeled secondary antibodies. The anti-dsDNA antibody levels in eighty-one serum samples were determined and the results compared to those obtained with a standard method, *Crithidia luciliae* immunofluorescence. The Bioaffy™ based method appears promising, producing specific and reproducible results with less time and effort than the conventional assays.

Keywords

Antibody assay, systemic lupus erythematosus (SLE), autoantibody, anti-dsDNA antibody, Gyrolab Bioaffy™, *Crithidia luciliae* immunofluorescence (CLIF)

Supervisors

Mats Inganäs
Gyros AB

Scientific reviewer

Jan Carlsson
Department of Surface Biotechnology, Uppsala University

Project name	Sponsors
Language **English**	Security
ISSN 1401-2138	Classification
Supplementary bibliographical information	Pages **40**

Biology Education Centre Biomedical Center Husargatan 3 Uppsala
Box 592 S-75124 Uppsala Tel +46 (0)18 4710000 Fax +46 (0)18 555217

Development of an assay for quantification of anti-dsDNA autoantibodies using CD microlaboratories

Maria Thurfjell

Sammanfattning

Antikroppar används av immunförsvaret för att känna igen främmande ämnen som ska oskadliggöras. Vid autoimmuna sjukdomar är immunförsvaret felprogrammerat så att antikroppar mot kroppsegna ämnen, s.k. autoantikroppar, förekommer. Auto-antikroppar mot dubbelsträngat DNA (dsDNA) finns hos patienter med den autoimmuna sjukdomen systemisk lupus erythematosus (SLE). Förekomst av dessa antikroppar är ett viktigt kriterium när diagnosen SLE ställs, och antikroppsnivån bevakas sedan i behandlingssyfte.

I detta examensarbete har en metod för att bestämma koncentrationen av antikroppar mot dsDNA i serumprover utvecklats och utvärderats. Dagens standardmetoder är tidskrävande och besvärliga, och det vore därför önskvärt med ett snabbt, enkelt alternativ som är lika tillförlitligt. Tekniken bygger på att antikropparna i provet binder till dsDNA och därefter detekteras med fluorescensmärkta sekundära antikroppar, d.v.s. antikroppar som är specifika för antikroppar från människa i provet. Metodiken är utvecklad för CD-mikrolaboratorier från Gyros AB, Uppsala.

Utvärdering av metoden visade på hög reproducerbarhet och bra mätområde. Resultat för 81 serumprover jämfördes med resultat för samma prover utvärderade med en av dagens standardmetoder. Genom val av lämplig koncentrationsgräns för positiv och negativ provklassificering kan hög specificitet erhållas med det nya analyssystemet. För ytterligare utvärdering av metoden hade det varit önskvärt att analysera fler prov och jämföra resultaten med diagnostiska patientdata.

Examensarbete 20 p
Civilingenjörsprogrammet i molekylär bioteknik

Uppsala universitet januari 2006

Contents

Abbreviations

BAA	Bridging antibody assay
bp	Base pairs
BSA	Bovine serum albumin
CLIF	*Crithidia luciliae* immunofluorescence
dsDNA	Double-stranded DNA
ELISA	Enzyme-linked immunosorbent assay
FRET	Fluorescent resonance energy transfer
HSA	Human serum albumin
IAA	Indirect antibody assay
IgG	Immunoglobulin G
kb	Kilobases (1000 bp)
LIF	Laser-induced fluorescence
PBS	Phosphate-buffered saline: 15 mM $NaPO_4$ pH 7.4, 0.15 M NaCl, 0.02% NaN_3
PBS-T	PBS with 0.01% Tween® 20
SLE	Systemic lupus erythematosus
ssDNA	Single-stranded DNA

1 Introduction

1.1 Systemic lupus erythematosus and anti-dsDNA antibodies

Systemic lupus erythematosus (SLE) is an autoimmune rheumatic disease, affecting approximately 1 in 2250 Swedes. SLE patients, of whom 80-90% are female, typically develop the disease between 20 and 40 years of age. Fatigue, weight loss and fever are general symptoms; affected organs include joints, skin, kidneys, central nervous system, heart and lungs. Alternating periods of remission and relapse are characteristic of the disease [1,2].

The American College of Rheumatology lists 11 diagnostic criteria for SLE, stating that for diagnosis, at least 4 should be fulfilled [3,4]. The presence of antibodies against double-stranded DNA (anti-dsDNA antibodies) in SLE patient sera was first described in 1957 [5]. Since presence of anti-dsDNA antibodies is specific for SLE, it constitutes one of the most important criteria, and tests for these antibodies have high diagnostic value. Approximately 60-80% of SLE patients have detectable anti-dsDNA antibodies, depending on the assay method utilized [5,6]. Anti-dsDNA antibodies are not only diagnostic, but also involved in SLE pathogenesis. Together with extracellular DNA, they form immunocomplexes that are deposited in end organs, such as the kidneys, causing inflammation and organ damage [6]. The serum levels of anti-dsDNA antibodies often increase before disease flares, making monitoring of these levels critical in disease management [7].

SLE treatment is adjusted based on symptoms and severity, and ranges from administration of anti-inflammatory drugs to administration of immunosuppressive drugs. New treatments are continuously researched [1,2]. Recently, injection of an IgG antibody with a mutated Fc region that confers increased binding affinity to Fc receptors resulted in decreased concentrations of endogenous IgG antibodies in mice [8]. The authors suggest that engineered antibodies could enhance antibody clearance in antibody-mediated diseases such as SLE. Such therapy could be monitored by antibody quantification.

1.2 Detection of anti-dsDNA antibodies

Today, the three standard laboratory tests for quantification of anti-dsDNA antibodies are enzyme-linked immunosorbent assay (ELISA), Farr assay and *Crithidia luciliae* immunofluorescence (CLIF), which are briefly described below.

- In a typical anti-dsDNA antibody ELISA, purified mammalian or bacterial dsDNA is immobilized in microtiter plate wells and incubated with diluted serum sample. Bound IgG antibodies are detected with enzyme-linked anti-human IgG antibodies and a substrate that changes color when exposed to the enzyme [5].
- The Farr assay is a radioimmunoassay using radiolabeled antigen. Antibody-antigen complexes are precipitated with ammonium sulfate or polyethylene glycol and the fraction of precipitated antigen is determined at different serum dilutions [5].
- The CLIF assay is based on the detection of antibodies binding to the kinetoplast DNA of the protozoan *Crithidia luciliae*. Serum dilutions are incubated with *C. luciliae* organisms, followed by incubation with

fluorescence labeled secondary antibodies. The fluorescence level for each dilution is determined through immunofluorescence microscopy [5].

There are disadvantages associated with all of the assays described above. ELISA tests can give false positive results, possibly due to ssDNA contamination. The Farr assay requires use of radioactivity and may also have problems with dsDNA purity. The CLIF method can generate false positives in patients with anti-histone antibodies, due to histones present in the kinetoplast [9]. Additionally, these methods are rather time consuming, which makes the development of a time efficient assay with good performance appealing.

1.3 Bridging antibody assay vs. indirect antibody assay

A bridging antibody assay (BAA) utilizes the ability of antibodies to bind two antigens simultaneously. One antigen binding site binds an immobilized antigen (capture reagent) and the other binds a labeled antigen (detection reagent), so that the antibody forms a bridge between them (Figure 1a). With this technique, the specific antibody-antigen interaction that is under investigation is used both for capture and detection. The assay does not discriminate between antibody classes or isotypes, nor does it discriminate between different species. These features may simplify comparison of antibody profiles between species.

As a comparison, the commonly used indirect antibody assay (IAA) uses a labeled antibody specific for the Fc region of the antibody being assessed for detection (Figure 1b), and thus detects a certain type of antibody, e.g. IgG. This can be advantageous or disadvantageous, depending on the application. A clear disadvantage, however, when compared to the BAA, is that any nonspecifically bound antibodies will also be detected. The typical anti-dsDNA antibody ELISA and CLIF assay described in section 1.2 are examples of IAAs.

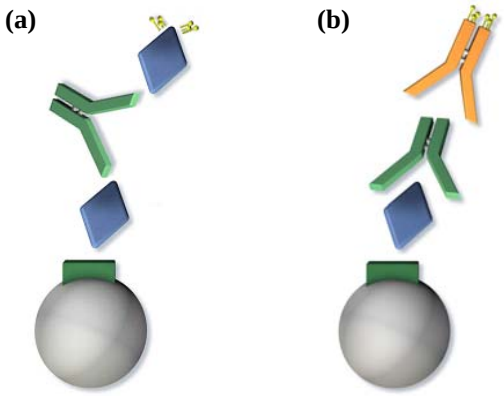

Figure 1. Antibody assays.
(a) Bridging antibody assay (BAA). Detection is performed using labeled antigen.
(b) Indirect antibody assay (IAA). Detection is performed using labeled secondary antibody.
Illustration used with permission from Gyros AB.

Successful use of a bridging ELISA for detection of anti-erythropoietin antibodies in human sera has been reported [10]. In this case, the second antigen was digoxigenin labeled and detection was performed using an anti-digoxigenin antibody coupled to horseradish peroxidase. Another bridging ELISA, an immunogenicity assay for monoclonal antibodies, used horseradish peroxidase coupled to the second antigen [11]. This assay is more similar to the BAA principle described above, since the second antigen is directly used as a detection reagent (through addition of horseradish peroxidase substrate). Also, a recent BAA for detection of antibodies against therapeutic human monoclonal antibodies was reported to have high sensitivity and reproducibility [12]. Here, biotin- and ruthenium-conjugated drug compounds were used for capture and detection, respectively.

IAAs for detection of anti-dsDNA antibodies have also been reported to function satisfactory. One ELISA uses biotinylated plasmid bound to streptavidin-coated plates as capture reagent and peroxidase-linked goat anti-human IgG for detection. This assay has been reported to perform well and to produce fewer false positives than other ELISAs using heterogeneous DNA [9]. Modern ELISAs are suggested to have diagnostic accuracies similar to the Farr assay [13], which together with the CLIF assay is the most widely accepted method for detection of anti-dsDNA antibodies.

1.4 Gyrolab Bioaffy™

Gyros AB has developed a compact disc (CD) microlaboratory, Gyrolab Bioaffy™, for protein quantification at the nanoliter scale. The plastic CD is 12 cm in diameter and consists of 14 segments, with 8 microstructures in each (Figure 2). Thus, parallel processing of 112 samples on one CD is possible. The analysis process is performed in an automated fashion and detected with laser-induced fluorescence (LIF) using a special instrument, the Gyrolab™ Workstation LIF. A biotinylated capture reagent is first immobilized onto streptavidin-coated polystyrene beads in the capture column, followed by addition of analyte and fluorescent detection reagent, with intermittent washing steps. Gyrolab Bioaffy™ was originally developed for sandwich immunoassays, in which antibodies are utilized both for antigen capture and detection, but can readily be adapted for antibody quantification in bridging or indirect antibody assays.

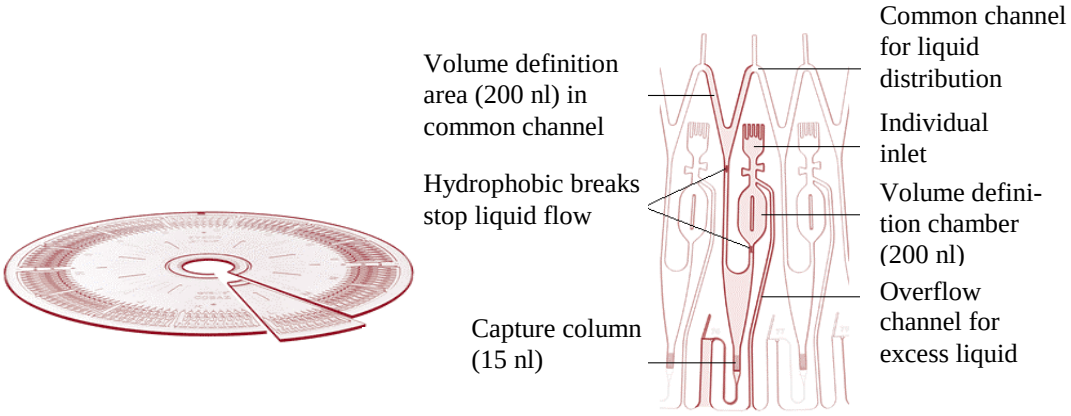

Figure 2. Gyrolab Bioaffy™ CD (left) with detailed microstructure (right). *Illustration used with permission from Gyros AB.*

Liquids can be added to an entire segment or to a specific microstructure. The liquids are drawn into the CD channels by capillary action, and propagated through the channels and columns by centrifugal force. Exact control over added volumes is achieved through volume definition chambers and hydrophobic breaks. Altering the rotational speed controls the flow-rate. The fluorescent signal of bound detection reagent is detected in the LIF station integrated in the workstation.

Gyrolab Bioaffy™ uses considerably smaller volumes of samples and reagents than conventional assay methods, and offers a high degree of automation. Up to five CDs can be analyzed in one run. Only 2.5 µl each of capture and detection reagents are needed per segment (serving eight microstructures), and only 420 nl of analyte per microstructure.

2 Aim

The goal of this degree project was to develop an assay for quantification of anti-dsDNA antibodies using Gyrolab Bioaffy™. Both bridging and indirect antibody assay formats were evaluated. For the BAA, biotinylated dsDNA was used to capture the antibodies, which were then detected with Cy™5 labeled dsDNA. The IAA utilized the same biotinylated dsDNA as capture reagent, but detection was performed with Alexa Fluor® 647 labeled secondary antibodies. The performance regarding diagnostic sensitivity and specificity was compared to a standard laboratory test, the CLIF assay used at the Department for Clinical Immunology, Uppsala University Hospital. The aim was to develop a Gyrolab Bioaffy™ method that was as diagnostically accurate as the CLIF assay, but less labor intense.

3 Materials and methods

3.1 Capture and detection reagents for bridging antibody assay

Two types of DNA molecules were evaluated as antigens. One was a 3.4 kb bacterial plasmid and the other was a linear 30 bp DNA molecule. When designing an assay for detection of anti-dsDNA antibodies, it is imperative that the DNA used does not contain any single-stranded regions. Presence of ssDNA can cause false positives, since anti-ssDNA antibodies are found in patients with autoimmune rheumatic diseases other than SLE and also in some normal individuals [5,14]. Two independent antigen systems were evaluated to increase the chances of a serviceable assay. Use of a streptavidin-biotin immobilized plasmid has previously been shown to be beneficial with regards to dsDNA purity in ELISA [9]. Use of a short synthetic DNA molecule as antigen appears to be a novel design for anti-dsDNA antibody detection.

3.1.1 Plasmid

Bacterial (*E. coli*) plasmid DNA was obtained from DIARECT (Freiburg, Germany). According to the supplier, the antigen is highly purified and should contain no antigenic protein contaminants, which could potentially cause false positives. The antigen specification stated that the plasmid could be used for SLE analysis, including functionality with the reference antibody described in section 3.3.1 below. The plasmid DNA was labeled with biotin or Cy™5 using *Label* IT® nucleic acid labeling kits (Mirus Bio, Madison, WI), which according to the manufacturer adds approximately one biotin or Cy™5 molecule per every 20-60 bp of dsDNA. Biotinylation of DNA of up to one biotin molecule per 30 bp has been reported not to affect the antigenicity of the DNA [9]. Labeling was performed according to the manufacturer's instructions. Briefly, DNA was incubated with reagent (1:1 ratio (v:w) of reagent to DNA) for 1 hour at 37°C before purification in enclosed G50 microspin purification columns. The concentration after labeling was approximately 0.1 mg/ml or 45 nM.

3.1.2 Oligomer

30 bp oligonucleotides were obtained from TIB MOLBIOL (Berlin, Germany) (for sequences, see Appendix 1). The lyophilized oligonucleotides were dissolved in distilled water to a concentration of 20 μM. 5'-biotinylated and 5'-Cy™5-labeled oligonucleotides were hybridized with unlabeled complimentary oligonucleotides to form dsDNA oligomers labeled at one end with biotin or Cy™5, for capture and detection, respectively. Unlabeled oligonucleotide was used in excess, to minimize the amount of labeled single-stranded DNA. Single-stranded oligonucleotides without biotin labels in the capture reagent should not bind to the column, and single-stranded oligonucleotides without Cy™5 labels in the detection reagent are not likely to affect the results.

Different proportions of Cy™5-labeled and unlabeled oligonucleotides were examined for the hybridization. It was assumed that the biotinylated sequence would have the same affinity for the unlabeled sequence as the Cy™5-labeled one, so that the results could be considered general. The concentration of labeled oligonucleotide was 0.1 μM and that of unlabeled complementary oligonucleotide was between 0 and 1.6 μM, in a final volume of 50 μl annealing buffer (20 mM Tris-acetate, 2 mM magnesium acetate, pH 7.6). The mixed oligonucleotides were incubated at 95°C for 5 minutes followed by at least 15 minutes at room temperature.

The hybridizations were evaluated by fluorescent resonance energy transfer (FRET). 50 µl Quant-iT™ PicoGreen® dsDNA reagent (Molecular Probes, Eugene, WA), a fluorescent dye that binds to dsDNA, was added to 10 µl hybridization samples in black fluorimeter plate wells, followed by fluorescence detection in a microplate reader. All samples were analyzed in duplicate.

FRET can occur between fluorophors with overlapping emission and excitation peaks. In this case, the microplate reader emits light with the excitation wavelength of the PicoGreen® fluorophor. When the PicoGreen® reagent emits light, Cy™5 is in its turn excited, and energy with the emission wavelength of Cy™5 can be detected. This occurs when the two fluorophors are physically close; in this case, when the DNA is double-stranded (Figure 3).

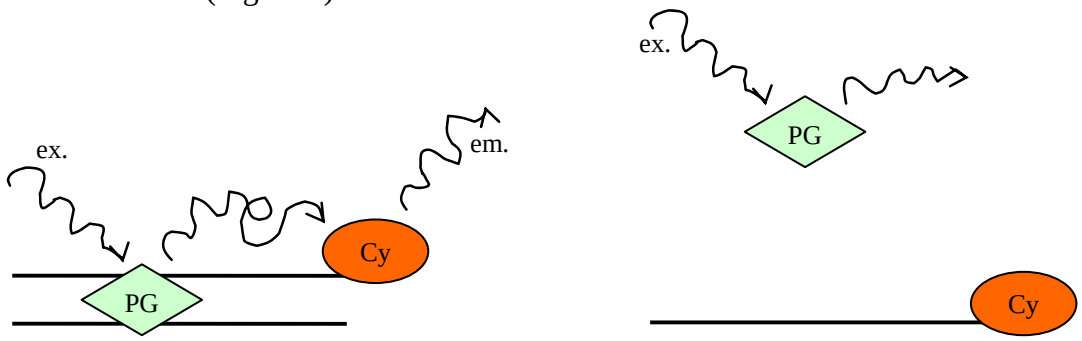

Figure 3. Schematic representation of FRET. When the donor (PicoGreen®) is bound to the dsDNA it is close enough to the acceptor (Cy™5) for energy transfer to occur (left). When the donor is unbound, it cannot deliver its emission energy to the acceptor, and no Cy™5 emission is detected (right).

For the final hybridization, a concentration ratio with 4 times more of the unlabeled oligonucleotide was chosen (see FRET results in Figure 7). The concentrations were 1.25 µM and 5 µM for the labeled and unlabeled sequences, respectively. Thus, the concentration of biotinylated and Cy™5-labeled oligomers could be estimated to 1.25 µM. The final volume was 160 µl.

3.1.3 Bovine serum albumin

Bovine serum albumin (10% BSA solution, VWR International, Stockholm, Sweden) was biotinylated with EZ-Link® Sulfo-NHS-LC-Biotin (Pierce Biotechnology, Rockford, IL). The 10 mM biotin reagent was added in 12-fold molar excess to a 1 mg/ml BSA solution, and the mixture was incubated for 1 hour at room temperature. According to the biotin manufacturer, this should yield incorporation of 3-5 biotins per BSA molecule. Protein desalting spin columns (Pierce Biotechnology) were used according to the manufacturer's instructions to remove free biotin and exchange the buffer to PBS. The final volume was approximately 330 µl, and the protein concentration was verified to remain 1 mg/ml by spectrophotometry at 280 nm. The biotinylated BSA was mixed with the biotinylated dsDNA in the capture reagent to decrease nonspecific interactions between samples and capture column.

A second biotinylation was performed, aimed to yield 1 biotin per BSA molecule. The procedure above was followed, with biotin reagent added in 6.7-fold molar excess to one vial and 4-fold to another. Later results (section 4.2.1) showed that the 4-fold ratio was closest to producing 1 biotin per BSA molecule. These biotinylated BSA solutions, used for a capture reagent immobilization study (section 3.5.1), are referred

to as sparsely biotinylated BSA in this report. The final volumes were approximately 100 μl and the concentrations 1 mg/ml, in both cases.

3.2 Capture and detection reagents for indirect antibody assay

3.2.1 Capture reagents

The biotinylated plasmid and oligomer preparations from sections 3.1.1-3.1.2 were used as capture reagents in this assay format as well as in the BAA. However, no BSA was added to the capture reagent.

3.2.2 Human serum albumin

Lyophilized human serum albumin (HSA) (Sigma-Aldrich, Stockholm, Sweden) was reconstituted in PBS without NaN_3, to a concentration of 1 mg/ml. Possible IgG contamination was removed by passage through a HiTrap™ Protein G HP column (GE Healthcare, Uppsala, Sweden). The concentration of the effluent was determined by spectrophotometry at 280 nm to 0.52 mg/ml. 100 μg HSA solution was concentrated to 1 mg/ml using a Nanosep® 30K Omega™ centrifugal device (Pall Corporation, East Hills, NY), which allows liquid and small molecules to pass through but retains molecules larger than 30 kDa, such as HSA. Biotinylation and desalting was performed as for the BSA in section 3.1.3, with biotin in 12-fold molar excess. The buffer was exchanged for PBS with NaN_3 during the desalting step. The final volume was approximately 110 μl, and the concentration was 0.79 mg/ml. This biotinylated HSA was used as capture reagent in specificity studies in sections 3.7.2-3.7.3.

3.2.3 Detection reagents

Two detection reagents were examined, a goat anti-human IgG $F(ab')_2$ fragment (Code number 109-006-008, Jackson ImmunoResearch, Soham, United Kingdom) and a mouse anti-human IgG monoclonal antibody (Catalogue number 9040-01, SouthernBiotech, Birmingham, AL) (Figure 4).

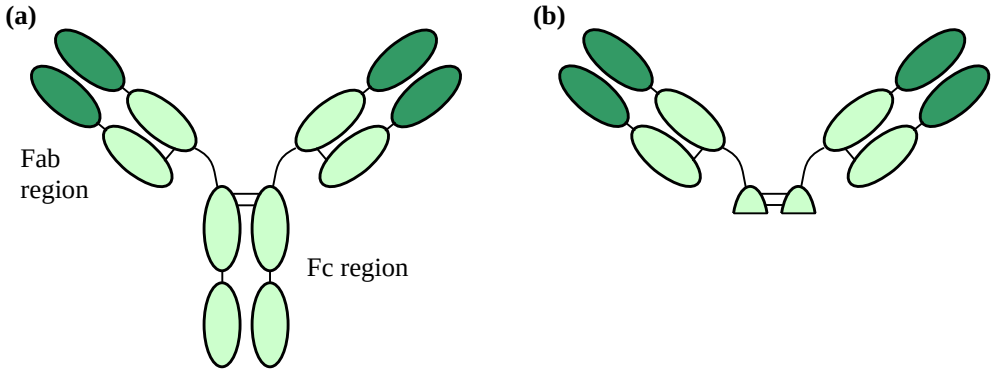

Figure 4. Schematic representation of **(a)** IgG molecule and **(b)** $F(ab')_2$ fragment. Variable, antigen binding domains in darker green. Both the $F(ab')_2$ fragment and the monoclonal antibody used for detection are specific for the Fc region of human IgG.

Labeling of the $F(ab')_2$ fragment with Alexa Flour® 647 (Molecular Probes, Eugene, WA) was performed according to the manufacturer's instructions. 100 μg $F(ab')_2$ fragment in 0.1 M sodium bicarbonate was mixed with the dye. After incubation for 1 hour at room temperature, the labeled fragments were purified using a spin column enclosed in the kit. The final volume was approximately 90 μl. The protein concentration was calculated to 4.5 μM and the degree of labeling to 2.7 moles dye per mole protein, after spectrophotometry at 280 and 650 nm. 100 μl of 1 μM Alexa-

labeled monoclonal antibody was kindly provided by Ulrika Lindberg, Gyros AB. The labeling of this antibody was performed following the same procedure as for the $F(ab')_2$ fragment, yielding 4.6 moles of dye per mole protein.

3.3 Analytes

Before use, serum samples were vigorously vortexed followed by centrifugation for 15 minutes at 4000 rpm. The bottom layer was avoided when pipetting. This procedure decreases the risk of clogging the column.

3.3.1 Reference antibody

Serum Wo/80, the international standard for anti-dsDNA antibody [15], was obtained from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service to be used for assay development. The vial was reconstituted with 500 µl of distilled water, as instructed in the information leaflet, to an anti-dsDNA antibody concentration of 200 international units (IU) per ml.

3.3.2 Negative control

Serum from a healthy blood donor (internal Gyros AB supply) was used as a negative control throughout the study.

3.3.3 Serum samples

Eighty-one CLIF tested serum samples were kindly provided by Dr. Bo Nilsson and Elisabeth Wijkström at the Department for Clinical Immunology, Uppsala University Hospital. CLIF test results were provided after Gyrolab Bioaffy™ evaluation was performed, specifying fourteen negative and sixty-seven positive samples (Appendix 2).

3.4 Assay procedure

Assays in this project were performed using the workflow below, adapted from Gyrolab™ Workstation User Guide [16]. The process is described in more detail in sections 3.4.1-3.4.4 below.

1. Prepare lists, e.g. sample lists
2. Create batch
3. Prepare solutions and microtiter plates
4. Start-up and prime Gyrolab™ Workstation
5. Prepare Gyrolab™ Workstation Control Software to run batch
6. Load Gyrolab™ Workstation
7. Start run
8. Finish run and unload Gyrolab™ Workstation
9. Data analysis

3.4.1 Preparations

For each run, reagent and transfer lists are prepared. In the reagent list, which specifies where samples are located on microtiter plates, samples are defined as capture/detection reagents, blanks, controls etc. Concentrations are listed for standards. For unknown samples, a sample list is prepared. These definitions simplify subsequent data analysis. The transfer list describes to which microstructures in the CD the samples, standards and reagents should be transferred (Figure 5). Standards and unknown samples are normally analyzed in triplicate. When the batch for the run is created, the lists are combined with a method (see section 3.4.2) to provide run

instructions for Gyrolab™ Workstation Control Software. After the workstation has been primed with pump liquid and wash solution, the run can be started.

Segment (M)	Structure (M)	Inlet (M)	Op_Capture reagent addition	Op_Analyte addition	Op_Detection reagent addition
1	Common left	Common inlet			Cy5-DNA
1	Common right	Common inlet			
1	1	Structure inlet	C1_1	Wo80	
1	2	Structure inlet	C1_2	Wo80	
1	3	Structure inlet	C1_3	Wo80	

Figure 5. Excerpt of transfer list from a capture reagent titration run (section 3.5.1). Here, capture reagents (C1_1-C1_3) and analyte (Wo80) were loaded individually into the microstructures, whereas the detection reagent (Cy5-DNA) was loaded in the common inlet of the segment.

3.4.2 Bioaffy™ 1C v3

In this project, the Bioaffy™ 1C v3 method (Appendix 3) was consistently used. First, the columns were washed twice to recondition the streptavidin-coated beads. Biotinylated capture reagent was added and immobilization was achieved through interaction with the streptavidin. After two washes, the samples were added to the microstructures. After two more washes and background detection (see section 3.4.3), the detection reagent was added. Four washes (to ensure removal of unbound detection reagent) and a final detection completed the run. All washes were performed with PBS-T.

3.4.3 Detection

Gyrolab™ Workstation LIF contains a LIF detector, with a 633 nm HeNe laser suitable for detection with the fluorophor Alexa Fluor® 647 (Alexa). In this project, Cy™5, which has nearly identical absorption and fluorescence maxima, was used instead of Alexa in the BAA due to the fact that Cy™5-labeled oligonucleotides were commercially available. In the IAA, Alexa was used for detection, as is the standard proce-dure for Bioaffy™ assays.

During detection, the CD is scanned while rotating. Two detection rounds are carried out, one before and one after detection reagent addition, so that the response values can be adjusted for background fluorescence (see method in Appendix 3). The fluorescence signal is amplified in a photo multiplier tube (PMT) and integrated to represent the total response. If the amplification level is too high, the detector will be saturated. If the amplification level is too low, the sensitivity decreases. The detection is performed with PMT levels of 1, 5 and 25% to ensure good quality values for at least one setting. In this project, the detection with a PMT setting of 1% was used for result evaluation unless otherwise stated.

3.4.4 Data analysis

Gyrolab Bioaffy™ data is preferably analyzed with Gyrolab™ Evaluator and Gyrolab™ Viewer software. The Gyrolab™ Evaluator handles the raw data, generating standard curves and statistics (Figure 6). The Gyrolab™ Viewer displays column profiles, showing the distribution of the fluorescence signal (Figure 6). The raw data can be viewed in a two-dimensional or three-dimensional format.

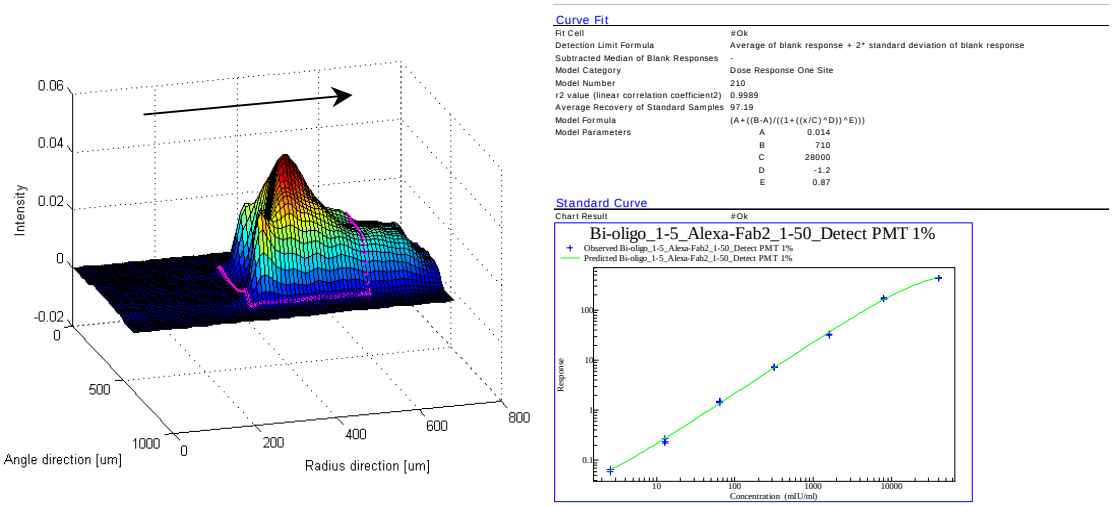

Figure 6. Examples of information displayed by Gyrolab™ Viewer and Gyrolab™ Evaluator. To the left, a three-dimensional column profile from Gyrolab™ Viewer. The column begins at approximately 400 μm (varies somewhat between microstructures). The arrow (added) illustrates the direction of liquid flow, in the radius direction of the CD. The area within the pink border is integrated. This integrated volume constitutes the response; it is proportional to the amount of bound detection reagent and thus the amount of analyte. To the right, a standard curve from Gyrolab™ Evaluator with curve fit data. For example, the model and the r^2 value are stated.

3.5 Bridging antibody assay development

3.5.1 Titration of capture reagent concentration

The concentration of capture reagent is important for bridge formation. If the concentration is too high, both arms of the analyte antibody can bind the immobilized antigen so that binding of detection antigen, and thus detection, is prevented. If, on the other hand, the concentration is too low, the amount of bound analyte will be insufficient to generate a response [11].

In order to find the best conditions, different concentrations of biotinylated dsDNA were evaluated as capture reagents. Biotinylated BSA was added to the solutions to help saturate the streptavidin column and prevent nonspecific surface interactions. The BSA concentration had to be optimized as well. If the BSA concentration is too high, the dsDNA will be out-competed so that the amount of capture reagent on the column is insufficient. If the concentration is too low, the column will not be saturated and nonspecific binding can occur.

Different concentrations of biotinylated dsDNA and BSA in DNA diluent (5 mM Tris pH 7.6, 1 M NaCl, 0.5 mM EDTA, 0.05% Tween® 20) were mixed 1:1 and evaluated in order to find the optimal combination. For these initiating assay runs, a 1:100 dilution of reference antibody in PBS with 1% BSA was used as analyte. The dilution of the detection reagent was 1:5 or 1:10 for the plasmid and 1:40 for the oligomer (in DNA diluent).

Due to problems in obtaining acceptable responses, a study of the capture reagent immobilization was performed for the plasmid assay. The biotinylated plasmid was titrated with monovalently biotinylated BSA and detected with 40 nM Alexa-labeled streptavidin (Molecular Probes, Eugene, WA). This approach cannot be used for immobilized oligomer, since each oligomer molecule is labeled with exactly one biotin, which is used for immobilization and hence not available for detection. For

this study, the Bioaffy™ 1C v3 method was modified so that detection (i.e. background detection, detection reagent addition and final detection) followed immediately after capture reagent addition and subsequent washes. Method steps 13-18 in Appendix 3 were omitted. No analytes were used.

3.5.2 Titration of detection reagent concentration

The effect on detection by different concentrations of Cy™5-labeled dsDNA, in DNA diluent, was studied. A low background is desirable, but at the same time the concentration must be high enough to allow measurements for high analyte concentrations; the detection reagent must be in excess compared to the analyte. Thus, the intended measurement range is important when optimizing the detection reagent concentration. To determine the optimal concentration of Cy™5-labeled dsDNA in this study, different concentrations were evaluated with serially diluted reference antibody. The capture reagent concentration determined in section 3.5.1 was used.

3.6 Indirect antibody assay development

3.6.1 Column particle evaluation

When designing an IAA, the risk of detecting nonspecifically bound antibodies needs to be taken into special consideration. In an attempt to address this issue, a small particle evaluation was performed. Serum samples from 5 normal donors (internal Gyros AB supply) were analyzed on the commercially available Gyrolab Bioaffy™ CD, with standard beads, and on a separate, but identical type, CD with HEMA Bio 1000 E beads. As comparison, the Wo/80 reference serum and the anti-dsDNA negative control serum were also analyzed on both CDs. The capture reagent (biotinylated oligomer) was diluted 1:10. The detection reagent, Alexa-labeled $F(ab')_2$ fragment, was diluted 1:100 in PBS with 1% BSA. Sera were serially diluted 1:5, 1:20 and 1:80 in PBS with 1% BSA. The streptavidin level on the HEMA Bio particles was 5 mg per gram particle, which is comparable to the level in the conventional particles[1]. The capacity of the HEMA Bio beads for capture reagent immobilization is higher than that of the standard beads, due to higher porosity. For this particular application, this was not a problem since the absolute responses were not of interest. Instead, relative responses (i.e. differences between the sera) were compared for the two particle types.

3.6.2 Titration of capture reagent concentration

The concentration of the capture reagent is less critical for IAAs than for BAAs. Here, the goal is to saturate the column, and as long as the capture reagent concentration is high enough to achieve saturation, no fine-tuning is necessary. The biotinylated dsDNA was not mixed with biotinylated BSA in the IAA format, since saturation of the column should be accomplished by the dsDNA itself and BSA presence can cause false positives if samples contain anti-albumin antibodies.

For the plasmid assay, biotinylated plasmid was diluted 1:2, 1:5 and 1:10 in DNA diluent and evaluated as capture reagent for analysis of the Wo/80 reference serum and the negative control serum, with both sera diluted 1:10, 1:50 and 1:100. Alexa-labeled $F(ab')_2$ fragment, diluted 1:100 in PBS with 1% BSA, was used as detection reagent.

[1] Personal communication, Dr. Anna Carlmark, Gyros AB (26 Oct. 05)

A corresponding study was performed for the oligomer assay. The same dilutions of sera and detection reagent were used. For the oligomer capture reagent, dilutions of 1:5, 1:10 and 1:20 were investigated.

3.6.3 Titration of detection reagent concentration

For the detection reagent, the considerations regarding concentration are the same as in the BAA (see section 3.5.2). The concentration must be low enough to yield a low background, but high enough not to limit the response maximum. The capture reagent concentration determined in section 3.6.2 was used. Alexa-labeled anti-human IgG $F(ab')_2$ fragment was diluted 1:50, 1:100 and 1:200 in PBS with 1% BSA and used to detect Wo/80 reference serum, diluted 5-fold from 1:5 to 1:78,125. Alexa-labeled anti-human IgG monoclonal antibody was diluted 1:10, 1:20 and 1:40 and used for detection of 5-fold Wo/80 dilutions from 1 (undiluted) to 1:15,625. The dilution series used in the monoclonal antibody titration was compensated for low serum content by addition of negative control serum to a serum dilution of 1:25.

3.7 Indirect antibody assay performance

3.7.1 Measurement range and reproducibility

The measurement range and the response reproducibility of the IAA – within and between CDs – were studied by generation of standard curves from 5-fold dilutions of Wo/80 reference serum (as described in section 3.6.3), and analyzed in triplicate on four CDs. For dilutions above 1:25 (i.e. 1:125 to 1:78,125), the low serum content was compensated by addition of negative control serum to a dilution of 1:25. In each case, the same dilutions were used for all four CDs.

3.7.2 Specificity

In order to verify the absence of ssDNA on the column, detection of bound single-stranded biotinylated oligonucleotide was performed using Cy™5-labeled complementary oligonucleotide. Oligomer hybridizations with ratios of unlabeled to labeled complementary oligonucleotides between 2 and 16 were evaluated as capture reagent. The concentration of labeled oligonucleotide was kept constant and the same as in the final assay. The Bioaffy™ 1C v3 method was modified as in section 3.5.1 to exclude analyte addition. Detection was performed with Cy™5-labeled ssDNA (0.2 μM in DNA diluent) (TIB MOLBIOL, Berlin, Germany), complementary to the biotinylated sequence (the same sequence as for the unlabeled oligonucleotide, Appendix 1).

The specificity of the assay was further investigated by evaluation of the samples on columns saturated with HSA. HSA was chosen since it is present in human blood and thus should not generate an immune response. However, anti-albumin autoantibodies have been reported [17], and the HSA columns may therefore not be totally inert. Nonetheless, they were considered the best choice for the specificity assessment. Biotinylated HSA was diluted in PBS-T to a concentration of 0.1 mg/ml and used as capture reagent. No other modifications to the assay conditions were made. As an extension of the investigation, a corresponding study of a selection of samples diluted in PBS with 1% HSA was performed.

3.7.3 Double columns

During the specificity study described in section 3.7.2, questions regarding response generated by filtered IgG-containing complexes arose (see section 4.3.4). To address this issue, a second column was loaded on top of the existing column in the CD

microstructures. Two column particles were evaluated for this second column: standard Bioaffy™ particles and Superdex™ Peptide particles (GE Healthcare, Uppsala, Sweden). These beads were not streptavidin-coated and thus should not bind the capture reagent. The purpose of an extra column was to add an inline purification step, trapping IgG-containing complexes and other debris that could potentially cause a false signal if trapped in the regular column. In order to investigate the influence of the double column setup on assay specificity, biotinylated oligomer or HSA was used as capture reagent. Ten serum samples diluted 1:25 in PBS with 1% HSA were analyzed in parallel with Wo/80 anti-dsDNA reference serum diluted 5-fold from 1 (undiluted) to 1:15,625 in PBS with 1% HSA (dilutions above 1:25 with added negative control serum, as previously described).

3.7.4 Serum dilutions

It is common to obtain response vs. concentration plots with different slopes for different samples in immunoassays, due to affinity differences between antibodies. This can pose a problem when sample responses are compared with the same standard curve. In order to investigate whether the problem applies for this assay, five dilutions each of eight serum samples were analyzed against a Wo/80 standard curve. Samples were diluted 5-fold from 1 (undiluted) to 1:3,125 in PBS with 1% BSA. Dilutions above 1:25 were made in negative control serum diluted 1:25, both for samples and standard. For each sample, five dilutions were analyzed. Dilutions 1 to 1:625 were evaluated for samples with an anti-dsDNA antibody concentration less than 200 IU/ml (as determined in section 3.8). For samples with a concentration of more than 200 IU/ml, dilutions 1:5 to 1:3,125 were evaluated.

3.8 Evaluation of serum samples

Serum samples that had previously been tested with the CLIF method (sixty-seven positive, fourteen negative) were evaluated with the IAA, using biotinylated oligomer (diluted 1:5) as capture reagent and Alexa-labeled anti-human IgG monoclonal antibody (diluted 1:20) as detection reagent. Samples were diluted 1:25 in PBS with 1% BSA. For each run, a standard curve was generated as described in section 3.7.1. When several CDs were run in the same batch, calibration controls were included on CDs without complete standard curves for reproducibility verification. Two separate evaluations were performed; one on standard single columns and one on double columns, where standard Bioaffy™ particles without streptavidin were loaded on top of the existing columns as described in section 3.7.3.

3.9 Receiver operating characteristic plots

In order to assess the Gyrolab Bioaffy™ assay accuracy, receiver operating characteristic (ROC) plots were generated for the single and double column results, considering the CLIF classification of negative and positive samples as the truth. The generation and use of ROC plots is discussed in detail in the National Committeé for Clinical and Laboratory Standards (NCCLS) document GP10-A [18] and described in brief below. The NCCLS guideline defines clinical accuracy, sensitivity and specificity as follows:

- Clinical accuracy: the ability of a diagnostic test to discriminate between two or more clinical states.
- Sensitivity: true positive fraction; ability of a test to correctly identify disease at a particular decision threshold.

- Specificity: true negative fraction; ability of a test to correctly identify the absence of disease at a particular decision threshold.

ROC plots visually present the sensitivities and specificities for the decision threshold spectrum. For each threshold (cut-off value between positive and negative results, in this case), the sensitivity and specificity are calculated as follows:

- Sensitivity: number of true positive results divided by number of true positive and false negative results.
- Specificity: number of true negative results divided by number of true negative and false positive results.

The ROC plot is obtained by plotting sensitivity (true positive fraction) on the y axis and 1 - specificity (false positive fraction) on the x axis. The closer to the upper left corner that the curve passes, the more accurate the test is. This is, of course, based upon the conditions used for classifying the test results – in this case, the CLIF assay. Note that a data point in the upper left corner would represent a cut-off with 100% sensitivity and 100% specificity, so that every single test result is classified correctly. On the other extreme, a test with no discriminating ability results in a diagonal ROC plot following a straight line between the lower left and upper right corners.

4 Results

4.1 Initiating reagent study – oligomer hybridization

The goal of the study was to minimize the amount of labeled ssDNA and thus the risk of exposing ssDNA on the column. For the capture reagent, non-biotinylated ssDNA should not bind to the streptavidin column and instead be removed by washes before analyte addition (see section 3.4.2 for assay method). Non-fluorescent ssDNA in the detection reagent in the BAA setup could, if it binds to the anti-dsDNA antibodies (most anti-dsDNA antibodies also bind ssDNA [5]), cause a lower signal level by competing for binding with the labeled dsDNA, but this is not likely to influence the relative detection results.

Cy™5-labeled oligonucleotide was hybridized with increasing concentrations of unlabeled complementary sequence. The hybridizations were evaluated with fluorescent resonance energy transfer (FRET), in order to find a concentration ratio where as much of the labeled oligonucleotide as possible hybridized to form labeled dsDNA.

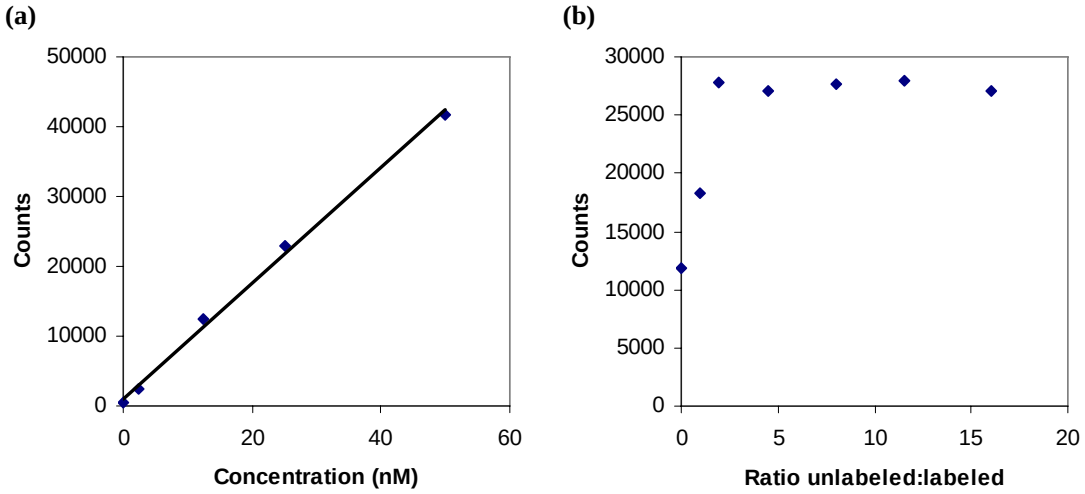

Figure 7. FRET results.
(a) The FRET assay was linear in the test range. The concentration refers to the concentration of Cy™5-labeled oligonucleotide. The concentration of the unlabeled oligonucleotide was consistently 8 times higher. 50 µl of PicoGreen® reagent was added to each 40 µl sample.
(b) Hybridizations with 0.1 µM Cy™5-labeled sequence and increasing concentrations of unlabeled sequence. 50 µl of PicoGreen® reagent was added to each 10 µl sample.

The FRET signal reached a plateau as the surplus of unlabeled oligonucleotide increased (Figure 7). When the plateau was reached, addition of more unlabeled sequence did not result in more dsDNA. Virtually all labeled sequence had hybridized with unlabeled complementary molecules to form double-stranded oligomers. A concentration ratio on the plateau, with 4 times more of the unlabeled oligonucleotide, was chosen for the final hybridization.

4.2 Bridging antibody assay

4.2.1 Capture reagent concentration

The assay development was started with a determination of the optimal conditions for the capture reagents. Serial dilutions of biotinylated dsDNA and BSA were mixed 1:1 and evaluated with a 1:100 dilution of reference serum. For the plasmid, a first screening was performed with dilutions ranging from 1 (undiluted) to 1:80 and BSA

dilutions from 1 to 1:16. Cy™5-labeled plasmid was diluted 1:10 and used as detection reagent.

The column profiles for this run showed long, broad peaks with low intensity (Figure 8), which implied that the BSA:dsDNA ratio was too high so that the dsDNA immobilization was hindered. One should consider that the plasmid has a molecular weight of 2.24 MDa, compared to 66 kDa for BSA, so that the plasmid diffuses considerably slower in the column. However, lowering the ratio to a mixture of dsDNA diluted 1:5 and BSA diluted 10-fold from 1:200 to 1:200,000 did not improve results.

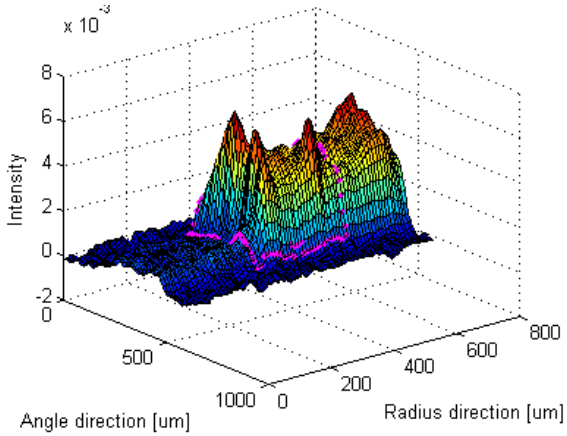

Figure 8. Column binding profile for undiluted biotinylated plasmid mixed 1:1 with biotinylated BSA, diluted 1:8, as capture reagent. PMT 5%.

In order to study the capture reagent immobilization directly, plasmid dsDNA was mixed with sparsely biotinylated BSA and used as capture reagent. The dsDNA dilution was 1:5 and the BSA was diluted 10-fold from 1:200 to 1:200,000. The modified Bioaffy™ 1C v3 method (Appendix 3, steps 13-18 omitted) was used, and detection was performed with 40 nM Alexa-labeled streptavidin without prior analyte addition. Since each plasmid is labeled with several biotins (one for every 20-60 bp) and all biotins on the BSA should be involved in column streptavidin interactions if the BSA is monovalently biotinylated, all signal should derive from immobilized plasmids.

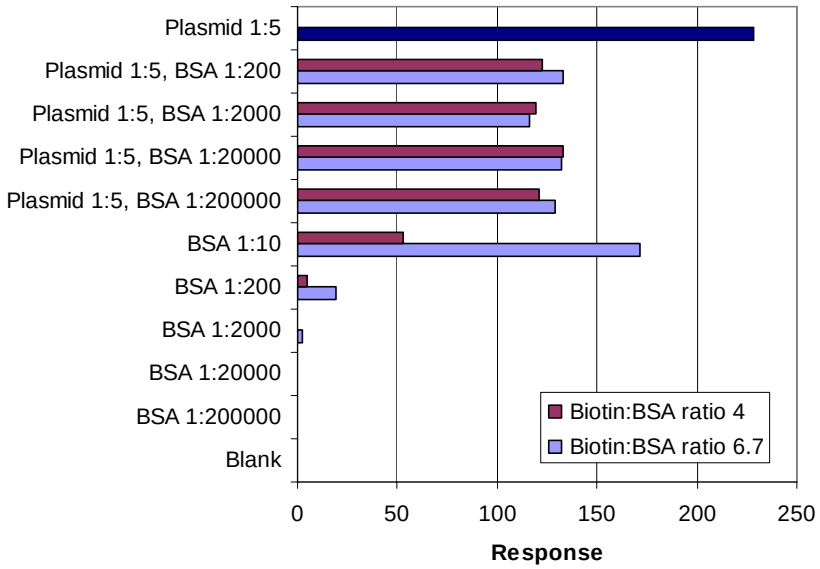

Figure 9. Streptavidin-Alexa detection of capture reagent immobilization. The BSA contribution to the response in the mixtures is insignificant (BSA diluted 1:200-1:200,000).

The response for the dsDNA mixed with BSA was markedly higher than for the BSA alone, with the exception of BSA diluted 1:10 (Figure 9). Thus, some BSA molecules

were labeled with more than one biotin, but the response contribution of the BSA in the dsDNA mixtures was insignificant. Based on these results, it was suspected that the low responses previously obtained were due to excessive antigen concentration on the column. In addition, the column profiles showed slowly declining peaks. To favor antigen enrichment in the first part of the column, the capture reagent spin (Appendix 3, step 8) was changed so that the flow-rate decreased. A screening was then performed with plasmid dilutions ranging from 1:5 to 1:80 and BSA dilutions from 1:10 to 1:160, i.e. higher BSA concentrations than in the immobilization study above, resulting in less dsDNA on the column. Cy™5-labeled plasmid diluted 1:5 was used for detection. Responses (absolute and compared to the negative control) are listed in Table 1. No further enrichment in the beginning of the column was apparent in the column profiles, but nevertheless, the modified version of 1C v3 was used for the plasmid capture reagent for the remainder of the project.

Table 1a. Response values (PMT 5%) for plasmid capture reagent titration. The biotinylated dsDNA and BSA dilutions were mixed 1:1 and used as capture reagent. Analyte was Wo/80 reference serum, diluted 1:100. The highest values are shown in bold, red font.

B-dsDNA	**B-BSA dilution**				
dilution	1:10	1:20	1:40	1:80	1:160
1:5	14	**19**	**17**	16	16
1:10	13	15	14	14	16
1:20	11	11	12	12	15
1:40	8.5	10	8.6	9.2	8.6
1:80	6.3	7.0	7.5	7.8	7.0

Table 1b. Response ratio between Wo/80 reference and negative control for plasmid capture reagent titration. The biotinylated dsDNA and BSA dilutions were mixed 1:1 and used as capture reagent. The values corresponding to the highest values in Table 1a are shown in bold, red font.

B-dsDNA	**B-BSA dilution**				
dilution	1:10	1:20	1:40	1:80	1:160
1:5	2.4	**3.0**	**2.6**	2.7	2.6
1:10	2.2	2.6	2.3	2.4	2.8
1:20	2.2	1.9	2.3	2.1	2.8
1:40	2.4	2.3	1.8	1.9	1.5
1:80	1.4	1.3	1.6	1.3	1.3

As shown in Table 1, decreasing the dsDNA concentration did not increase the response. Instead, the capture reagents consisting of dsDNA diluted 1:5 and BSA diluted 1:20 and 1:40 were chosen for further evaluation, since they had the highest absolute responses with a relatively high response ratio between the reference serum and the negative control serum (bold, red values in Table 1). With a dilution of 1:5, it is possible to generate approximately 1,400 data points with 50 µl biotinylated capture reagent stock solution (45 nM), corresponding to 5 µl of unlabeled plasmid DNA.

For the oligomer, the first run was performed with dsDNA dilutions ranging from 1 (undiluted) to 1:160, mixed 1:1 with BSA dilutions from 1 to 1:64. Cy™5-labeled oligomer was diluted 1:40 and used as detection reagent. No specific binding was observed (Figure 10). To verify that this was a capture reagent problem, oligomer capture reagent was used with plasmid detection reagent (diluted 1:5). This setup resulted in no response difference between reference serum and blank. Consequently, the use of oligomer as capture reagent in the BAA was abandoned.

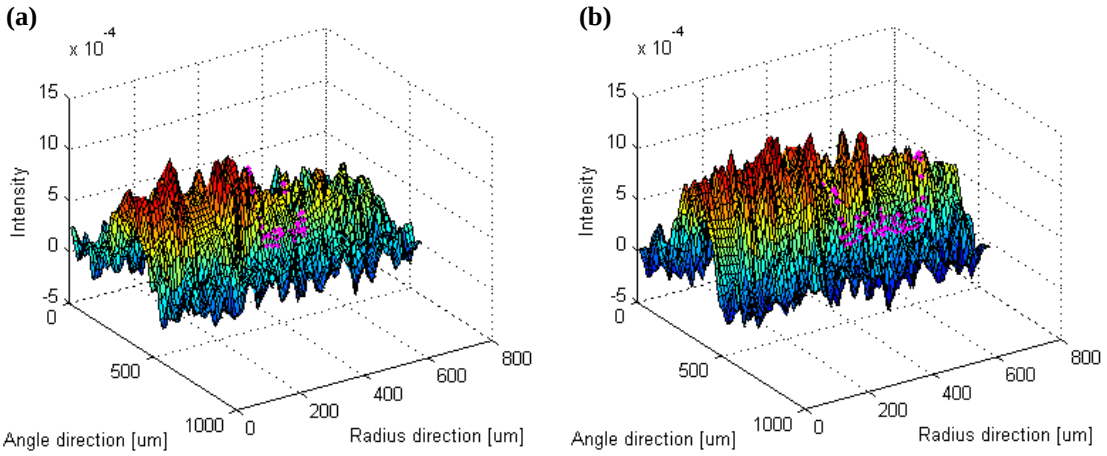

Figure 10. No difference was seen in the column binding profiles for **(a)** negative control serum and **(b)** Wo/80 reference serum. Biotinylated dsDNA (diluted 1:10) mixed 1:1 with biotinylated BSA (diluted 1:16) was used as capture reagent. Please note the scaling of the intensity axis with 10^{-4} (PMT 5%), inferring practically no signal.

4.2.2 Detection reagent concentration

In order to find the optimal detection reagent concentration, serial dilutions were tested with the plasmid capture reagent concentrations determined in section 4.2.1 (dsDNA diluted 1:5 mixed 1:1 with BSA diluted 1:20 or 1:40). Cy™5-labeled plasmid was diluted 2-fold from 1:5 to 1:40 and used for detection. The response for serial dilutions of the reference antibody and negative control serum was determined for each detection reagent concentration. The goal was to find the concentration of detection reagent that yielded the lowest background (control serum) response. However, the general appearance of the resulting diagrams was similar for all concentrations; one example is shown in Figure 11. Signal-to-noise ratios were consistently highest for the 1:5 serum dilutions (listed in Table 2).

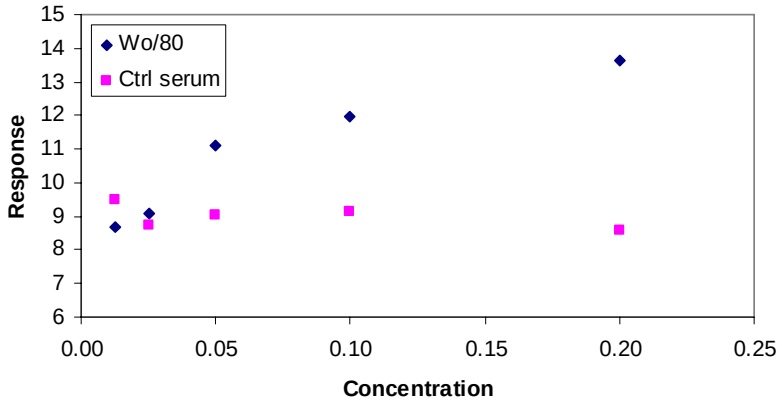

Figure 11. Evaluation of detection reagent concentration for the plasmid. For this particular dataset, the capture reagent was biotinylated dsDNA diluted 1:5 and mixed 1:1 with biotinylated BSA diluted 1:20. The detection reagent was Cy™5-labeled dsDNA diluted 1:5. A concentration of 0.1 corresponds to a serum dilution of 1:10. PMT 5%.

The Cy™5-labeled oligomer was also examined as detection reagent, with plasmid capture reagent consisting of dsDNA diluted 1:5 and mixed with BSA diluted 1:20. No signal was obtained for dilutions 1:5 to 1:40 (see column profiles in Figure 12). Due to the poor signal-to-noise ratios (Table 2), and in light of the IAA performance (see section 4.3), development of the BAA was discontinued and all efforts focused on the IAA development.

Table 2. Response ratio between Wo/80 reference and negative control, both diluted 1:5. Capture reagents were biotinylated plasmid diluted 1:5 and mixed 1:1 with biotinylated BSA diluted 1:20 or 1:40.

Cy™5-dsDNA dilution	B-BSA dilution	
	1:20	1:40
1:5	1.6	1.4
1:10	1.4	1.4
1:20	1.6	1.8
1:40	1.6	0.9

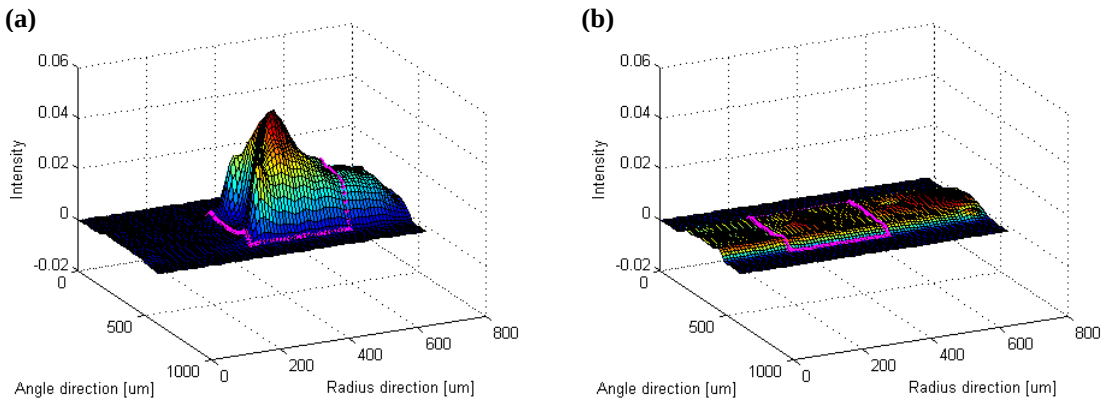

Figure 12. Column profiles (PMT 5%) after detection with **(a)** plasmid and **(b)** oligomer, both diluted 1:5. Capture reagent was biotinylated plasmid diluted 1:5 mixed with biotinylated BSA diluted 1:20. Analyte was Wo/80 reference serum, diluted 1:2.

4.3 Indirect antibody assay

4.3.1 Column particle evaluation

The indirect assay development was started by determining whether solid or porous beads were preferable. This was done by comparing the results of analyses of 5 serum samples from normal donors using either standard particles or HEMA Bio particles in the Gyrolab Bioaffy™ CD. The Wo/80 anti-dsDNA international standard and the negative control used in the BAA development were analyzed in parallel with these samples. The biotinylated oligomer was used as capture reagent (diluted 1:10). Sera were diluted 1:5, 1:20 and 1:80 and detected with Alexa-labeled F(ab')$_2$ fragment (diluted 1:100).

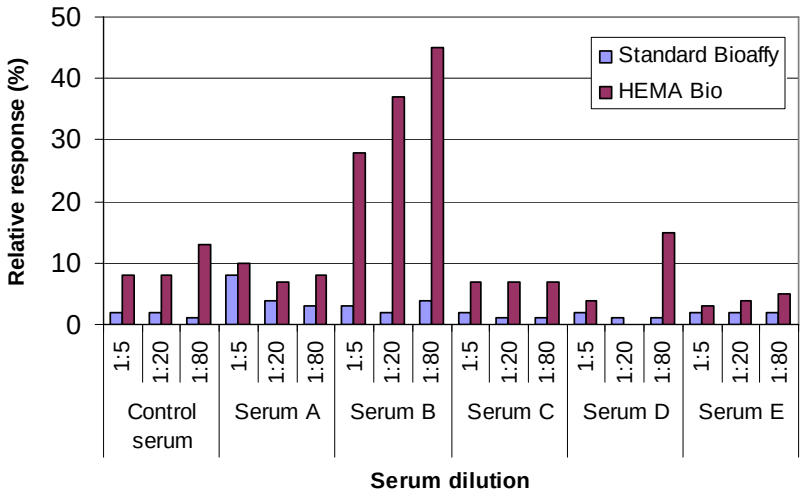

Figure 13. Responses in percent of Wo/80 response for sera diluted 1:5, 1:20 and 1:80. Solid and porous particles (conventional Bioaffy™ and HEMA Bio, respectively) were used. Capture reagent was biotinylated oligomer.

For the normal solid beads, serum A yielded the highest response and could potentially be suspected of nonspecific interactions. The macroporous HEMA Bio beads appeared overall to cause more nonspecific IgG binding, with a particularly high response for serum B. The relative response (compared to the Wo/80 response) was higher for all sera on the HEMA Bio particles (Figure 13). In general, the column profiles for the HEMA Bio run were more enriched in the beginning of the columns. This could indicate some sort of "sieving" effect, where antibodies were trapped by the porous particles (see contour plots in Figure 14) without association to the capture reagent.

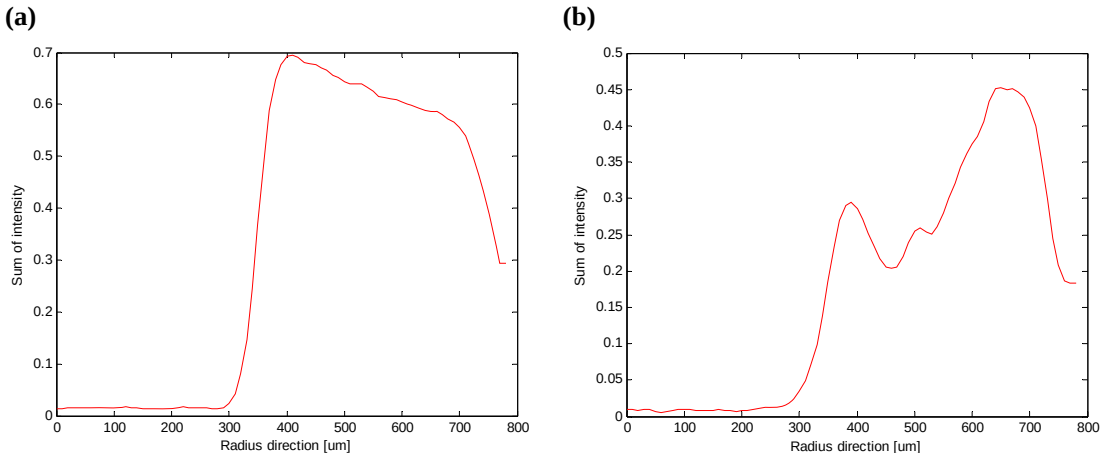

Figure 14. Contour plots for negative control serum on **(a)** HEMA Bio particles and **(b)** standard Bioaffy™ particles. Please note the difference in intensity (y) axis scaling.

Based on the observation of nonspecific binding to the HEMA Bio particles, it was concluded that the assay would be developed on the conventional Bioaffy™ particles.

4.3.2 Capture reagent concentration

The plasmid capture reagent concentration was titrated to ensure saturation of the column without waste of material. Wo/80 reference serum and negative control serum, diluted 1:10, 1:50 and 1:100, were analyzed. A F(ab')$_2$ fragment detection reagent dilution of 1:100 was used. The column profiles for the control serum (not shown) indicated that a high capture reagent concentration was preferable (increased saturation of the column). The difference between a capture reagent dilution of 1:2 and 1:5 was particularly noticeable. However, the response values were not similarly affected (Table 3).

Table 3. Response values (PMT 5%) for plasmid capture reagent titration. Detection was performed with Alexa-labeled F(ab')$_2$ fragment, diluted 1:100.

B-dsDNA	**Wo/80 dilution**			**Control serum dilution**		
dilution	1:10	1:50	1:100	1:10	1:50	1:100
1:2	588	131	64	18	5.8	3.4
1:5	562	112	57	15	4.9	3.1
1:10	503	87	48	19	6.1	3.9

The oligomer capture reagent concentration was titrated likewise. For the reference serum, the responses and column profiles were similar for all capture reagent concentrations. For the negative control serum, the column profiles (not shown) showed signs of unsaturated columns for the lower capture reagent concentrations, but the response values were only slightly affected (Table 4).

Table 4. Response values (PMT 1%) for oligomer capture reagent titration. Detection was performed with Alexa-labeled F(ab')$_2$ fragment, diluted 1:100.

B-dsDNA	**Wo/80 dilution**			**Control serum dilution**		
dilution	1:10	1:50	1:100	1:10	1:50	1:100
1:5	252	56	29	3.2	0.9	0.4
1:10	243	52	25	3.2	0.4	0.7
1:20	216	45	23	5.2	1.5	0.9

The response ratio between the Wo/80 reference serum and the negative control serum was consistently lower for the plasmid capture reagent (for raw data, se Tables 3-4). Also, the column profiles were more enriched in the beginning of the column when using the oligomer capture reagent (Figure 15), which is preferred for the data analysis. Consequently, it was concluded that the oligomer would be used as capture reagent for the remaining assay development. Based on the column profile appearance, a dilution of 1:5 was decided upon. With this dilution, a volume of 160 µl biotinylated oligomer would yield approximately 2,240 data points.

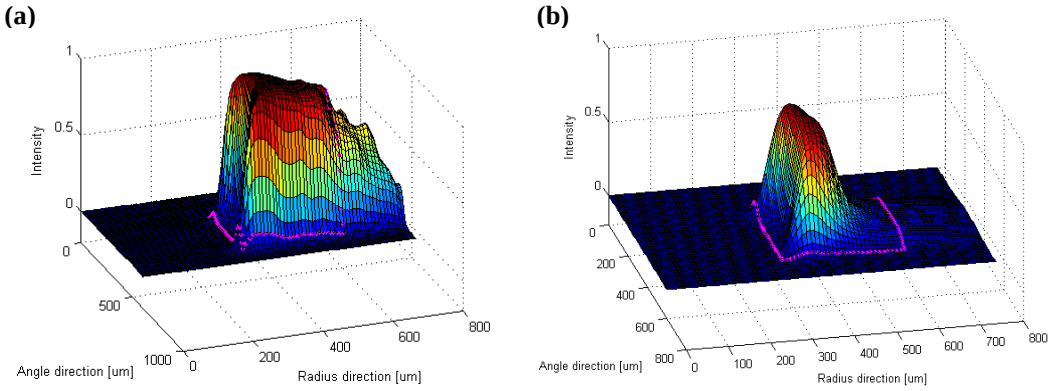

Figure 15. Column profiles for Wo/80 serum diluted 1:10. Detection was performed with Alexa-labeled F(ab')$_2$ fragment, diluted 1:100. The difference between the two capture reagents, **(a)** plasmid (diluted 1:2), PMT 5%, and **(b)** oligomer (diluted 1:5), PMT 1%, is evident. A slow peak decline is typical for plasmid peaks, whereas the preferred enrichment in the beginning of the column (preventing signal loss) can be seen for oligomer peaks.

4.3.3 Detection reagent concentration

Goat anti-human IgG F(ab')$_2$ fragment, labeled with Alexa, was titrated to determine the optimal detection reagent concentration. A dilution series of Wo/80 reference serum was analyzed with biotinylated oligomer diluted 1:5 as capture reagent. Blank responses (PBS with 1% BSA) decreased with lowered detection reagent concentration (data not shown), and consequently, the signal-to-noise ratio increased. The response curve for the highest detection reagent dilution was similar to those of the other two (Figure 16a). Thus, it was concluded that for the F(ab')$_2$ fragment detection reagent, a dilution of 1:200 would be used. With this dilution, it is possible to generate approximately 50,000 data points with 90 µl Alexa-labeled F(ab')$_2$ fragment (4.5 µM).

Mouse anti-human IgG monoclonal antibody, labeled with Alexa, was also titrated with a dilution series of Wo/80. In this case, dilutions above 1:25 (i.e. 1:125 to 1:15,625) were compensated for low serum content by addition of negative control serum. The signal-to-noise ratios were higher for the two lower detection reagent concentrations, and after considering the standard curves (Figure 16b) it was

determined that the best detecting conditions were obtained with the 1:20 dilution. With this dilution, it is possible to generate approximately 5,600 data points with 100 µl Alexa-labeled monoclonal antibody (1 µM).

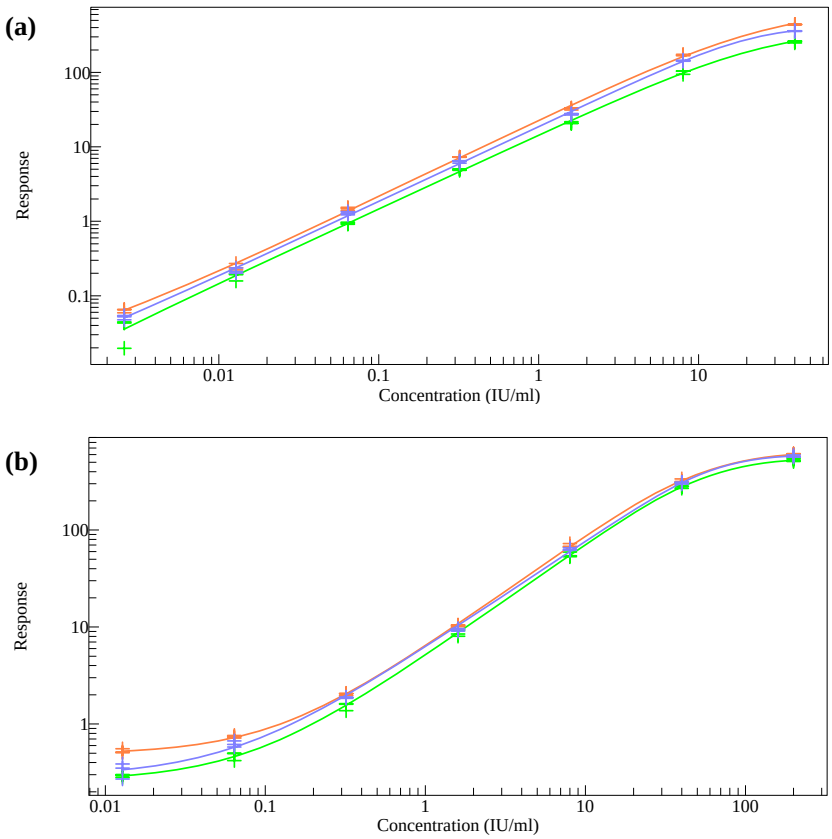

Figure 16. Serial dilutions of Wo/80 reference serum detected with **(a)** $F(ab')_2$ fragment diluted 1:50 (red), 1:100 (blue), and 1:200 (green); and **(b)** monoclonal antibody diluted 1:10 (red), 1:20 (blue), and 1:40 (green). For the monoclonal antibody titration in **(b)**, the Wo/80 dilutions were compensated for low serum content in dilutions above 1:25, resulting in higher background. With the same compensation, the curves in **(a)** would not have been linear for the low concentrations.

4.3.4 Performance

For each detection reagent, 5-fold dilutions of Wo/80 reference serum (high dilutions compensated for low serum content) were analyzed on four different CDs, in order to study measurement range and reproducibility. The resulting standard curves were in each case overlapping, with a coefficient of variance (CV) below 5% for all dilutions except one (Figure 17). Within CDs, the CV was below 10% for all dilutions.

The measurement range was estimated from the standard curves in Figure 17 to cover approximately two and three orders of magnitude on the response axis for the $F(ab')_2$ fragment and monoclonal antibody, respectively. The background response seen at lower concentrations prevents a wider measurement range. However, for diagnostic purposes, anti-dsDNA assays should have low sensitivity to minimize the risk of false positives. Thus, the background is not likely to be a problem.

After comparison of the results obtained with the two detection reagents, it was concluded that the monoclonal antibody would be used, since it gives a larger measurement range.

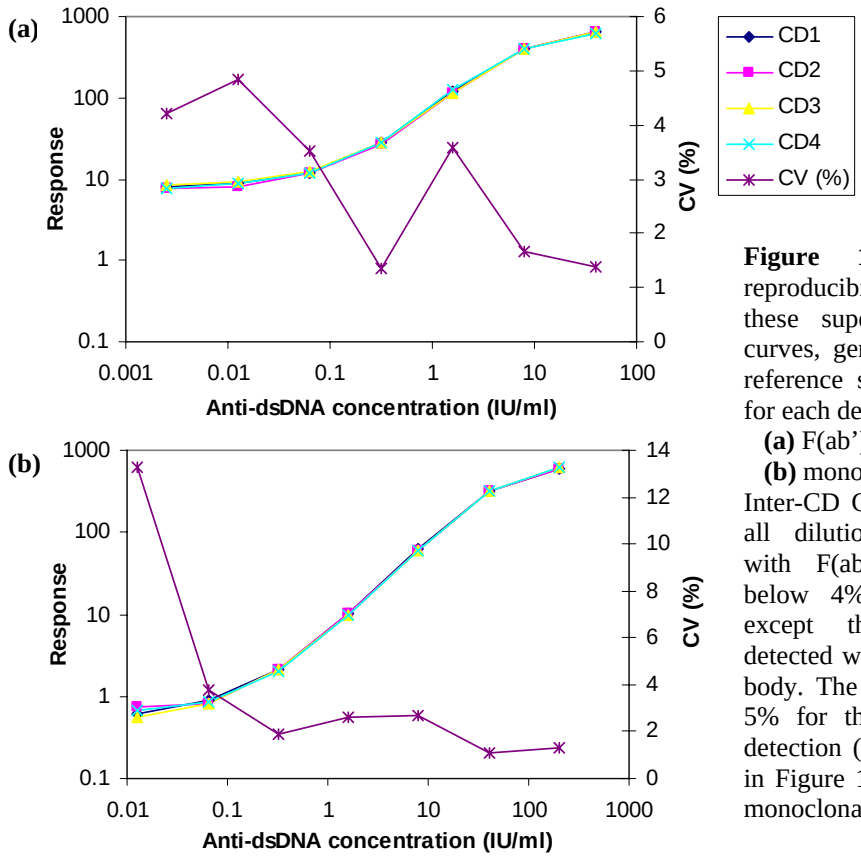

Figure 17. Good assay reproducibility is illustrated by these superimposed standard curves, generated with Wo/80 reference serum on four CDs for each detection reagent:
(a) $F(ab')_2$ fragment,
(b) monoclonal antibody.
Inter-CD CV is below 5% for all dilutions when detected with $F(ab')_2$ fragment, and below 4% for all dilutions except the highest when detected with monoclonal antibody. The PMT settings were 5% for the $F(ab')_2$ fragment detection (i.e. not the same as in Figure 16a) and 1% for the monoclonal antibody detection.

The presence of ssDNA on the column was investigated for four different hybridizations with ratios of unlabeled to labeled oligonucleotide between 2 and 16. As comparison, a blank (DNA diluent) and a positive control (biotinylated oligonucleotide only) were also analyzed. For all capture reagents containing DNA, the concentration of biotinylated oligonucleotide was 0.25 μM, as in the developed assay. Detection was performed with Cy™5-labeled ssDNA, complementary to the biotinylated oligonucleotide, without prior analyte addition. The results (Figure 18) indicate that for the concentration ratio used in the assay, with 4 times more of the unlabeled sequence, there is hardly any ssDNA in the column since there is no notable response decrease for higher ratios. The responses for the hybridizations (ratios 2-16) constitute a low percentage of the ssDNA response: 5.0 % for the 2-fold ratio and 2.9-3.4% for the others. These results support the conclusions drawn from the FRET study (section 4.1), that a 4-fold surplus of unlabeled oligonucleotide is sufficient to ensure low ssDNA contamination while efficiently generating labeled dsDNA.

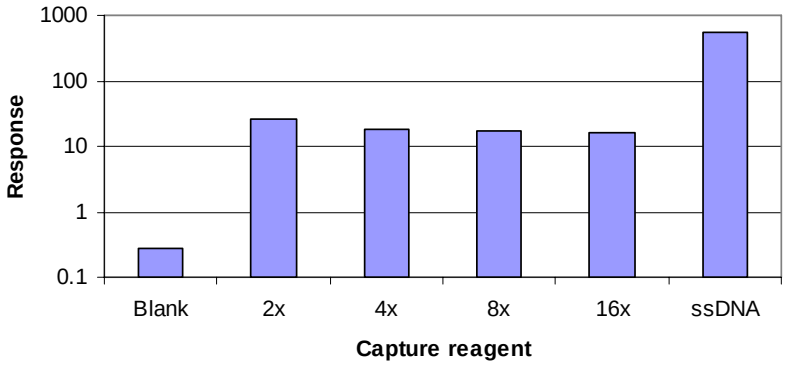

Figure 18.
Unlabeled : labeled ratios 2-16, blank and ssDNA; all detected with Cy™5-labeled ssDNA. Please note that the response scale is logarithmic.

In order to further investigate the specificity of the assay, serum samples (diluted 1:25 in PBS with 1% BSA) were analyzed on columns with immobilized HSA instead of

dsDNA to determine the level of nonspecific binding of serum components and/or detection reagent. Alexa-labeled anti-human IgG monoclonal antibody, diluted 1:20, was used for detection. Responses were generally low, with some exceptions (Figure 19). For comparison, a response of 35 (the highest response) would result in an anti-dsDNA concentration of approximately 100 IU/ml with the conditions used in section 3.8 for the final evaluation of serum samples.

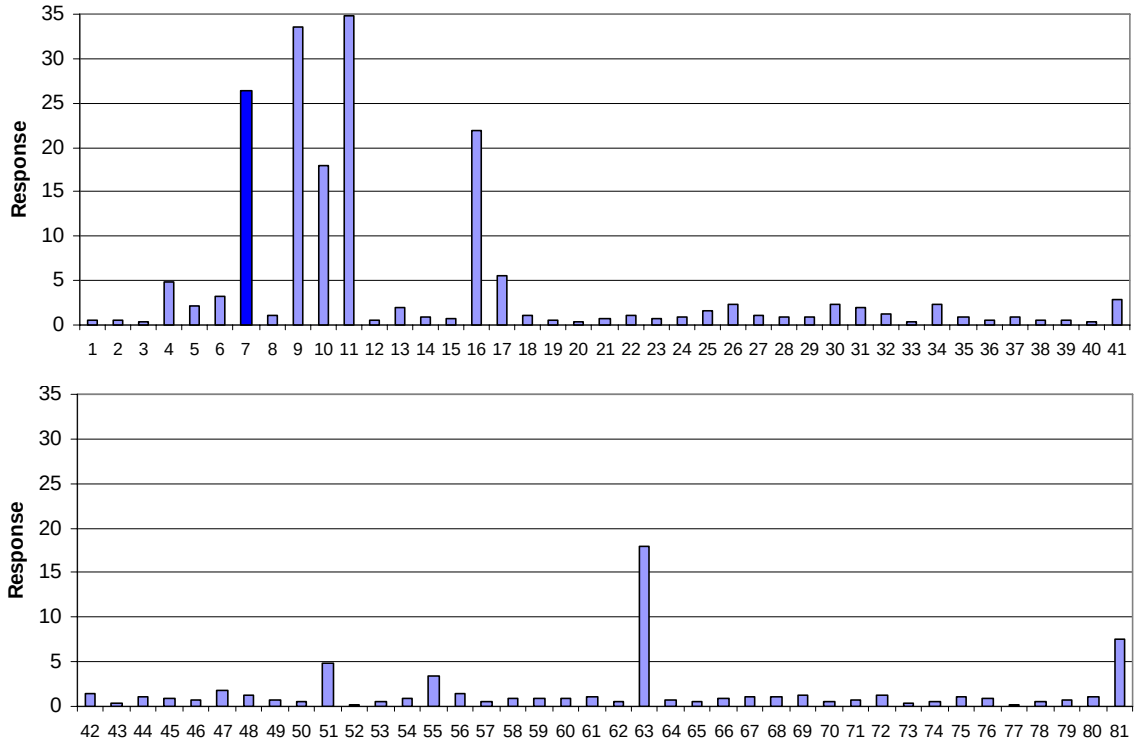

Figure 19. Average responses for serum samples, diluted 1:25, with 0.1 mg/ml biotinylated HSA as capture reagent. The bar in darker blue indicates a sample with exceptionally high response variation – responses were 71, 4 and 4 yielding 26 in average response.

Interestingly, it was immediately apparent that the samples generating a high response on the HSA columns were not the same as those yielding high anti-dsDNA responses (compare Figures 19 and 27). Thus, high anti-dsDNA responses could not be explained by nonspecific column interactions also detected with HSA columns. Instead, the anti-HSA response was sometimes higher than the anti-dsDNA response, possibly due to anti-albumin antibodies. The seventeen samples with the highest responses were further investigated by analysis on HSA columns, diluted 1:25 in PBS with 1% HSA instead of 1% BSA. The difference in response for the two diluents is illustrated in Figure 20. Most samples demonstrated a response decrease when diluted in PBS with 1% HSA instead of 1% BSA, as could be expected. However, a few samples displayed the opposite behavior.

The overall response decrease seen when sera are diluted in PBS containing HSA can be interpreted as neutralization of anti-HSA antibodies by the soluble HSA. Sera 9 and 16, where the response decrease is clear, showed signs of low affinity anti-HSA antibodies when diluted in PBS containing BSA, supporting this interpretation (Figure 21a). For serum 11, which generated the highest response when diluted in PBS with 1% HSA, a “sieving” effect was suspected (Figure 21b).

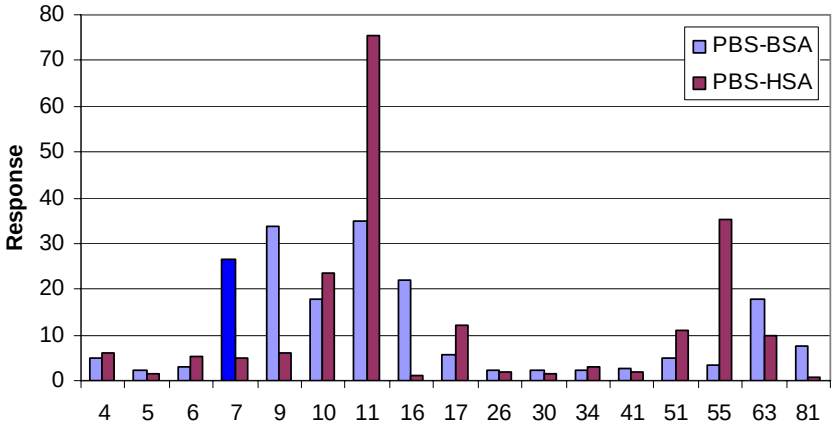

Figure 20. Average responses for 17 samples diluted in PBS with 1% BSA or HSA. Capture reagent was biotinylated HSA. The bar in darker blue once again indicates the sample with high response variation (see legend of Figure 19).

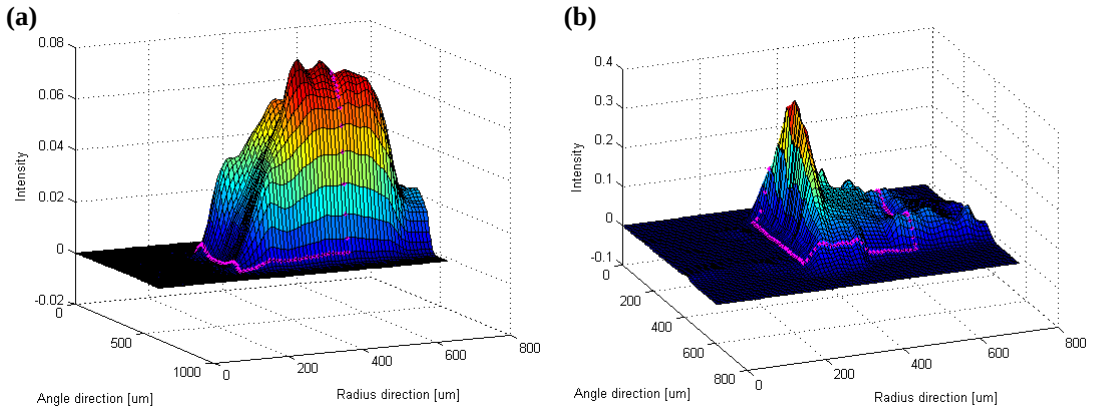

Figure 21. Column profiles of anti-HSA responses for **(a)** serum 9 diluted in PBS with 1% BSA and **(b)** serum 11 diluted in PBS with 1% HSA. Note the gradual accumulation of the response in **(a)**, possibly indicating low affinity antibodies, and the sharp enrichment in the beginning of the column in **(b)**, indicating a sieving effect.

A second column was loaded on top of the existing one in order to add an inline sample purification step. Two different particles were evaluated, standard Bioaffy™ and Superdex™ Peptide, both without streptavidin. Ten serum samples, diluted 1:25 in PBS with 1% HSA, were analyzed for each double column setup with both oligomer and HSA as capture reagents. A standard curve was in each case generated from 5-fold dilutions of Wo/80 reference serum, also diluted in PBS with 1% HSA.

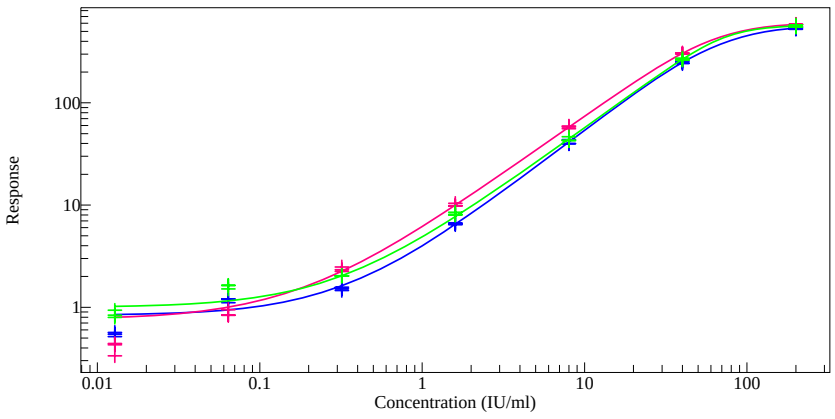

Figure 22. Standard curves generated on single (red) and double columns, consisting of standard Bioaffy™ (green) and Superdex™ (blue) particles.

A comparison of these standard curves with a standard curve from a single column run is shown in Figure 22. Please note that the single column standard curve originates in Wo/80 dilutions in PBS with 1% BSA. The standard curves show good

correlation, indicating that there is no nonspecific capture of anti-dsDNA antibodies from the Wo/80 serum by either the Bioaffy™ or Superdex™ secondary columns.

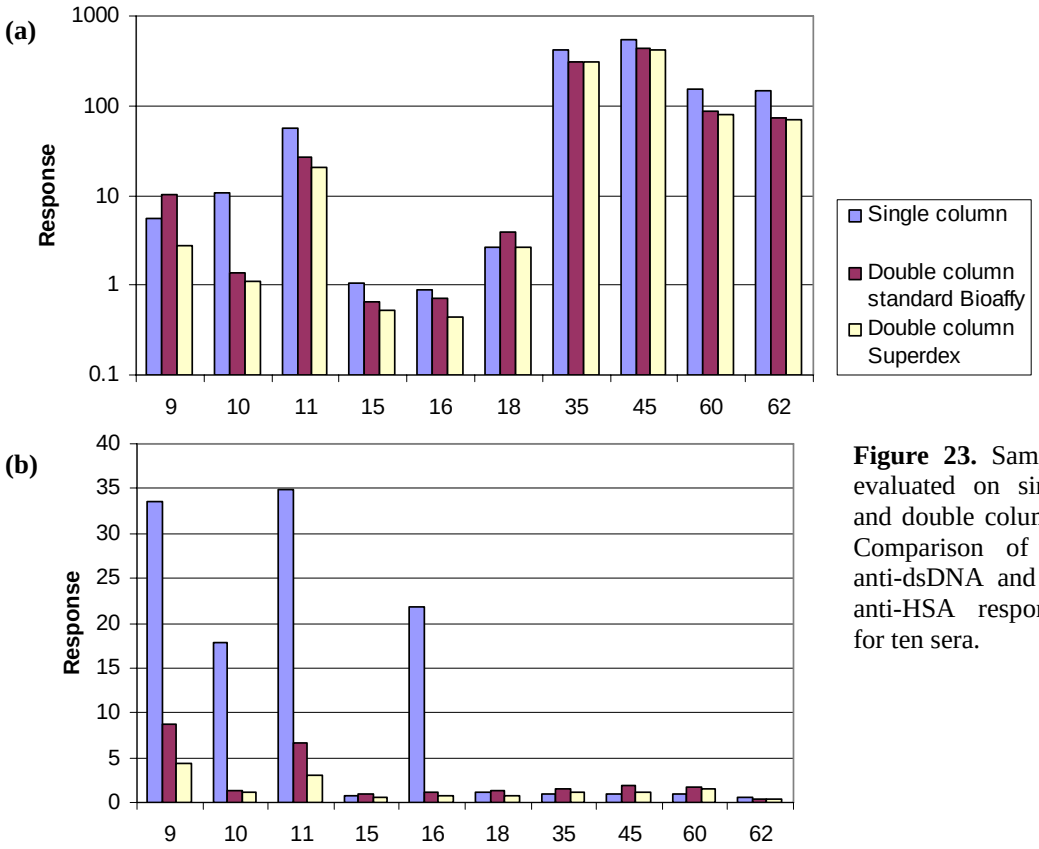

Figure 23. Samples evaluated on single and double columns. Comparison of **(a)** anti-dsDNA and **(b)** anti-HSA responses for ten sera.

A corresponding comparison between sample results is shown in Figure 23. Here also, sera were diluted in PBS with 1% BSA in the single column assay and in PBS with 1% HSA in the double column assay. The anti-dsDNA result was lower for some sera when double columns were used (Figure 23a), which could imply clearance of nonspecific interactions. For the control assay with immobilized HSA, the difference between single and double columns was dramatic in some cases where the anti-HSA response on the single column was high (Figure 23b). The column profiles clearly show that material, as intended, has been trapped in the first column (see Figure 24 for an example).

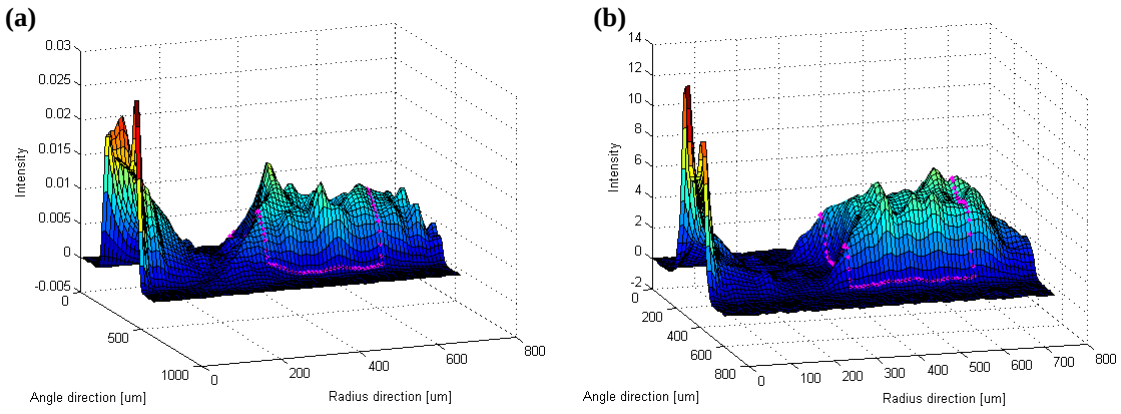

Figure 24. Column profiles of HSA response for serum 11 with **(a)** standard Bioaffy™ and **(b)** Superdex™ double columns. The second column, with capture reagent, starts at approximately 300 μm. The first column begins outside of the detection area, leading to cut-off profiles. For the corresponding single column profile, see Figure 21b.

The conclusion from the double column study was that an extra column loaded on top of the existing one results in increased assay specificity, as seen in the decreased anti-HSA responses. Reproducible results were obtained for both double column setups. Consistent with the results using Wo/80 serum (Figure 22), the difference in anti-dsDNA response is minimal, indicating that the double column assay does not interfere with the specific dsDNA interactions.

In order to investigate the effect of sample dilution on the assay results, 5-fold dilutions of eight sera were analyzed on standard Bioaffy™ CDs (single column). For five sera (1, 8, 13, 22, and 36), dilutions 1 to 1:625 were evaluated. For three sera (28, 47, and 49), dilutions 1:5 to 1:3,125 were analyzed. Dilutions were chosen based on the anti-dsDNA results in Figure 27. Capture reagent was biotinylated oligomer, diluted 1:5. Detection was performed using Alexa-labeled monoclonal antibody, diluted 1:20. The two samples with slightly deviating slopes (sera 8 and 13, Figure 25) had low anti-dsDNA antibody concentrations, with the highest dilutions falling below the level of detection of the assay. The results of the sample dilution assay (Figure 25) indicated that the risk of obtaining incorrect results due to individual dilution effects was small.

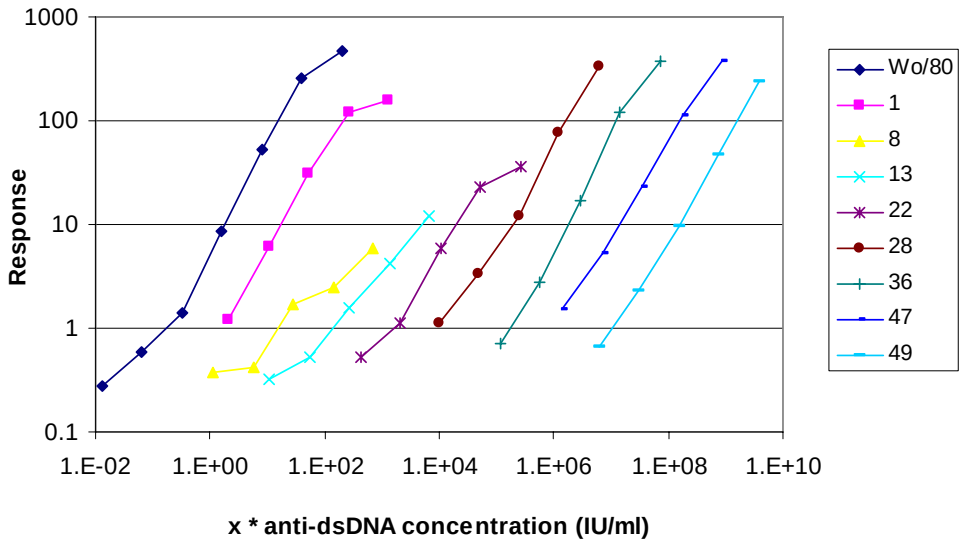

Figure 25. Responses for Wo/80 reference serum and eight serum samples. For the samples, concentrations are calculated based on results for the 1:25 dilutions. Concentrations are multiplied with an increasing factor "x" (1, 10, 100, etc.) to separate the curves horizontally.

Anti-dsDNA concentrations determined for the different dilutions are presented in Figure 26. The general agreement was good. The highest dilution (1:3,125) diverged somewhat, but for this dilution even extremely small variations in response would have a big impact, since the result is multiplied by 3,125. It should be noted that the concentrations obtained for dilutions surrounding the 1:25 dilution, which was used for final sample evaluation, were particularly stable.

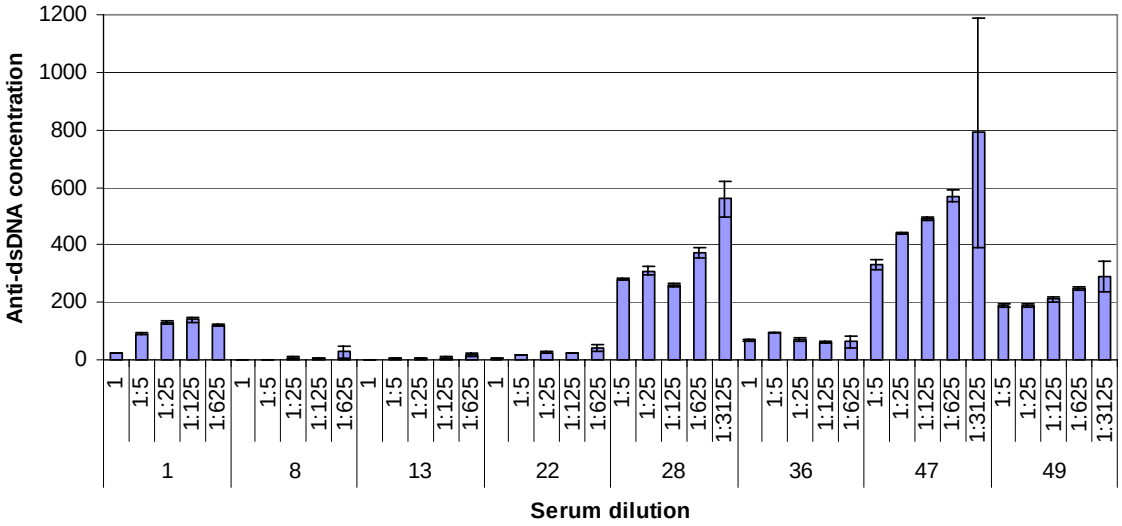

Figure 26. Anti-dsDNA antibody concentrations as determined by Gyrolab™ Evaluator based on stated sample dilutions. Error bars indicate standard deviations.

4.3.5 Evaluation of serum samples

81 serum samples were diluted 1:25 in PBS with 1% BSA and analyzed on single columns with biotinylated oligomer (1:5) as capture reagent and Alexa-labeled anti-human IgG monoclonal antibody (1:20) as detection reagent. The same assay was also performed on double columns, where the second column consisted of standard Bioaffy™ particles without streptavidin. Results are presented in Figure 27.

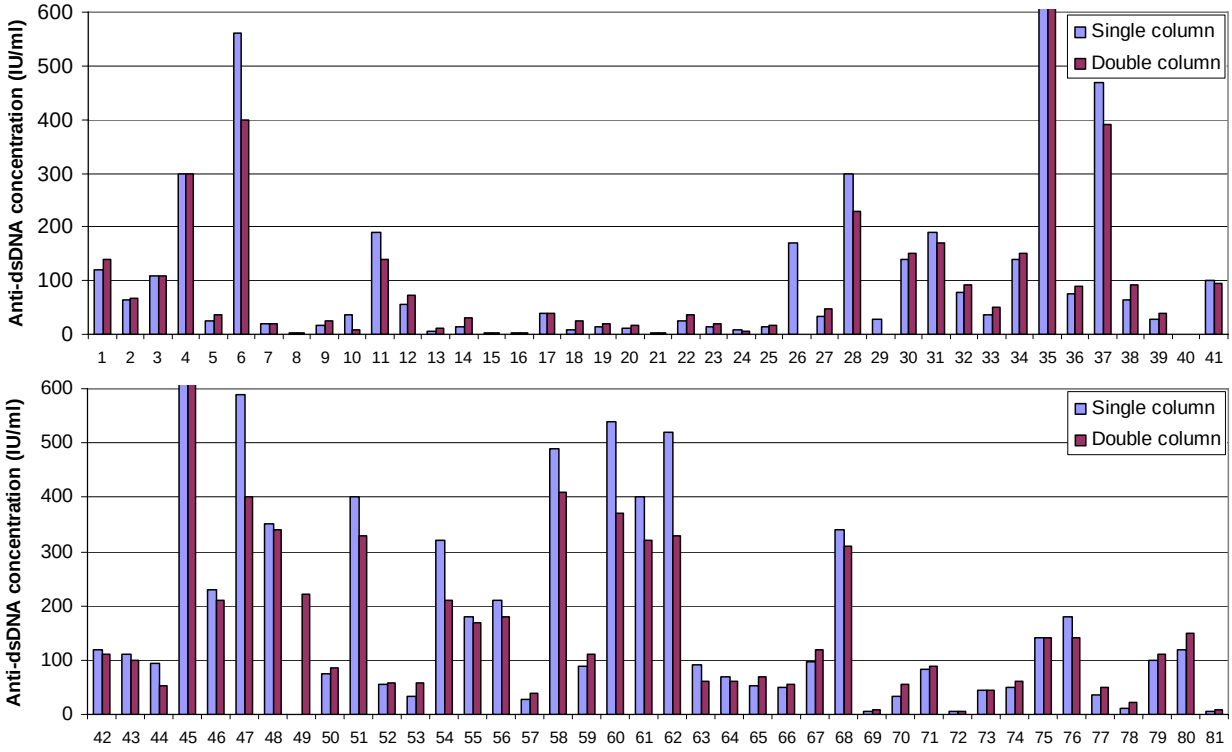

Figure 27. Anti-dsDNA antibody concentration of serum samples. For the single column assay, no result was obtained for serum 49 due to an error in sample distribution. For the double column assay, no results were obtained for sera 26 and 29 due to problems with signal interpretation. Serum 40 yielded a result below the measurement range, i.e. lower than 0.3 IU/ml, on both single and double columns. Results for serum 35 were 1,500 and 1,300 IU/ml on the single and double columns, respectively. Serum 45 yielded a result of 2,400 IU/ml on the single column, while above the measurement range, i.e. higher than 5,000 IU/ml, on the double column (y axes scaled only to 600 IU/ml to increase visibility of low concentration results).

The correlation between single and double column results was high, with a correlation coefficient of 0.98 (Figure 28). For higher concentrations, the double column setup tended to generate lower results than the single column setup.

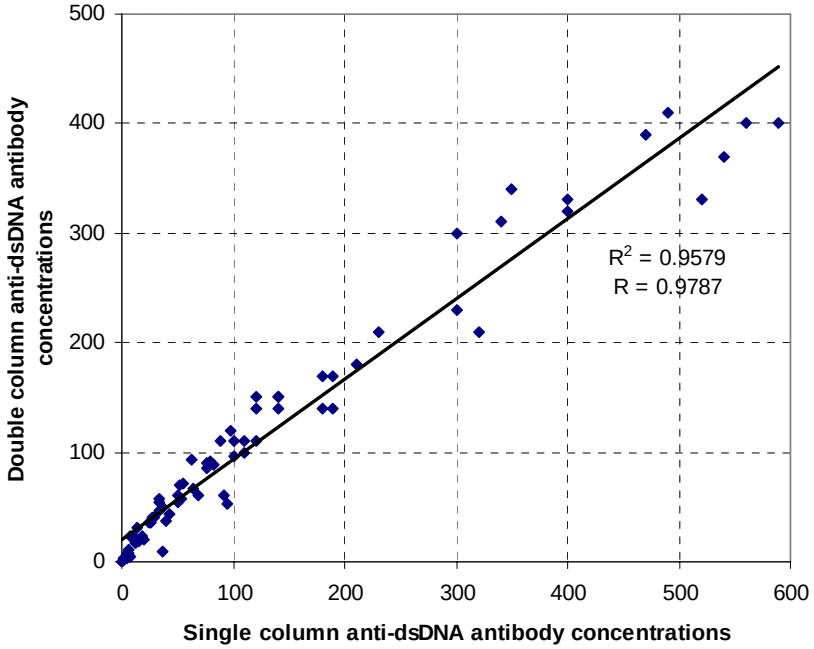

Figure 28. Correlation plot for anti-dsDNA antibody concentrations (in IU/ml) of samples evaluated on single and double columns. Samples 26, 29, 35, 45 and 49 are omitted. An r^2 value of 0.9579 for the linear regression corresponds to a correlation coefficient of approximately 0.98.

4.3.6 Comparison with CLIF

Results of the CLIF assay performed at the Department for Clinical Immunology, Uppsala University Hospital are listed in Appendix 2. Gyrolab Bioaffy™ results, separated in positive and negative as determined in the CLIF assay, are presented in Figure 29.

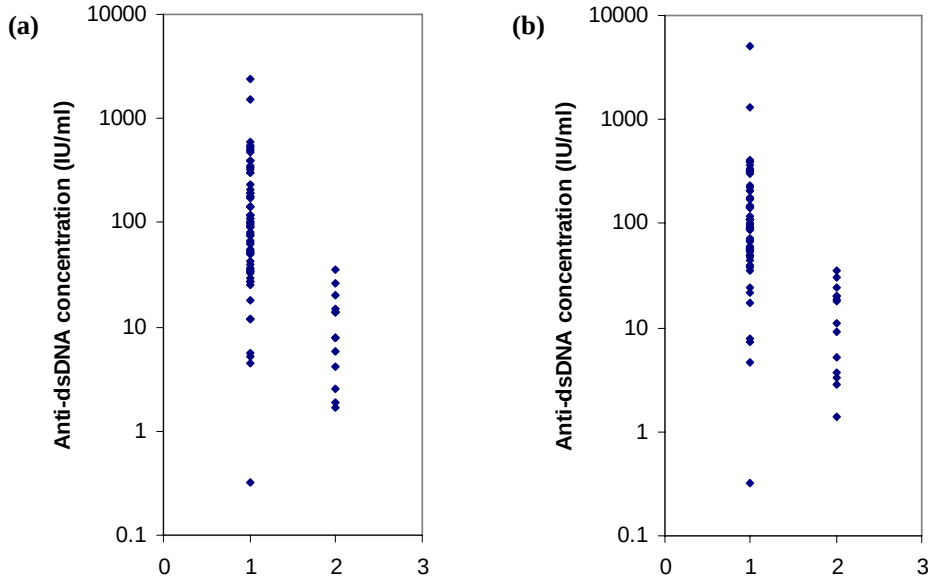

Figure 29. Gyrolab Bioaffy™ results, sorted by positive (1) and negative (2) CLIF results, for the **(a)** single and **(b)** double columns.

It is immediately apparent that the Gyrolab Bioaffy™ results correlated with the CLIF results to some degree. A closer look at the individual results revealed that for the single column assay, 14 positive samples were quantified as having a lower anti-

dsDNA antibody concentration than the negative sample with the highest concentration. For the double column assay, that number was 7.

Since the negative and positive groups in Figure 29 partially overlap, the discriminating ability of the Gyrolab Bioaffy™ assay (as compared with the CLIF assay) is not perfect. The relationship between Gyrolab Bioaffy™ and CLIF with regards to sensitivity and specificity is illustrated in the receiver operating characteristic (ROC) plots in Figure 30. These indicate that the test is in good agreement with the CLIF assay, since the curves pass close to the upper left corner. A specificity of 100 % can be obtained by choosing a cut-off value of 38 IU/ml. The two curves are nearly identical.

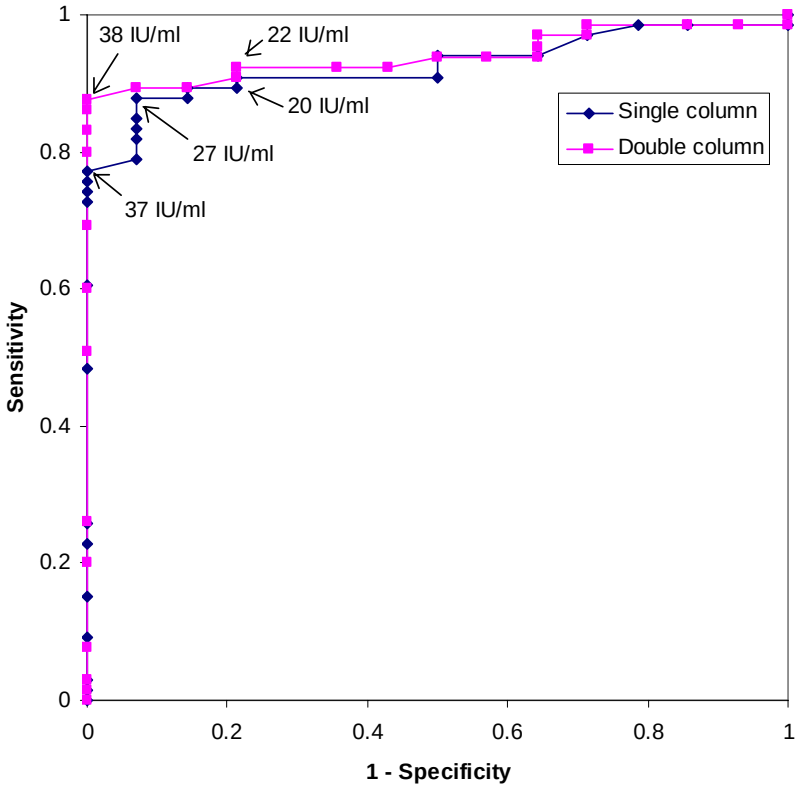

Figure 30. ROC plots for the single and double column Gyrolab Bioaffy™ assays, based on the CLIF classification of negative and positive samples. Selected cut-offs are indicated below and above the curves for the single and double column set-ups, respectively.

5 Discussion

Measurement of anti-dsDNA autoantibodies is important for diagnosis and monitoring of SLE. The three standard laboratory tests are CLIF, Farr and ELISA [5]. All of these assays are time consuming and, at times, labor intensive. Therefore, the development of a rapid, reliable anti-dsDNA antibody assay with little "hands-on" time is desirable.

The intention of this degree project was to develop an assay using Gyrolab Bioaffy™ CD microlaboratories for quantification of anti-dsDNA autoantibodies and to characterize its performance. Two assay formats were investigated: bridging antibody assay (BAA) and indirect antibody assay (IAA). For schematic representations, see Figure 1. For each format, two antigens were examined: one a 3.4 kb plasmid and the other a 30 bp synthetic DNA molecule. The plasmid was chosen because its specification states functionality with the Wo/80 reference antibody used as a standard in the project. However, the molecular weight of the plasmid is higher than what is commonly used in the Bioaffy™ system. Therefore, a parallel evaluation of a smaller antigen was performed. The 30 bp oligomer was prepared by hybridization of single-stranded synthetic oligonucleotides.

For SLE diagnosis, it is important that the antigen used in assays is free from ssDNA contamination since anti-ssDNA antibodies are not specific for SLE patients. The closed circular form of the plasmid was thought to decrease the risk of ssDNA. The dsDNA content of the oligomer preparation was monitored using FRET. The oligonucleotide sequences used for the hybridization were chosen so that hairpin formation was unlikely (kindly controlled by Dr. Nigel Tooke, Biotage). If hairpins do form, the presence of ssDNA stretches is unavoidable.

Originally, it was expected that a BAA utilizing antigen (dsDNA) for both capture and detection would perform best. Since the specific antigen-antibody interaction of interest is used for detection, low background was expected. Therefore, the initial efforts were focused on BAA development. However, acceptable signal-to-noise ratios were not obtained in this research project. The lack of success in the BAA development could be due to several reasons. One possibility is affinity problems. The low capture antigen concentration necessary for monovalent binding of the antibody inherently results in an assay sensitive to antibody affinity. The stability of the first interaction is of utmost importance, since loss of that interaction results in lost signal.

For the plasmid antigen, size-dependent diffusion is probably important. Immobilization must occur in a short time span, and slow diffusion rates would hinder the movement of the plasmid to the bead surface, thereby preventing efficient loading of the antigen onto the beads. Another possibility is that the plasmid size could promote bivalent binding of the antibodies to a single plasmid, even if the plasmids are well separated in space, thus preventing bridge formation. However, some signal was obtained for the BAA when using plasmid as antigen, albeit with low signal-to-noise ratio. For the oligomer antigen BAA, no response could be achieved, even if the plasmid was used as capture antigen and the oligomer solely for detection.

Another parameter is the time allowed for the interaction, i.e. the time between analyte and detection reagent additions. The capture antigen concentration and

interaction time can be viewed as two axes spanning an area where detection of antibodies through bridging can be achieved. It is possible that the assay method used in this project was not optimal for avoidance of bivalent binding of sample antibodies to the capture antigen, so that the best conditions were not encountered.

As an alternative approach for detection of anti-dsDNA antibodies, an IAA was developed using the oligomer as capture reagent and a secondary antibody as detection reagent. The final assay setup is illustrated in Figure 31. The possibility of bivalent and thus stronger antigen-antibody interactions decreased the risk of sample antibodies being washed away, and the capture antigen concentration optimization became a matter of ensuring saturation of the solid phase. The oligomer antigen was chosen for the final assay, since it resulted in higher signal-to-noise ratios than the plasmid antigen. It can also be noted that the antigen preparation for this assay is easy to perform, only requiring a straightforward hybridization of pre-labeled synthetic oligonucleotides.

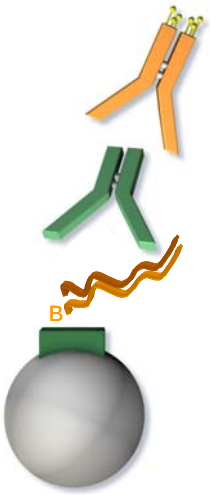

Figure 31. Indirect antibody assay (IAA). First, biotinylated oligomer is immobilized on the streptavidin-coated column beads. Second, sample anti-dsDNA antibodies (green) are allowed to interact with the antigen. Last, Alexa-labeled anti-human IgG antibodies (orange) interact with sample antibodies, producing a fluorescent signal.
Illustration (partly modified) used with permission from Gyros AB.

Good assay reproducibility was demonstrated by analysis of the same standard curve on four different CDs. Inter-CD CVs were below 5% except for the highest dilution where responses varied somewhat. Thus, results in the low concentration range (responses of 1 and below for PMT 1%) should not be considered as absolute. However, a response of 1 corresponds to an anti-dsDNA concentration of approximately 2.5 IU/ml (see standard curve in Figure 22, sample dilution 1:25), which clearly qualifies as a negative result in this assay. Consequently, the possible response variation in the lower range is probably not clinically relevant.

Absence of significant levels of ssDNA contamination of the capture antigen was indicated by direct detection with Cy™5-labeled ssDNA complementary to the biotinylated oligonucleotide used in the capture reagent hybridization. An increased proportion of unlabeled oligonucleotide in the hybridization mixtures did not significantly lower the response, suggesting that the ssDNA contamination is probably minor. This result demonstrated that the assay would be specific for anti-dsDNA antibodies.

The specificity of the assay was further investigated by evaluation of samples on columns with HSA as capture reagent instead of dsDNA. HSA was chosen since the anti-HSA responses were supposed to be low and thus could be viewed as “blank”

responses. The anti-HSA responses were indeed low, and further decreased when double columns were used for inline sample pre-treatment. This indicated that the anti-dsDNA responses obtained in the final assay were generated by specific interactions between dsDNA and antibodies. The double column approach was an attempt to separate nonspecific column-related interactions from the specific antigen-antibody interaction under investigation. The fact that the anti-HSA responses were lowered by the addition of a second column suggest that an inline sample purification approach could eliminate signals derived from nonspecific interactions. The standard curves generated on single and double columns showed good correspondence, indicating that the specific anti-dsDNA interactions were not influenced by the pre-treatment.

When designing an anti-dsDNA antibody assay, high diagnostic specificity is more important than high diagnostic sensitivity – that is, occasional false negatives can be tolerated if a method produces very few false positives. If a cut-off value of 38 IU/ml is set, a specificity of 1.0 is obtained with sensitivities of 0.76 and 0.88 for the single and double column assays, respectively, as compared to the CLIF assay. Thus, the ratio of patients with a positive CLIF result and a positive Bioaffy™ result to all patients with a positive CLIF result is 0.76 or 0.88 for a cut-off value of 38 IU/ml. Decreasing the cut-off value gives a higher sensitivity but lowers the specificity.

In the ROC plots in Figure 30, the double column plot lies slightly above and to the left of the single column plot, suggesting a higher accuracy of the double column method compared to CLIF. Still, the difference between the plots is small and cannot be used to conclude that a double column assay is more accurate. Instead, the correlation plot (Figure 28) indicates that the single and double column results correspond well ($r = 0.98$). A larger sample set with a higher proportion of negative samples would decrease the jaggedness of the ROC plots and possibly clarify single and double column assay correlation. The study on anti-HSA responses suggests a higher double column significance. To evaluate the double column approach further, investigations of other antigen-antibody pairs would be informative. It is also possible that the small difference between single and double column assay results in the anti-dsDNA case reflects an uncomplicated sample group. A larger sample group analyzed on both single and double columns might reveal elimination of false positives by the double column setup, and possibly allow a lowered cut-off value between negative and positive results.

When the samples are separated by CLIF titer (Figure 32), the difference in quantification between Bioaffy™ and CLIF is evident. Some sera resulting in a low titer in the CLIF assay yield high anti-dsDNA antibody concentrations in the Bioaffy™ assay, and vice versa. At a first glance, one is tempted to conclude that the Bioaffy™ method results in serious diagnostic errors. However, the classification of positive and negative results shows better correlation. Also, one must remember that a fundamental difference between the two methods is that the Bioaffy™ method operates with a constant flow of samples, wash solution and reagents, whereas the CLIF assay allows stationary incubation. In addition, the time period where antigen-antibody interactions can occur is much shorter in the Bioaffy™ case. Hence, the affinity of the antibodies is more important. Low affinity antibodies are, if they even bind, easier to wash away and will give a lower signal than high affinity ones. Consequently, the CLIF assay may give a better measurement of the total amount of

anti-dsDNA antibodies, whereas the Bioaffy™ assay quantifies the anti-dsDNA antibodies with regards to the concentration of the high affinity antibodies. Occurrence of antibodies with high affinity is a prognostic marker and associated with severe SLE [19,20], indicating that the Bioaffy™ information could be of high clinical interest.

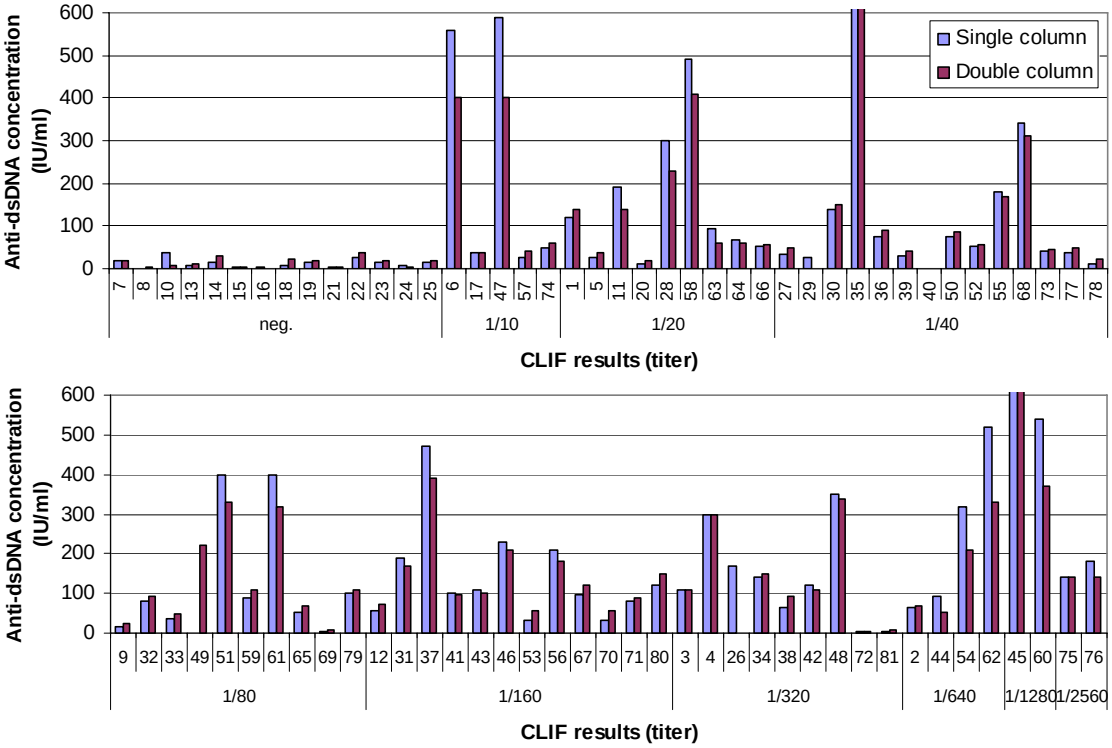

Figure 32. Gyrolab Bioaffy™ results sorted by CLIF titer. For sera 26, 29, 35, 40, 45, and 49, see legend of Figure 27.

When considering the sensitivity and specificity determined with CLIF results as the gold standard, one should recognize that the CLIF assay is not perfect. It has been reported to have a sensitivity of 62% and a specificity of 99% for SLE [21]. A direct comparison of Bioaffy™ results with patient diagnoses (SLE or non-SLE) would have been informative, but was regrettably not possible due to lack of diagnostic information.

In conclusion, the anti-dsDNA assay developed in this project appears promising. It requires considerably less time and effort than conventional assays. The classification of results as positive or negative correlate well with the CLIF assay, even though individual quantifications can differ greatly between the two methods. Future evaluation of this method should consider including a comparison with patient diagnosis.

6 Acknowledgments

First and foremost, I wish to thank my supervisor, Dr. Mats Inganäs at Gyros AB, for giving me the opportunity to carry out this degree project and for supporting me all the way through to completion, and all Gyros AB employees who have patiently answered questions and made me feel very welcome. I would also like to thank Dr. Nigel Tooke at Biotage AB for much appreciated assistance with oligonucleotide design and hybridization studies, and Dr. Bo Nilsson and Elisabeth Wijkström at the Department for Clinical Immunology, Uppsala University Hospital, for providing patient serum samples for analysis and assay evaluation. I am also grateful to Professor Jan Carlsson at the Department of Surface Biotechnology, Uppsala University, and Dr. Helena Laven at the Department of Oncology-Pathology, Karolinska University Hospital, for reading and reviewing this report.

7 References

1. Reumatikerförbundet website (Sjukdom & forskning, reumatiska sjukdomar) http://www.reumatikerforbundet.org/start.asp?sida=3929 (6 Sep. 2005)
2. American College of Rheumatology website (Patients & public, patient education, disease-specific information) http://www.rheumatology.org/public/factsheets/sle_new.asp?aud=prs (6 Sep. 2005)
3. Tan E.M., Cohen A.S., Fries J.F., Masi A.T., McShane D.J., Rothfield N.F., Schaller J.G., Talal N., and Winchester R.J. (1982) The 1982 revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum.* **25**, 1271-1277
4. Hochberg M.C. (1997) Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum.* **40**, 1725
5. Hahn, B.H. (1998) Antibodies to DNA. *N. Engl. J. Med.* **338**, 1359-1368
6. Förger F., Matthias T., Oppermann M., Becker H., and Helmke K. (2004) Clinical significance of anti-dsDNA antibody isotypes: IgG/IgM ratio of anti-dsDNA antibodies as a prognostic marker for lupus nephritis. *Lupus* **13**, 36-44
7. Sherer Y., Gorstein A., Fritzler M.J., and Shoenfeld Y. (2004) Autoantibody explosion in systemic lupus erythematosus: more than 100 different antibodies found in SLE patients. *Semin. Arthritis Rheum.* **34**, 501-537
8. Vaccaro C., Zhou J., Ober R.J., and Ward E.S. (2005) Engineering the Fc region of immunoglobulin G to modulate in vivo antibody levels. *Nat. Biotechnol.* **23**, 1283-1288
9. Emlen W., Jarusiripipat P., and Burdick G. (1990) A new ELISA for the detection of double-stranded DNA antibodies. *J. Immunol. Methods* **132**, 91-101
10. Hoesel W., Gross J., Moller R., Kanne B., Wessner A., Müller G., Müller A., Gromnica-Ihle E., Fromme M., Bischoff S., and Haselbeck A. (2004) Development and evaluation of a new ELISA for the detection and quantification of antierythropoietin antibodies in human sera. *J. Immunol. Methods* **294**, 101-110
11. Bourdage J.S., Lee T.N., Taylor J.M., Willey M.B., Brandt J.T., and Konrad R.J. (2005) Effect of double antigen bridging immunoassay format on antigen coating concentration dependence and implications for designing immunogenicity assays for monoclonal antibodies. *J. Pharm. Biomed. Anal.* **39**, 685-690
12. Moxness M., Tatarewicz S., Weeraratne D., Murakami N., Wullner D., Mytych D., Jawa V., Koren E., and Swanson S.J. (2005) Immunogenicity testing by electrochemiluminescent detection for antibodies directed against therapeutic human monoclonal antibodies. *Clin. Chem.* **51**, 1983-1985
13. Wasmuth J., Grün B., Terjung B., Homrighausen A., and Spengler U. (2004) ROC analysis comparison of three assays for the detection of antibodies against double-stranded DNA in serum for the diagnosis of systemic lupus erythematosus. *Clin. Chem.* **50**, 2169-2171
14. von Mühlen C.A. and Tan E.M. (1995) Autoantibodies in the diagnosis of systemic rheumatic diseases. *Semin. Arthritis Rheum.* **24**, 323-358

15. Feltkamp T.E.W., Kirkwood T.B.L., Maini R.N., and Aarden L.A. (1988) The first international standard for antibodies to double stranded DNA. *Ann. Rheum. Dis.* **47**, 740-746
16. Gyros AB (2005) *Gyrolab™ Workstation User Guide*. Version 7.1
17. Trendelenburg M., Lutz H.U., Tissot J.D., Moll S., Hoffmann T., and Schifferli J.A. (2003) Cryoglobulin/albumin complexes in a patient with severe autoimmune syndrome. *Scand. J. Rheumatol.* **32**, 367-373
18. NCCLS. Assessment of the clinical accuracy of laboratory tests using receiver operating characteristic (ROC) plots; approved guideline. NCCLS document GP10-A (ISBN 1-56238-285-3). NCCLS, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087, USA.
19. Smeenk R.J., van den Brink H.G., Brinkman K., Termaat R.M., Berden J.H., and Swaak A.J. (1991) Anti-dsDNA: choice of assay in relation to clinical value. *Rheumatol. Int.* **11**, 101-107
20. Swaak T. and Smeenk R. (1985) Detection of anti-dsDNA as a diagnostic tool: a prospective study in 441 non-systemic lupus erythematosus patients with anti-dsDNA antibody (anti-dsDNA). *Ann. Rheum. Dis.* **44**, 245-251
21. Seltsam A., Schwind P., Abraham K., Hiepe F., Cygan S., Aebischer I., and Salama A. (2002) Rapid detection of autoantibodies to dsDNA with the particle gel immunoassay (ID-PaGIA). *Ann. Rheum. Dis.* **61**, 367-369

Appendix 1.

The following oligonucleotides were synthesized by TIB MOLBIOL.

- Sequence 1: 5'-GAGAT GAACA AGCAC CAGAT GACAA CTAAA-3' 5'-biotinylated.
- Sequence 2: 5'-GAGAT GAACA AGCAC CAGAT GACAA CTAAA-3' 5'-Cy™5-labeled.
- Sequence 3: 5'-TTTAG TTGTC ATCTG GTGCT TGTTC ATCTC-3'

Note that sequence 1 and 2 are identical except for the labels, and that sequence 3 is complementary to sequences 1 and 2. A 5'-Cy™5-labeled version of sequence 3 was also synthesized.

The sequence was kindly suggested by Dr. Nigel Tooke, Biotage, and evaluated by him in a primer design program (Oligo) to ensure that it had no tendencies for hairpin formation.

Appendix 2.

Results of CLIF assay performed at the Department for Clinical Immunology, Uppsala University Hospital. Results are given in serum titers. Negative results are denoted “Neg.”.

1. 1/20	18. Neg.	35. 1/40	52. 1/40	69. 1/80
2. 1/640	19. Neg.	36. 1/40	53. 1/160	70. 1/160
3. 1/320	20. 1/20	37. 1/160	54. 1/640	71. 1/160
4. 1/320	21. Neg.	38. 1/320	55. 1/40	72. 1/320
5. 1/20	22. Neg.	39. 1/40	56. 1/160	73. 1/40
6. 1/10	23. Neg.	40. 1/40	57. 1/10	74. 1/10
7. Neg.	24. Neg.	41. 1/160	58. 1/20	75. 1/2560
8. Neg.	25. Neg.	42. 1/320	59. 1/80	76. 1/2560
9. 1/80	26. 1/320	43. 1/160	60. 1/1280	77. 1/40
10. Neg.	27. 1/40	44. 1/640	61. 1/80	78. 1/40
11. 1/20	28. 1/20	45. 1/1280	62. 1/640	79. 1/80
12. 1/160	29. 1/40	46. 1/160	63. 1/20	80. 1/160
13. Neg.	30. 1/40	47. 1/10	64. 1/20	81. 1/32
14. Neg.	31. 1/160	48. 1/320	65. 1/80	
15. Neg.	32. 1/80	49. 1/80	66. 1/20	
16. Neg.	33. 1/80	50. 1/40	67. 1/160	
17. 1/10	34. 1/320	51. 1/80	68. 1/40	

Appendix 3.

Operation sequence for the Bioaffy™ 1C v3 method:

1. Initial needle wash
2. Particle wash 1
3. Particle wash spin 1
4. Particle wash 2 structure
5. Particle wash 2 common
6. Particle wash spin 2
7. Capture reagent addition
8. Capture reagent spin
9. Capture reagent wash 1
10. Capture reagent wash spin 1
11. Capture reagent wash 2
12. Capture reagent wash spin 2
13. Analyte addition
14. Analyte spin
15. Analyte wash 1
16. Analyte wash spin 1
17. Analyte wash 2
18. Analyte wash spin 2
19. CD alignment 1
20. Detect background PMT 1%
21. Detect background PMT 5%
22. Detect background PMT 25%
23. Spin out
24. Detection reagent addition
25. Detection reagent spin
26. Detection reagent wash 1
27. Detection reagent wash spin 1
28. Detection reagent wash 2
29. Detection reagent wash spin 2
30. Detection reagent wash 3
31. Detection reagent wash spin 3
32. Detection reagent wash 4
33. Detection reagent wash spin 4
34. CD alignment 2
35. Detect PMT 1%
36. Detect PMT 5%
37. Detect PMT 25%