UPPSALA
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UPTEC X 20006
Examensarbete 30 hp
Juni 2020

Development and comparison of three immunoassay formats to screen for total anti-adeno-associated virus serotype 2 antibodies in human serum using the Gyrolab immunoassay platform

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Abstract

Development and comparison of three immunoassay formats to screen for total anti-adeno-associated virus serotype 2 antibodies in human serum using the Gyrolab immunoassay platform

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Recombinant adeno-associated virus vectors are one of the most promising gene delivery tools for applications within gene- and cell therapy. The high level of wild-type adeno-associated virus infections in humans is a limitation due to the pre-existing immunity against the vector or its transgene product. An important tool to develop effective and safe therapies is the ability to measure the pre-existing immune response against the virus capsids in humans.

This master thesis at Gyros Protein Technologies aimed to investigate if the Gyrolab immunoassay system can be used to screen for pre-existing anti-capsid immunity in human sera by optimizing and evaluate three different assay formats: an indirect assay, a generic anti-AAV adsorption assay and a bridging assay. The evaluation focused on immunity against adeno-associated virus serotype 2. All immunoassay formats performed well and depending on application, the different formats offers different advantages. The generic anti-AAV adsorption assay offers the ability to easily screen for several viral serotypes without having to label the capsid, and the bridging assay provides high sensitivity. When screening 31 individual human sera, 58% were positive using the indirect assay and the genetic anti-AAV adsorption assay and 65% using the bridging assay format. Provided, is automated and high throughout immunoassays where 16 individuals can be screened in one-two hours. It is shown that all three immunoassay formats can be used to screen for anti-adeno-associated virus antibodies, even though further optimization, cut off development and a larger data set is needed to obtain a fully sophisticated screening tool.

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Ämnesgranskare: Staffan Svärd
Examinator: Erik Holmqvist
ISSN: 1401-2138, UPTEC X 2006

Populärvetenskaplig sammanfattning

Många av våra mest komplexa sjukdomar beror på mutationer i funktionella gener. En metod för att bota denna typ av sjukdomar är föra in främmande genetiskt material som kompenserar för de skadade generna. Detta kan göras antingen genom att föra in hela celler som innehåller och uttrycker de gener som önskas, eller att få in generna i patientens egna celler, kallat gen- eller cellterapi (Brown 2015, Santiago-Ortiz 2016). Eftersom att många virus naturligt infekterar och för in främmande gener i värdorganismens genom, kan dessa modifieras och användas för att föra in önskat främmande genetiskt material. En grupp av virus det forskas mycket på, som vektor inom gen- och cellterapi, är adeno-associerade virus (Santiago-Ortiz & Schaffer 2016).

Det finns många hinder att undkomma för att få en fungerade gen- och cellterapi med virus som vektor, till exempel adeno-associerade virus. En av dem är det redan existerande immunförsvaret många har mot adeno-associerade virus. Eftersom att adeno-associerade virus ofta infekterar människor naturligt vid en tidig ålder kan immunförsvaret attackera vektorviruset och/eller dess produkt och göra terapin ineffektiv eller farlig. Under utvecklingen av gen- och cellterapi med adeno-associerade virus som vektor, är det därför viktigt att kunna mäta antikroppar mot viruset (Martino & Markusic 2020). Det finns ett stort behov av känsliga, robusta och optimerade immunoassays och ett stort problem är att kunna jämföra mätningar från olika studier.

I denna studie presenteras tre optimerade immunoassays på plattformen Gyrolab för att mäta antikroppar i serum som binder till adeno-associerade virus typ 2. De kan användas som screening-verktyg för att snabbt kunna undersöka om personer har antikroppar mot viruset. Med dessa verktyg kan man på en-två timmar screena 16 individer för immunrespons mot viruset.

Content

Abbreviations

a-	Alexa fluor 647 labelled
AAV	Adeno-Associated Virus
b-	Biotinylated
BSA	Bovine Serum Albumin
CD	Compact Disc
ELISA	Enzyme-Linked Immunosorbent Assay
F(ab')2	The two Fab regions of an antibody
Fab	Fragment antigen-binding
Fc	Fragment crystallizable
GAAA	Generic Anti-AAV Adsorption Assay
Ig	Immunoglobulin
MW	Molecular Weight
NC	Negative Control
PBST	Phosphate Buffered Saline + 0.02% Tween
PMT	PhotoMultiplier Tubes
PP/ml	Particles per ml
RMP	Rounds Per Minute
RU	Response Units
SD	Standard Deviation
ε	Extinction coefficient
TAb	Total Antibody
NAb	Neutralizing Antibody

1 Introduction

The aim of this Master thesis was to evaluate three different immunoassay formats on the Gyrolab platform to screen for pre-existing antibodies against adeno-associated virus (AAV) type 2 capsids in human sera.

1.1 Background

1.1.1 Why screen for pre-existing immune response against AAVs?

The introduction of foreign genetic material into target cells or tissues for therapeutic purposes, gene therapy, has large potential to treat many diseases such as various types of cancer, monogenic diseases and cardiovascular diseases. One of the main challenges is to deliver the genetic material to the target cells in a safe and efficient manner. Since viruses naturally recognize and infect cells they make the perfect candidates as vector for *in vivo* gene delivery. However, to engineer and improve their delivery properties can be challenging (Santiago-Ortiz & Schaffer 2016).

An alternative strategy to gene therapy is cell therapy, where live whole cells are infused into a patient. Often the cells used are the patient's own stem cells, and if that patient is being treated for disease causing mutations, the stem cells will contain the same mutations. One possible solution is to genetically modify the stem cells with, for example, AAVs as vector (Brown & Hirsch 2015).

Adeno-associated viruses are one of the most investigated viral vectors for gene delivery due to their lack of pathogenicity and gene delivery efficacy (Naso *et al.* 2017). AAVs are small single stranded DNA dependoviruses, part of the *Parvovirus* family. Humans and mammals are the natural host for AAV infections, but are not associated with any diseases in their hosts (Martino & Markusic 2020). To date, 13 AAV serotypes and 108 isolated (serovars) have been identified and classified (Ronzitti *et al.* 2020). For AAVs to be able to replicate once inside the host cell, they need mediation from immunogenic helper viruses and proteins. The helper viruses cause inflammation, resulting in humoral and cell-mediated immune response against the AAV capsid proteins. A large challenge for sustainable gene and cell therapy in humans is the pre-existing immunity against AAVs (Martino & Markusic 2020). When using AAVs as gene delivery vectors, the recombinant viral capsids are derived from wild-type AAVs, meaning that they might be recognized by the pre-existing adaptive immune responses (Martino & Markusic 2020).

1.1.2 Human immune responses to AAVs

The extensive anti-AAV immunity in human populations are limiting the preclinical and clinical studies on AAV gene delivery. The ability to measure the pre-existing immune responses in humans in a robust way is crucial for future development of finding vectors with

high transduction capabilities and gene expression as well as understanding the immune responses of the vectors and reducing the side effects (Goswami *et al.* 2019). The immune response against *in vivo* gene transfer using AAV is triggered by the viral capsid and the transgene product. Due to early exposure to wild-type AAVs, a significant proportion of humans develop humoral immunity against AAV capsids early in life. There is a high degree of conservation among AAV serotypes, and anti-AAV antibodies therefore have extensive cross reactivity between serotypes. Anti-AAV2 antibodies are the most prevalent, up to 60-70% (Mingozzi *et al.* 2013, Ronzitti *et al.* 2020).

Studying the pre-existing immunity against AAV capsids, in terms of gene delivery efficiency and safety, requires information about the pre-existing neutralizing antibodies/factors and the total antibodies (TAbs). Anti-AAV IgG antibodies from all subclasses have been found, correlating with the neutralizing antibody (NAb) titres (Falese *et al.* 2017). Some individuals carry non-neutralizing IgGs as well (Ronzitti *et al.* 2020). In this study three anti-AAV2 TAb assays are evaluated.

1.2 Theory

1.2.1 Antibodies

Antibodies or immunoglobulins (Igs) are heavy plasma proteins and serves as a part of the immune system for humans and animals by recognizing a variety of antigen. Antibodies are produced by B-cells and their function is as versatile as their composition. Among else, they identify and mediate neutralization or killing of foreign invaders such as pathogens, viruses or other infectious agents. (Casali & Schettino 1996, Wootla *et al.* 2014). Mammalian Igs are classified in IgM, IgD, IgG, IgE and IgA, where IgG is the most abundant in humans (Ma H *et al.* 2015). IgGs are monomeric and has the size of about 150 kDa. They consist of two light chains and two heavy chains (Figure 1a) which are connected via disulphide bonds. Each heavy chain consists of one variable domain, V_H, and three constant domains, C_H. Each light chain consists of one variable and one constant domain, V_L and C_L respectively (Ma H *et al.* 2015). One can divide a monomeric antibody into two parts; fragment antigen-binding (Fab) region and fragment crystallisable (Fc) domain (Figure 1b). The Fab region consist of both variable and constant domains, and the hyper mutations in the variable region allows the antibodies to recognize a wide range of antigens. The constant Fc region lets the antibody communicate with other biomolecules involved in the immune system (Hayes *et al.* 2014).

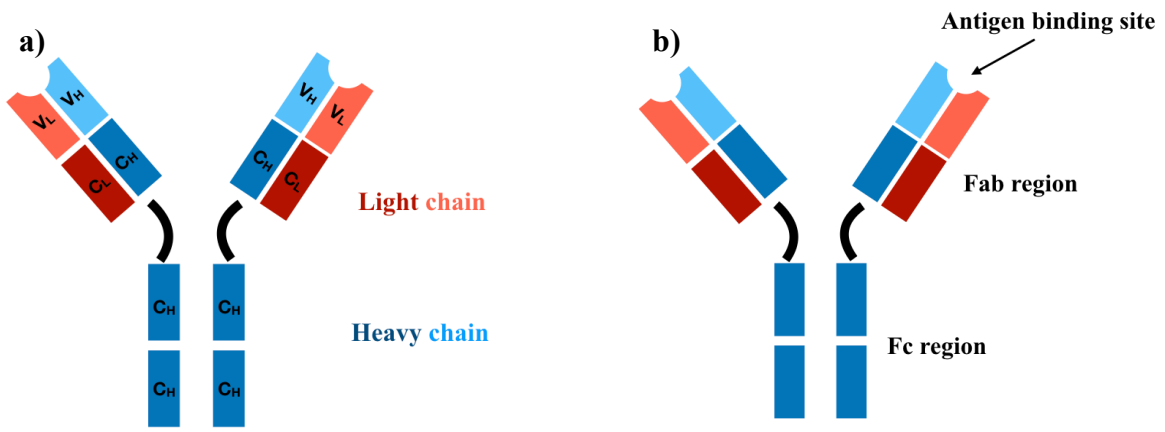

Figure 1. Schematic overview of the functional components of an antibody.

Monoclonal antibodies are produced from a single B cell clone and can only identify one type of epitope on the antigen. Polyclonal antibodies are produced in multiple B cell clones and recognizes multiple epitopes on the antigen (Wootla *et al.* 2014).

1.3 The Gyrolab® immunoassay platform

The Gyrolab immunoassay platform allows the user to perform its own experiment design, and is therefore an open platform with large degree of flexibility.

1.3.1 The Gyrolab® technology

The Gyrolab® immunoassay system utilize semi-automated, high throughput analysis by an affinity flow through format. The reagents and analyte are transferred automatically from a storage microplate onto a compact disc (CD) containing up to 112 identical microstructures with solid phase columns with 15 nl streptavidin-coated particles (Gyros Protein Technologies 2019a). The nanoliter microfluidics system is based on capillary action, centrifugal forces and hydrophobic stops (Andersson *et al* 2007). Typically, the immunoassay consists of a biotinylated capture reagent with affinity to the analyte. The analyte is then quantified by addition of a fluorescent labelled reagent which is detected by a scanning confocal laser-induced fluorescent detector (Gyros Protein Technologies 2019b, Andersson *et al* 2007).

1.3.2 Gyrolab® Bioaffy™ CDs

A Gyrolab® CD consists of up to 112 identical nanoliter-scale microfluidic structures with a 15 nl affinity capture column in each structure. The column consists of streptavidin-coated beads where the biotinylated reagent is immobilized during the run. All reagents and washing buffers are added automatically and both capillary and centrifugal forces are used to control the liquid flow in the CD in a precise matter.

There are currently six types of Gyrolab® Bioaffy™ CDs; 20 HC, 200, 200 HC, 1000, 1000 HC and mixing CD, where the number stands for sample volume applied over the column [nl]

and HC stands for High Capacity beads on the solid phase (Gyros Protein Technologies 2019a). The functional components of all CDs except the mixing CD can be seen in Figure 2.

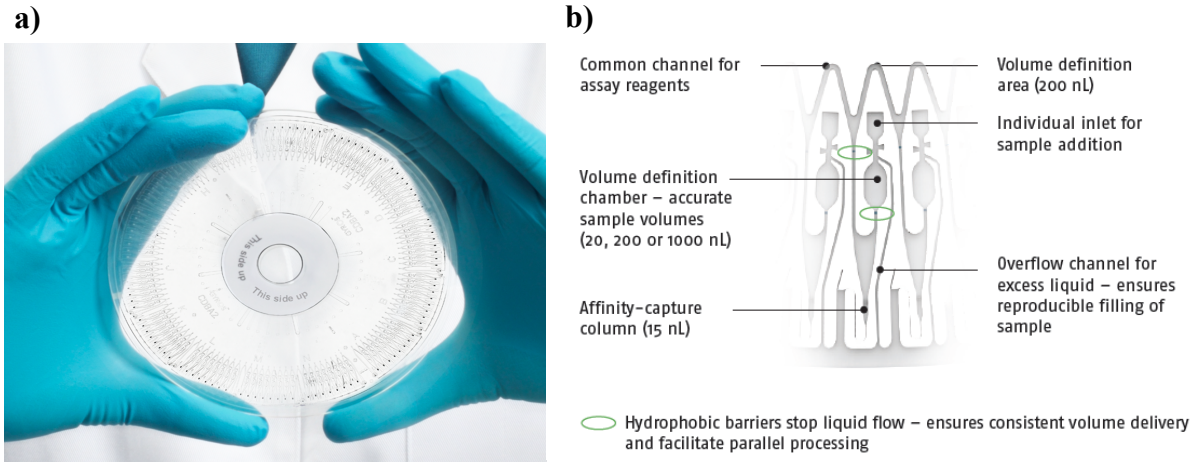

Figure 2. The design and functional component of the Bioaffy CDs.

Washing buffers and reagent are automatically added to the common channel and enters the channel by capillary forces and is stopped by a hydrophobic break. The CD is spun to create centrifugal forces overcoming the stop and adding the reagents to the column in defined volumes. The sample is automatically added to individual inlet and is filling the volume definition chamber by capillary forces. Again, a hydrophobic stop is used. To define the sample volume centrifugal forces are used to remove excess liquid. Even stronger centrifugal forces are then used to apply the sample to the column. To detect the fluorescence, the laser scan all columns (Andersson *et al* 2007).

The Gyrolab Mixing CD 96 uses a different technique than described above, where multiple analytes can be mixed on the CD automatically. Figure 3 shows the functional components of a Gyrolab Mixing CD 96.

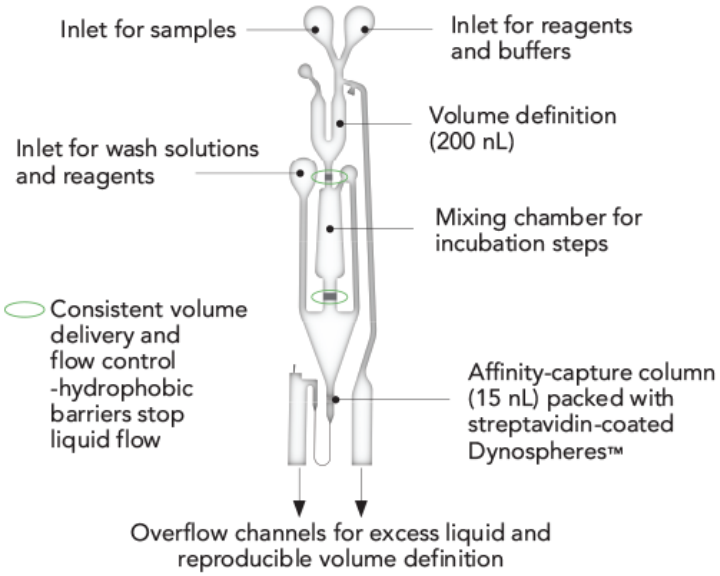

Figure 3. The functional components of the mixing CD. Illustration used with permission from Gyros Protein Technologies.

Sample enters by capillary action and a hydrophobic break stop the liquid flow. Sample volume is then defined to 200 nl in the volume definition chamber before spun into the mixing chamber. The second sample is volume defined to 200 nl and spun into the mixing chamber. To mix the samples, the CD is alternatingly spun.

1.3.3 Output

The output from the Gyrolab platform, visualized as binding profiles, are 3D plots of the intensity in two dimensions for each data point, shown in Figure 4. The different intensities are converted to response units (RU). An automatic evaluation of the raw fluorescent data is visualized in Gyrolab Viewer as a fluorescent profile for every data point (Andersson *et al* 2007) with x-axis along the flow of the reagent/analyte over the column, y-axis as the width of the column where fluorescence intensity is on the z-axis. The profile reflects the location where the capture reagent bind to the analyte. The profiles therefore somewhat reflect the affinity of the capture reagent (Honda *et al* 2005), but most often the column profiles are used to exclude outliers within replicates. The white square is the integration area, the part of the raw fluorescent data included in the response unit calculations. The response unit will reflect the concentration of bound analyte. To improve the dynamic range of the output data, different PMT (Photomultiplier tubes) settings can be used, 1, 5 or 25%. If for example 5% PMT result in a saturation, 1% PMT can be sufficient to cover the dynamic range wanted (Gyros Protein Technologies 2019c).

Examples of column profiles considered as specific signal and profiles considered to be outliers and would be excluded can be seen in Figure 4.

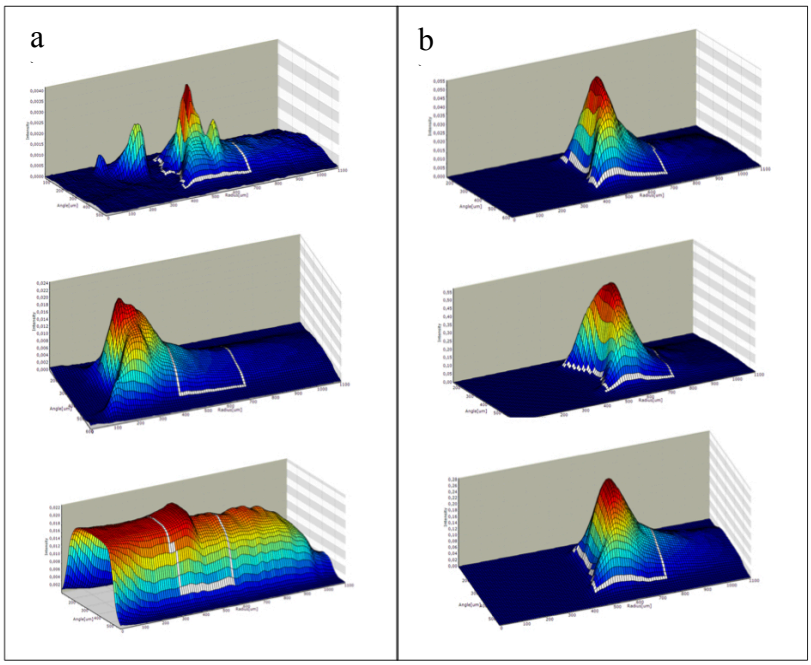

Figure 4. (a) Examples of column profiles considered non-specific outliers that would be excluded from the data set. (b) Examples of column profiles considered as specific binding.

2 Materials and methods

All experiments were performed using the Gyrolab immunoassay platform and executed according to Gyrolab User Guide P0020528 (Gyros Protein Technologies 2019d). In the measurements performed, no positive standard is available for standard curves since all human individuals have varying amounts and types of IgGs against the virus capsid. This leads to the quantification being relative. For the immunoassays performed using the Bioaffy 1000 CD a PMT-setting of 1% was used, and using the Gyrolab mixing 96 CD a PMT-setting of 5% was used. When the CD type is not mentioned, the Gyrolab 1000 CD is used.

2.1 Materials and consumables

In Table 1, Table 2, Table 3 and Table 4 the consumables, bioreagents, buffers, and instrument used in this study are listed.

Table 1. The consumables and respective supplier used in this study

Product	**Supplier**
Gyrolab Bioaffy™ 1000 CD	Gyros Protein Technologies
Gyrolab Mixing CD 96	Gyros Protein Technologies
Skirted 96-well PCR plate 0.2 ml	Thermo Fisher Scientific
Microtiter plate foil	Gyros Protein Technologies

Table 2. The bioreagents and respective supplier used in this study

Bioreagent	**Supplier**
Empty AAV1, 2, 3, 5, 8 and 10 capsids	Sirion Biotech
EZ-Link Sulpho NHS-LC-Biotin	Thermo Fisher Scientific
Mouse anti-human IgG Fc	Southern Biotech
Rabbit anti-mouse F(ab')2	Jackson ImmunoResearch
Alexa labelled monoclonal mouse anti-AAV2	Progen
Monoclonal mouse anti-AAV2	Progen
CaptureSelect™ Biotin Anti-AAVX Conjugate	Thermo Scientific
Human individual serum and serum pool	BioIVT (Seralab)
POROS™ CaptureSelect™ AAVX Affinity Resin	Thermo Fisher Scientific
Alexa Fluor™ 647 Antibody Labeling Kit	Thermo Fisher Scientific

Table 3. The buffers and respective supplier used in this study

Buffer	**Supplier**
Rexxip F	Gyros Protein Technologies
Phosphate Buffered Saline + 0.02% Tween (PBST)	Fisher Scientific

Table 4. The instruments and respective supplier used in this study

Instrument	Supplier
Gyrolab xPand	Gyros Protein Technologies
Gyrolab xPlore	Gyros Protein Technologies
Eppendorf® MiniSpin Plus	Eppendorf
Promax 2020	Heidolph
NanoPhotometer P330	IMPLEN
Centrifuge 5804R	Eppendorf
Heraeus™ Pico™ 17 Microcentrifuge	Thermo Fisher Scientific
Vortex	Merck
Centrifuge Z 100 M	Hermle LaborTechnik

2.2 Method overview

Most commonly used Gyrolab immunoassays consists of three bio-components

1. Capture reagent. The capture reagent is biotinylated to be able to bind to the streptavidin-coated beads on the solid phase affinity column and is the immobilizing component. The choice of capture reagent depends on the analyte.
2. Analyte. The analyte is what is quantified and has affinity towards the capture reagent and the detection reagent. In this study, a model antibody and polyclonal anti-AAV antibodies in human serum is used as analyte.
3. Detection reagent. The detection reagent has affinity towards the analyte and is Alexa Fluor 647-labelled. The fluorescence of the bound detection reagent is then detected to quantify the bound analyte.

In this study, three different immunoassay formats were investigated. A schematic overview over the different formats are shown in Figure 5.

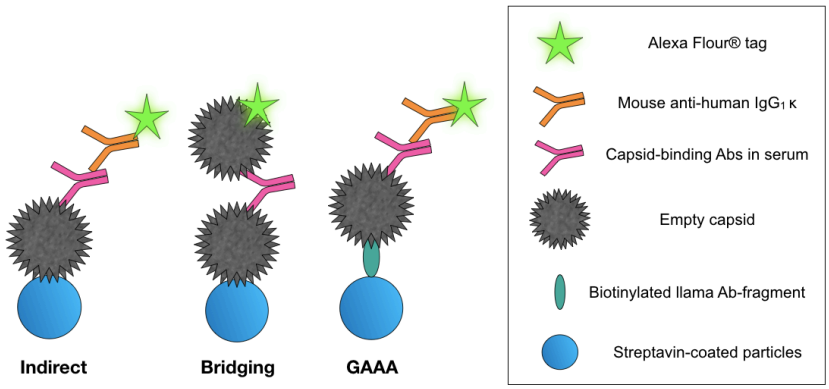

Figure 5. Schematic overview over the three assay formats evaluated in this project.

The empty AAV2 capsids used in this study are recombinantly expressed wild type capsids, containing all three virus proteins (VP): VP1, VP2 and VP3.

The first immunoassay format evaluated for anti-AAV2 immune response was an indirect immunoassay. Biotinylated empty AAV2 capsids act as capture reagent and the analyte was then detected by an Alexa labelled antibody with affinity towards the analyte. For the model system, a monoclonal mouse anti-AAV2 antibody was used as analyte detected by rabbit anti-mouse. When using serum as analyte, a mouse antibody with affinity towards human Fc-region was used at detection reagent.

The second immunoassay format evaluated was a bridging assay format where biotinylated and Alexa labelled anti-AAV2 capsids act as capture and detection reagent, respectively. The analyte was a monoclonal mouse anti-AAV2 antibody and antibodies detected in human serum.

The last assay format used was a so-called Generic Anti-AAV Adsorption assay (GAAA) where four bio-components were used. Biotinylated camelid V_HH (CaptureSelect™ Anti-AAVX conjugate) act as capture reagent. Un-labelled AAV capsids were mixed with the samples and incubated to allow anti-AAV antibodies present in the samples to bind to the capsids. The capsid-antibody complex was captured on the affinity column and detected with an anti-human IgG-Fc. The detection reagent was the same as used in the indirect assay format.

2.3 Labelling the reagents

The reagents used as capture and detect were labelled according to protocol C1 *Labelling of capture and detection reagents* in Gyrolab User Guide P0020528 (Gyros Protein Technologies 2019d).

2.3.1 Biotinylation

The biotinylation of empty AAV2 capsids used in the indirect and bridging assay format, was performed according to protocol C1.1 *Biotinylation of capture element* in Gyrolab User Guide P0020528 (Gyros Protein Technologies 2019d) except for the biotin-reagent ratio suggested. EZ-Link Sulpho NHS-LC-Biotin (Thermo Fisher Scientific), which reacts to the AAV2 amine groups, was dissolved in Milli-Q® to 1 mg/ml. 0.5 µl of 1 mg/ml EZ-Link Sulpho NHS-LC-Biotin was added to 100 µl 10^{13} PP/ml empty AAV2 capsids before 1 hour incubation. Hence, 5 µg biotin per ml 10^{13} PP/ml capsids was used. In the second biotinylation performed, 2 µl 1 mg/ml biotin was added to 200 µl 10^{13} PP/ml empty AAV2 capsids making the biotin-capsid ratio twice as high as the previous labelling, 10 µg biotin per ml 10^{13} PP/ml capsids. The absorbance at 280 nm was measured using a nanophotometer to obtain the protein concentration with MW = 3746 kDa and ε = 3746000, but the concentration was too low to measure. Hence, the concentrations used is expressed as dilutions of the labelled reagents.

2.3.2 Alexa Fluor 647 labelling

For the fluorophore labelling of reagent, Alexa Fluor™ 647 Antibody Labeling Kit (Thermo Fisher Scientific) was performed according to manufactures recommendations (Molecular Probes 2006) with the exceptions mentioned in this section.

500 µl of 500 µg/ml mouse anti-human IgG Fc antibody, used in the indirect and GAAA formats, was buffer exchanged to PBS and concentrated to 1043 µg/ml according to C1.3.3 *Concentrate reagent solution* in Gyrolab User Guide P0020528 (Gyros Protein Technologies 2019d). The remaining 194 µl 1043 µg/ml reagent was added to one vial of reactive dye and incubated for 1 hour. The protein concentration was measured using a nanophotometer at 280 nm. The final concentration of labelled reagent was 752 nM.

In the second labelling, 80 µl 700 µg/ml Rabbit anti-mouse F(ab')2, used in the model systems for the indirect and GAAA format, was added to a vial of reactive dye and incubated for 1 hour. The final protein concentration was 1000 nM.

Empty AAV2 capsids with the concentration of 10^{13} PP/ml were Alexa labelled with two different dye-capsids ratios. In the first labelling, the reactive dye in one vial was diluted in 100 µl Milli-Q and 4 µl of the solved dye was added to 100 µl capsids. In the second labelling the reactive dye was diluted in 25 µl Milli-Q and 8 µl was added to 200 µl. In conclusion, the second labelling had a fourfold increase in dye to capsid ratio. The absorbance at 280 nm was measured using a nanophotometer to obtain the protein concentration with MW = 3746 kDa and ε = 3746000, but was too low to measure.

2.4 Indirect assay format

Since the detection reagent used in the indirect assay format has specificity towards human IgG Fc-region the immune response against AAV2 detected consisted of IgGs. All Gyrolab runs the Bioaffy 1000 CD was used.

2.4.1 The capacity of the solid phase

Since the concentration of the biotinylated AAV2 capsids (b-AAV2) was unknown, the b-AAV2 labelled with 5 µg biotin per ml 10^{13} PP/ml capsids was diluted in series 1:2, 1:5, 1:10, 1:20, 1:80, 1:160 and 1:320 with Phosphate Buffered Saline + 0.02% Tween (PBST) buffer, to find what concentration that saturated the solid phase. The analyte step was exchanged to PBST and 25 nM Alexa labelled mouse anti-AAV2 antibodies as detection reagent diluted in Rexxip F.

2.4.2 Model system

Due to lack of a human reference, a model system was used as a first evaluation of the capacity of the indirect assay format. The AAV2 capsids labelled with 5 µg biotin per ml 10^{13} PP/ml capsids were diluted 1:5 in PBST. The analyte monoclonal mouse anti-AAV2 was diluted in series 0.17 - 20 000 pM in Rexxip F. The detection reagent, Alexa labelled rabbit

anti-mouse F(ab')2 antibody fragments, was diluted to a final concentration of 25 nM in Rexxip F. A blank was used with Rexxip F as analyte.

2.4.3 Optimization of the indirect assay format with human serum as analyte

As a first run with human serum, nine individual human sera were diluted to 1% in Rexxip F. Biotinylated AAV2 capsids (b-AAV2) labelled with 5 µg biotin per ml capsids was diluted 1:5 in PBST was used as capture component and 25 nM detect Alexa labelled mouse anti-human IgG Fc diluted in Rexxip F was used as detect. As a reference background, the sera were also run with the capture element replaced by Rexxip F.

To optimize the capture concentration in presence of serum, b-AAV2 labelled with 10 µg biotin per ml capsids was diluted 1:2, 1:4 and 1:8 in Rexxip F. As analyte, a serum with low positive responses to anti-AAV2 and a negative serum were serially diluted 1:4 - 1:186620 which were then detected using 25 nM Alexa labelled mouse anti-human IgG Fc. To optimize the detection concentration, the same experiment was performed using b-AAV2 diluted 1:2 and 25, 12.5 or 6.75 nM detect. A blank was used with Rexxip F as analyte.

To investigate what serum dilution to use in the screen, one high and one low positive serum was serially diluted 1:4 - 1:186620 with the capture element b-AAV2 diluted 1:2 in Rexxip F and 25 nM detect mouse anti-human IgG Fc diluted in Rexxip F. A blank was used with Rexxip F as analyte.

2.4.4 Screening cut off determination and full IgG screen

Serum from 31 human individuals were screened for AAV2 IgG immune response under the conditions of 25% serum, b-AAV2 diluted 1:2 and 25 nM detect mouse anti-human IgG Fc. The reagents and analyte were all diluted in Rexxip F. As a background reference, all measurements were performed without the capture element b-AAV2, replaced by Rexxip F. All data points were measured in triplicates. The limited number of serum samples available for this study, the high prevalence of positive individuals and the variability in the background signal between individuals made it challenging to establish a screening cut point. Hence, a strategy utilizing individual cut points were implemented. An individual screening cut off was determined to mean background +3SD.

2.5 Bridging assay format

Since the bridging assay used AAV2 capsids as both capture and detection reagent, all antibodies binding to the capsids was be detected. On all Gyrolab runs the Bioaffy 1000 CD was used.

2.5.1 Model system

Due to lack of a human reference, a model system was used as a first evaluation of the capacity of the indirect assay format. Empty AAV2 capsids were labelled with two different amounts of fluorescent dye (a-AAV2). In Alexa labelling 1, the dye to protein ratio was 4 times lower than for labelling 2. To investigate how the bridging assay performed and if the

Alexa labelling worked, a titre of monoclonal mouse anti-AAV2 antibodies 0.17-20 000 pM diluted in Rexxip F was run with capture b-AAV2 labelled with 5 µg biotin per ml 10^{13} PP/ml capsids diluted 1:5 in PBST. To detect the bound monoclonal antibodies, Alexa labelling 1 diluted 1:2 and Alexa labelling 2 diluted 1:2 and 1:4 was used, diluted in Rexxip F.

When using a high density solid phase in an ordinary bridging immunoassay there is a risk that both Fab-regions of the analyte antibody binds to the capture elements, blocking the binding site for the detecting molecule. To investigate if both Fab-regions bound to the capture element in this assay, various concentrations of biotinylated bovine serum albumin (b-BSA) (0, 15, 18, 23, 36, 65% (mol/mol)) were mixed with the capture element, b-AAV2 labelled with 5 µg biotin per ml 10^{13} PP/ml capsids. As analyte, a titre of monoclonal mouse anti-AAV2 antibodies 0.17-20 000 pM diluted in Rexxip F was run with 25 nM Alexa labelled rabbit anti-mouse F(ab')2 antibodies as detect reagent, diluted in Rexxip F.

2.5.2 Optimization of the bridging assay format with human serum as analyte

To find optimal run concentrations of serum, capture and detect, an individual that had previously shown high positive responses was diluted serially 1:4-1:16384 in Rexxip F. Both the capture reagent, b-AAV2 labelled with 5 µg biotin per ml 10^{13} PP/ml capsids, and the detection reagent, a-AAV2, was diluted 1:2 and 1:4 in PBST and Rexxip F respectively. The serum titre was run with the following dilution combinations of capture and detect:

- 1:2 capture – 1:2 detect
- 1:4 capture – 1:2 detect
- 1:2 capture – 1:4 detect
- 1:4 capture – 1:4 detect

Initially, the capture molecule was diluted in PBST. Due to unusually large variations between replicates, Rexxip F was evaluated as possible alternative dilution buffer for the capture element. A serum with high positive responses was serially diluted in Rexxip F 1:4 - 1:16384. The detect a-AAV2 was diluted 1:4 in Rexxip F and the capture reagent, b-AAV2 labelled with 10 µg biotin per ml 10^{13} PP/ml capsids were diluted 1:4 with Rexxip F or with PBST.

To challenge the system, two individuals with low positive responses were serially diluted 1:4 - 1:62500 run in the optimized conditions 1:2 detect a-AAV2 diluted in Rexxip F and 1:2 b-AAV2 labelled with 10 µg biotin per ml 10^{13} PP/ml capsids also diluted in Rexxip F.

2.5.3 Screening cut off determination and full Tab screen

Human sera from 31 individuals were screened for AAV2 TAb immune response under the conditions of 25% serum, b-AAV2 diluted 1:2 and 25 nM detect mouse anti-human IgG Fc. The reagents and analyte were all diluted in Rexxip F. As a background reference, all measurements were performed without the capture element b-AAV2, replaced by Rexxip F. All data points were measured in triplicates.

Due to low background levels or all individuals and low SD between replicates, a common screen cut off was set. The sera with a mean signal lower than the mean background+3SD were considered negative controls (NC). The mean signal of the NC was then used to calculate a common screening cut off using equation 1 used from the work of Frey *et al.* (1998).

$$Cut\ off = \bar{X} + SDt\sqrt{1 + (\frac{1}{n})} \qquad (1)$$

where $\bar{X}$ is the mean signal of the NC, *SD* is the standard deviation of the NC signals, and *n* is the number of independent NC values. The parameter *t* is the $(1 - \alpha)$th percentile of the one-tailed t-distribution with $v = n - 1$ degrees of freedom. Using Table 1 in the paper from Frey *et al* (1998), *t* = 3.180, with the number of negative controls *n* = 8 and a confidence level $(1 - \alpha)$ of 99.0%.

2.6 Generic Anti-AAV Adsorption assay format

The last immune assay format evaluated was a Generic Anti-AAV Adsorption assay (GAAA). Since the detection reagent used in the GAAA format has specificity towards human IgG Fc-region the immune response against AAV2 detected will consist of IgGs. The initial experiments were performed using Bioaffy 1000 CD, and the final CD used was the Gyrolab Mixing CD 96.

2.6.1 Model system

Due to lack of a human reference, a model system was used as a first evaluation of the capacity of the GAAA format. As analyte, a titre of monoclonal mouse anti-AAV2 antibody 0.17-20 000 pM diluted in Rexxip F were mixed with 500, 250, 125 and 50 pM empty AAV2 capsids. The capsid-antibody complexes were captured on the solid phase by 100 µg/ml CaptureSelect anti-AAVX diluted in PBST and detected with 25 nM Alexa labelled rabbit anti-mouse 2F(ab') fragment diluted in Rexxip F.

2.6.2 Initial optimization of the GAAA format with human serum as analyte

To investigate what serum to capsid ratios to use, two individuals that had previously shown positive responses were diluted to a final concentration of 5% or 1% in Rexxip F mixed with an AAV2 capsid titre with the final concentration of 0.12-500 pM diluted in Rexxip F. The stock concentration of empty capsids, 10^{13} PP/ml, corresponds to 16.6 nM. The concentration of the capture element anti-AAVX was 100 µg/ml diluted in PBST and 25 nM Alexa labelled mouse anti-human IgG Fc diluted in Rexxip F. The same experiment was performed for ten additional individuals, diluted to 1% in Rexxip F.

2.6.3 Background

The background levels from the runs in section 2.6.2 showed higher background than for the previous two assay formats evaluated. To investigate the cause of the background an experiment was performed without sera. In this experiment two controls were run

- No serum: 100 µg/ml anti-AAVX diluted in PBST as capture, 250 pM capsids without sera as analyte and 25 nM detect diluted in Rexxip F as detect
- No serum or capsids: 100 µg/ml anti-AAVX diluted in PBST as capture, Rexxip F as analyte and 25 nM detect diluted in Rexxip F as detect

To examine if the high background was due to an interaction between the capture element and serum, nine individual serum diluted to 1% in Rexxip F was run with

- No capsids: 100 µg/ml anti-AAVX diluted in PBST as capture and 25 nM detect diluted in Rexxip F as detect
- No capsids or capture: PBST as capture reagent and 25 nM detect diluted in Rexxip F as detect

2.6.4 Integrating the incubation step using a Gyrolab Mixing CD

Up to this point, the GAAA format had been performed using the Bioaffy 1000 CD using 1% PMT level, where serum and capsids were manually pre-mixed before added to the micro titre plate. To save hands-on time and have a controlled incubation time for the interaction between the anti-AAV2 antibodies in the sera and the AAV2 capsids, Bioaffy 96 mixing CD was used. On the Bioaffy 96 mixing CD the capsids and the sera are mixed automatically on the CD, with adjustable and controlled incubation time. In the experimental set up used, the reagent to analyte volume ratio was 2.5 times higher for the mixing CD. When using the mixing CD, 5% PMT level is used. To perform a first anti-AAV2 quantification on the new CD type, a positive serum was diluted to a final concentration of 1% in Rexxip F. A capsid titre with the final concentration of 0.17-1000 pM with a 2.5 times lower concentration of capture and detect than used for the Bioaffy 1000 CD was used to adjust for the larger volume applied. An incubation time of 15 minutes was set.

Since the serum and capsids were manually mixed when using the Bioaffy 1000 CD the incubation time varies and were longer than 15 minutes. To perform a small optimization on the incubation time, 2.5% positive serum diluted in Rexxip F was run with a capsid titre with the final concentration of 0.17-1000 pM, with 15 and 45 minutes incubation time.

2.6.5 Optimize analyte and detection concentration

To optimize the concentration of analyte and detection reagent using the Gyrolab Mixing CD, two sera that had previously shown low and high positive responses were run 5 and 10% diluted in Rexxip F, together with capsid titre with final concentration of 5000-0.32 pM diluted in Rexxip F. The concentration of the capture element anti-AAVX was 100 µg/ml diluted in PBST and the concentration of detect Alexa labelled mouse anti-human IgG Fc varied, shown in Table 5.

‘

Table 5. The run conditions for optimizing the concentration of analyte and detection reagent

Serum concentration [%]	Detection concentration [nM]
5	25
5	10
10	25
10	15
10	10
10	5

2.6.6 Screening cut off determination and full IgG screen

31 human individuals were screened for AAV2 IgG immune response with final analyte concentrations of 10% serum and 2500 pM AAV2 capsids. Reagent concentrations used were 5 nM detect diluted in Rexxip F and 100 µg/ml capture diluted in PBST. As a background reference, all measurements were performed without the capture element anti-AAVX, replaced by PBST. All data points were measured in triplicates. The limited number of serum samples available for this study, the high prevalence of positive individuals and the variability in the background signal between individuals made it challenging to establish a screening cut point. Hence, a strategy utilizing individual cut points were implemented. An individual screening cut off was determined to mean background +3SD.

2.6.7 Improve the signal to background ratio using POROS™ CaptureSelect™ AAVX Affinity Resin

One explanation for the high background on the GAAA format was that some components in the sera from certain individuals were interacting with the capture element, CaptureSelect anti-AAVX. The hypothesis was that adsorbing the sera with the same molecule as the capture molecule, could remove the interacting components in the sera and possibly increase the signal to background ratio. To investigate if the resin lowered the background, and what amount of resin to use, eigth individual sera screened in section 2.6.6 was purified with 10, 20 and 30% (v/v) POROS™ CaptureSelect™ AAVX Affinity Resin. First, the storage buffer in the resin was exchanges to Rexxip F. Then 10, 20 and 30% (v/v) resin was added to neat serum and incubated for 20 minutes. Thereafter, the samples were centrifuged for 4 minutes 2500 RPM before the purified sera was diluted to a final serum concentration of 10%.

31 individual sera were then purified in 20% (v/v) resin in the same conditions as the screen without resin purification, described in section 2.6.6.

2.6.8 Screen for immune response against multiple AAV serotypes

Since the capture molecule used in the GAAA format has affinity towards several AAV serotypes, five individuals with low positive immune response against AAV2 were screened for immune response against AAV1, AAV3, AAV5, AAV8 and AAV10 empty capsids. The sera were purified with 20% (v/v) resin and diluted to 10% purified serum. Just like previous screens 100 µg/ml anti-AAVX, 5 nM detect and 2500 pM capsids was used.

3 Results

3.1 Indirect assay format

The first assay format evaluated was an indirect assay format. Biotinylated empty AAV2 capsids were added to the streptavidin-coated beads on the solid phase and acted as the capturing element. IgG's in human serum with affinity towards the virus capsid were captured and detected by an Alexa labelled mouse anti-human IgG Fc antibody. A model over the indirect assay format can be seen in
Figure 6. In all runs, the Bioaffy 1000 CD was used.

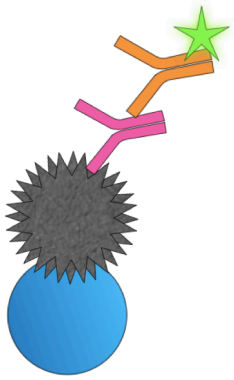

Figure 6. Schematic model of the indirect assay where the blue circle represents the streptavidin-coated particles on the solid phase, grey circles represent empty AAV2 capsids, pink represent the captured analyte antibody, orange the detection antibody and green star represents fluorescent Alexa tag.

3.1.1 Model system

Due to lack of a human reference, a model system was used were monoclonal mouse antibody with affinity towards AAV2 was used as analyte, and detected by an Alexa labelled rabbit anti-mouse F(ab')2 fragment. Empty AAV2 capsids were initially labelled with 5 µg biotin per ml 10^{13} PP/ml capsids. An experiment was performed to investigate if the biotinylation was successful and what concentration of the biotinylated capsids (b-AAV2) saturates the binding sites on the solid phase. The results showed that at a dilution of 1:2 b-AAV2, the capacity of the solid phase was not fully saturated (data not shown). The biotin to protein ratio was later increased to 10 µg biotin per ml capsid.

As a first evaluation of the indirect assay format, b-AAV2 diluted 1:5 was used to capture the analyte. As a starting point, the capacity was assessed as enough with a capture dilution of 1:5 to cut the reagent consumption. The analyte used in the model system was a titre of monoclonal mouse anti-AAV2 antibodies. To detect the captured antibodies, Alexa labelled rabbit anti-mouse F(ab')2 fragment was used.

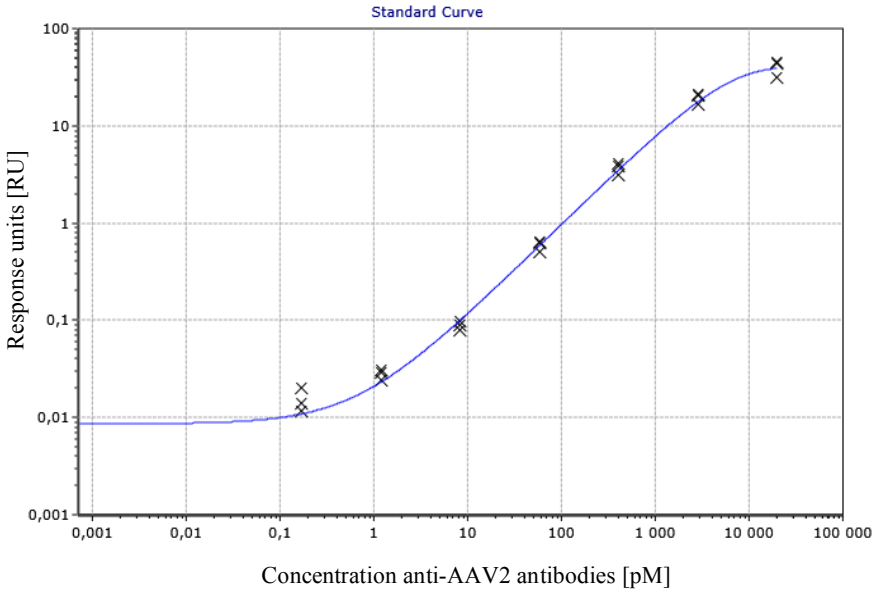

Figure 7. Model system where a titre of monoclonal mouse anti-AAV2 antibodies 0.17-20 000 pM are captured with biotinylated AAV2 (labelled with 5 µg biotin per ml capsids) diluted 1:5. The bound antibodies are detected using 25 nM Alexa labelled rabbit anti-mouse F(ab')2 fragment. Since the response increases with analyte concentration, the model antibody is captured and detected in this assay format.

As can be seen in Figure 7, the assay showed a dynamic range covering over four logs indicating sufficient capacity of the solid phase.

3.1.2 Optimization of the indirect assay with human serum as analyte

Nine individual human sera were diluted to 1%, run with b-AAV2 diluted 1:5 as capture and Alexa labelled mouse anti-human IgG Fc as detect. As a reference background, the sera were also run without the capture element, see Figure 8.

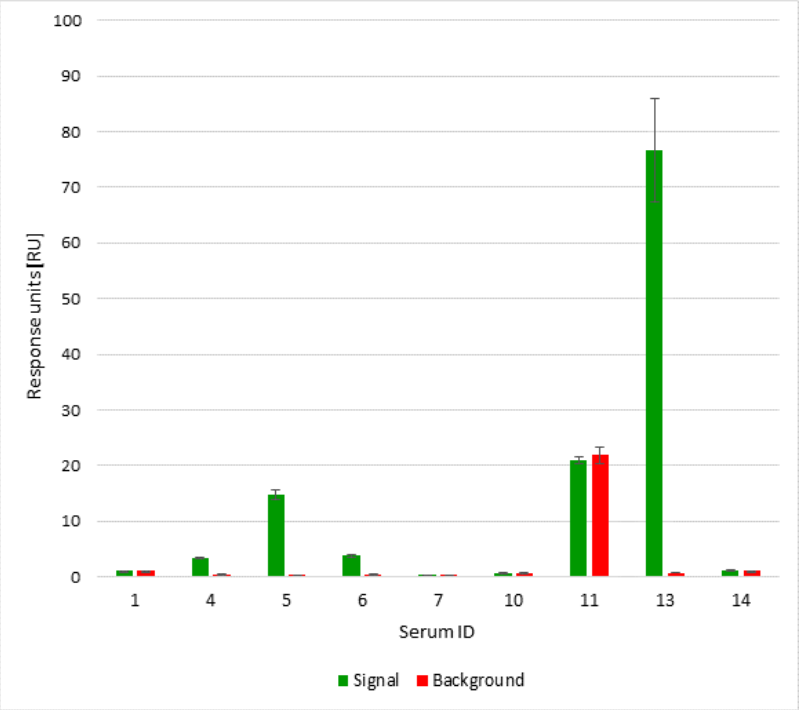

Figure 8. The green bars visualize 1% human serum ID 1, 4, 5, 6, 7, 10, 11, 12 and 14, run with b-AAV2 as capture molecule, diluted 1:5 and 25 nM detect Alexa labelled mouse antibody with specificity to human IgG Fc-region. The red bars are the measurements without the capture element, the background. The data is visualized as mean response ± SD.

ID 4, 5, 6 and 13 had significantly higher signal than background and are probably positive for IgGs against AAV2 capsids. Since serum ID 1, 7, 10, 11 and 14 shows similar responses with and without biotinylated AAV2 capsids, these are likely negative for AAV2 immune response or have such a high background that the positive signal cannot be detected. ID 11 has remarkably high background. The background varies between individuals.

To optimize the capture concentration in presence of serum, a titre of low positive serum ID 4 and negative ID 7 were run with b-AAV2 capsids labelled with 10 µg biotin per ml capsids diluted 1:2, 1:4 and 1:8 using 25 nM detect, seen in Figure 9. ID 7 had low signal and background (Figure 8), and were therefore used as a negative reference in the experiment.

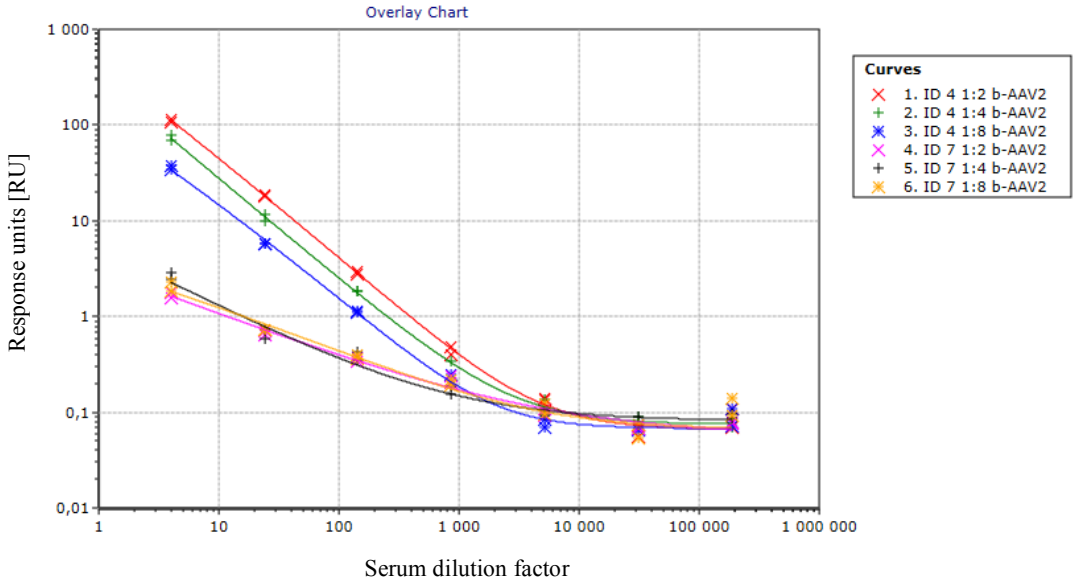

Figure 9. Capture dilution optimization. Empty AAV2 capsids labelled with 10 µg biotin per ml capsids, and run 1:2, 1:4 and 1:8 to capture the antibodies in sera. Low positive serum ID 4 and negative serum ID 7 were run in titre of 1:4 – 1:186620 and detected with 25 nM Alexa labelled mouse antibody with specificity to human IgG Fc-region. Highest S/B was achieved when using 1:2 b-AAV2 for ID 4.

Both positive ID 4 and the negative reference ID 7 reached similar responses at high sera dilutions, independent of capture concentration. Using capture b-AAV2 diluted 1:2 gave higher response than 1:4 and 1:8. This indicates that the signal/background ratio (S/B) were higher for higher concentration of capture molecule. A high S/B is wanted, since it increases the sensitivity for the assay in future screens. The responses for negative ID 7 are not affected by capture concentration, but the signal is serum-dose-depended.

Using the same two individual sera, detection concentration was optimized. Serum ID 4 and 7 were serially diluted, with b-AAV2 diluted 1:2 and 25, 12.5 or 6.75 nM detect, seen in Figure 10.

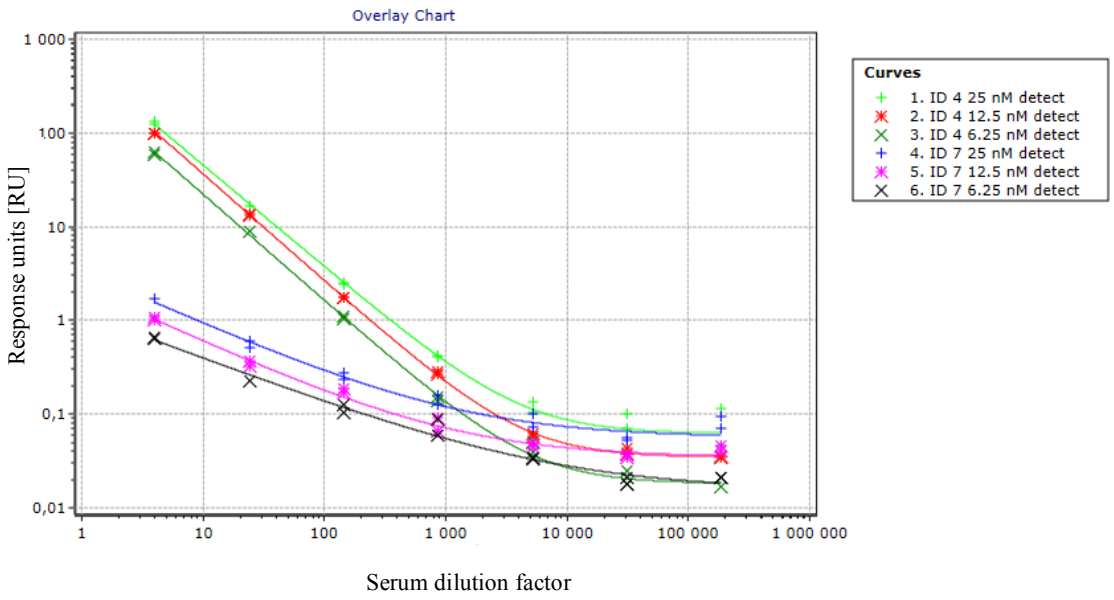

Figure 10. Detection concentration optimization. Empty AAV2 capsids labelled with 10 µg biotin per ml capsids, diluted 1:2, was used to capture the antibodies in sera. Low positive serum ID 4 and negative serum ID 7 were run in titre of 1:4 – 1:186620 and detected with 25, 12.5 and 6.75 nM Alexa labelled mouse antibody with specificity to human IgG Fc-region. Highest S/B was achieved when using 12.5 nM detect.

The titration curves for ID 4 and ID 7 using different concentrations of detect are visualised. Comparing the individuals at the same assay conditions showed that the biggest difference in response between ID 7 and ID 4, are achieved when using 12.5 nM detect.

Before performing a full screen for anti-AAV2 IgG immune response, the serum concentration was optimized. One serum titre of high positive (ID 13) and one low positive individual (ID 15) were run with capture b-AAV2 diluted 1:2 and 25 nM detect mouse-anti human Fc. To investigate the background, the experiment was also performed without the capture molecule, seen in Figure 11.

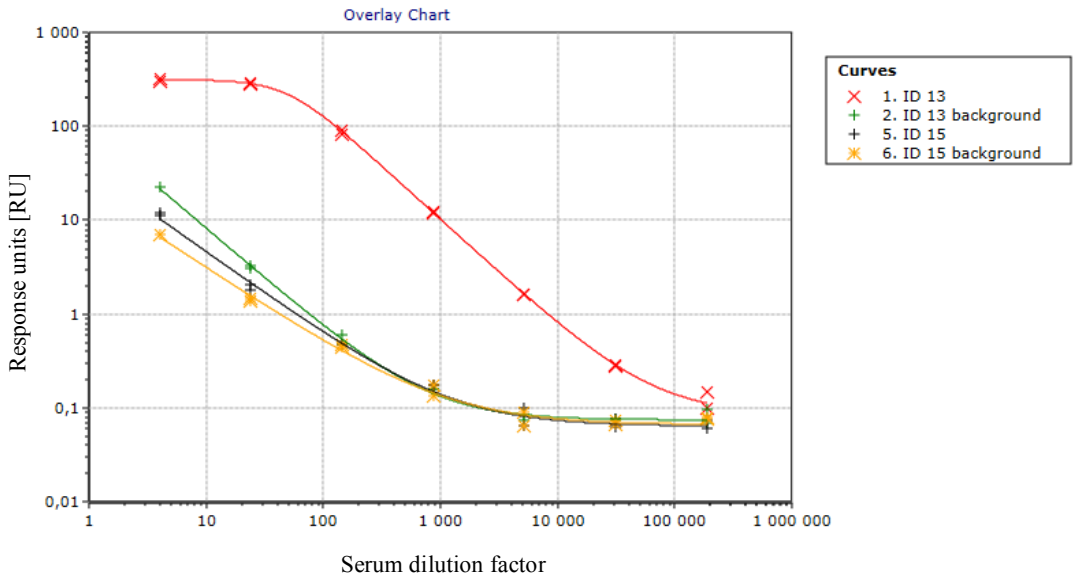

Figure 11. Serum dilution optimization. High positive serum ID 13 and low positive serum ID 15 was diluted in series of 1:4 – 1:186620, with 1:2 b-AAV2 and 25 nM detect. The curves labelled "background" are run without the capture element.

For the high positive serum ID 13, the biggest difference between signal and background is for the third lowest dilution of serum, 1:144. For the low positive serum, ID 15, diluting the serum 1:4 gives the biggest difference between signal and background. Since the aim of the screen is to be able to classify low positive serum, not quantify high responses accurately, the optimization will be based on the low positive sera. Hence, a serum concentration of 25%, was considered fit for purpose for this assay format.

3.1.3 Cut off determination and full screen

Human serum from 31 human individuals were screened for AAV2 IgG immune response using the conditions optimized in section 3.1.2, but using 25 nM mouse anti-human as detect.

The limited number of serum samples available for this study, the high prevalence of positive individuals and the variability in the background signal between individuals made it challenging to establish a screening cut point. Hence, a strategy utilizing individual cut points were implemented. An individual cut off is determined to: mean background +3SD.

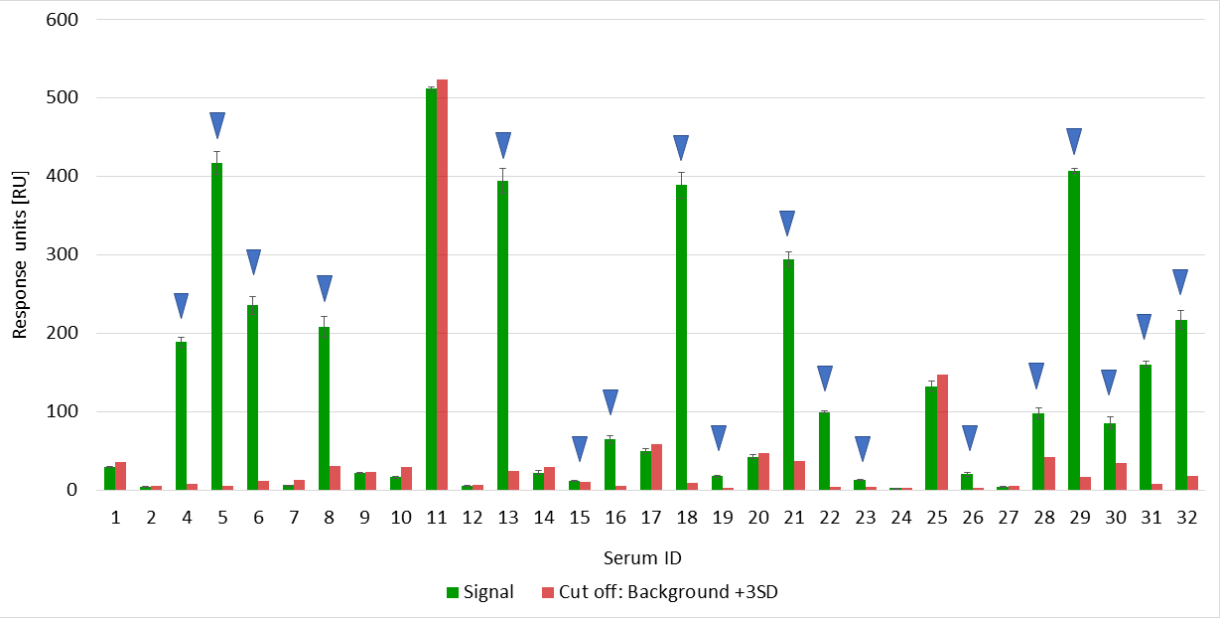

Figure 12. The signal data is visualized as mean response ± SD. The cut off is mean background +3SD.

Sera with a signal higher than the cut off were classified as positive for anti-AAV2 IgGs and are marked with blue triangles in Figure 12. 58% (18/31) of the individuals were classified as positive. ID 11 and ID 25, have significantly higher background than the other individuals. ID 3 is not shown since it was a serum pool.

3.2 Bridging assay

The second assay format evaluated to screen the pre-existing AAV2 immune response in human sera was a bridging assay, where biotinylated and Alexa labelled AAV2 capsids act as capturing and detection component respectively. Since all antibodies with affinity towards the AAV2 capsid will be captured and detected, this assay format will not only quantify IgGs with affinity towards AAV2, like in the indirect assay format, but all capsid-binding antibodies. A model of the bridging assay can be found in Figure 13. In all runs, the Bioaffy 1000 CD was used.

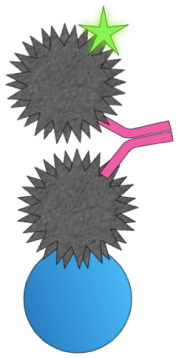

Figure 13. Schematic model of the bridging assay where the blue circle represents the streptavidin-coated particles on the solid phase, grey circles represent empty AAV2 capsids, pink represent the captured analyte antibody and green star represents fluorescent Alexa tag.

3.2.1 Model system

Empty AAV2 capsids were labelled with two different fluorescent dye to protein ratios. In Alexa labelling 1, the dye to protein ratio was 4 times lower than for labelling 2. To investigate how the bridging assay performs and if the Alexa labelling worked, a titre of monoclonal mouse anti-AAV2 antibodies was run with capture b-AAV2 diluted 1:5. To detect the bound monoclonal antibodies, Alexa labelling 1 diluted 1:2 and Alexa labelling 2 diluted 1:2 and 1:4 was used, shown in Figure 14.

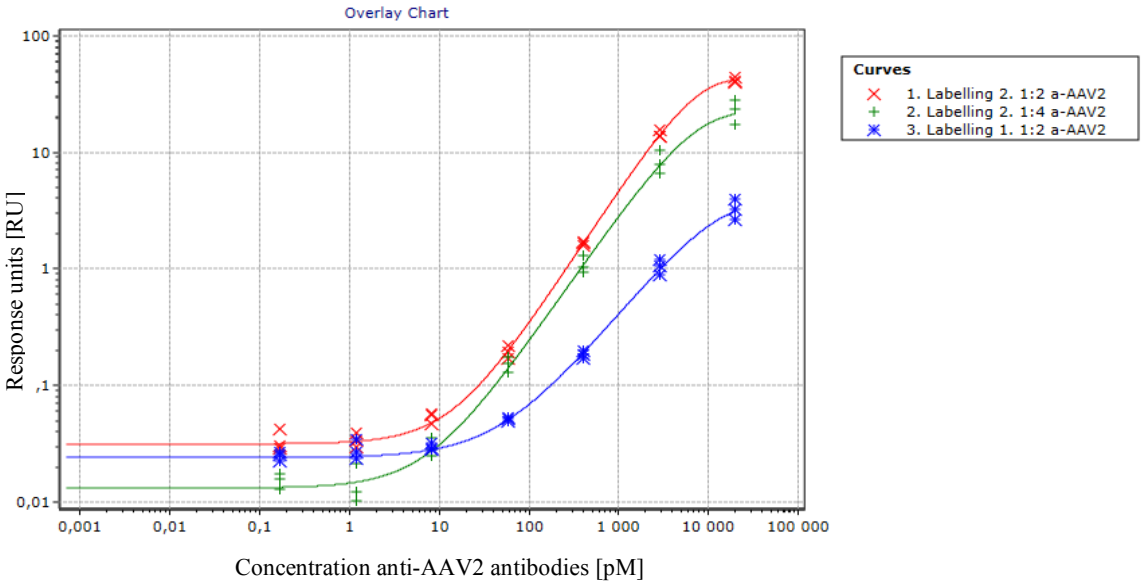

Figure 14. A titre of monoclonal mouse anti-AAV2 antibody 0.17-20 000 pM was run with 1:5 capture b-AAV2. To detect the bound monoclonal antibodies, two AAV2 capsids labelled with different amount of dye was used. In Alexa labelling 1 four times less dye was used than in Alexa labelling 2. Highest S/B was achieved when using Alexa labelling 2 diluted 1:2.

Since the responses increased with concentration of analyte, the analyte antibody is detectable in the bridging assay. Largest S/B, were achieved when using the a-AAV2 with 4 times higher dye to protein ratio (labelling 2) diluted 1:2.

When using a high density solid phase in an ordinary bridging immunoassay there is a risk that both Fab-regions of the analyte antibody binds to the capture elements, blocking the binding site for the detecting molecule. To investigate if both Fab-regions bind to the capture element in this assay, various concentrations of b-BSA were mixed with the capture element, b-AAV2. Using b-BSA to lower the density of the capture elements did not have an effect, indicating that both Fab-regions does not bind to capturing capsids, see Appendix 1.

3.2.2 Optimizing the bridging assay format with human sera

To find optimal run concentrations of serum, capture and detect, ID 13 that has previously showed positive responses, was diluted in series. Capture b-AAV2 was diluted 1:2 and 1:4 and detect a-AAV2 1:2 and 1:4. All combinations of capture and detect dilution were investigated, shown in Figure 15.

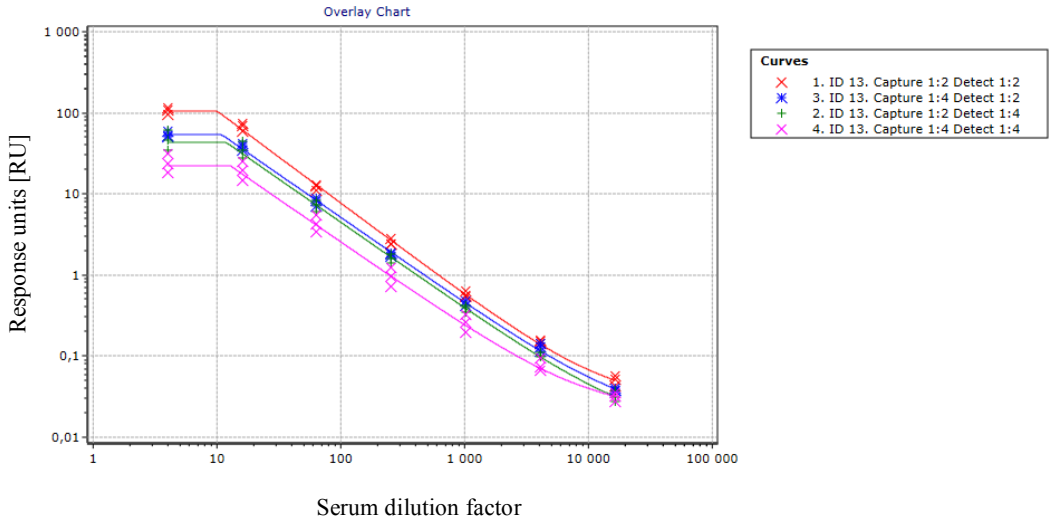

Figure 15. Serum ID 13 titre of 1:4→1:16384 with capture b-AAV2 and detect a-AAV2 diluted 1:2 and 1:4. All curves showed saturation between serum dilution 1:4 and 1:16, and the highest S/B is achieved when using 1:2 capture and 1:2 detect.

For all curves, saturation occurred when the serum was diluted between 1:4 and 1:16, with the highest S/B at serum dilution 1:4. The responses at low serum dilution (furthest left) differed a lot in the different conditions, while signal close to background, at high serum dilutions (furthest right), stayed somewhat constant. Highest responses and therefore highest S/B was achieved when using 1:2 capture and 1:2 detect. The second highest S/B was achieved when using 1:4 capture and 1:2 detect. It could be concluded that a high concentration of detect a-AAV2 was of greater importance than a high capture b-AAV2 concentration, to get as big difference between low and high dilution of serum. From this experiment, it could be concluded that using 1:2 capture and 1:2 detect gave the best performance on the high positive sera ID 13.

Usually, when using the Gyrolab platform, the capture molecule is diluted in PBST. In the experiment shown in Figure 14 and Figure 15, PBST was used as capture dilution buffer. Due to unusually large variations between replicates (for example pink curve in Figure 15), Rexxip F buffer from Gyros Protein Technologies was evaluated as possible dilution buffer for the capture element. It turned out that using Rexxip F gave lower SD and was therefore used for diluting b-AAV2 from now on (data not shown).

It was concluded in Figure 15 that diluting capture and detect 1:2 gave highest S/B for a high positive serum and therefore might give a more sensitive assay. To challenge the system, two individuals that had previously shown low positive responses, ID 15 and 16, were serially diluted with 1:2 capture and detect, shown in Figure 16. This was done to see if low immune responses were detectable in the bridging assay under the optimized conditions.

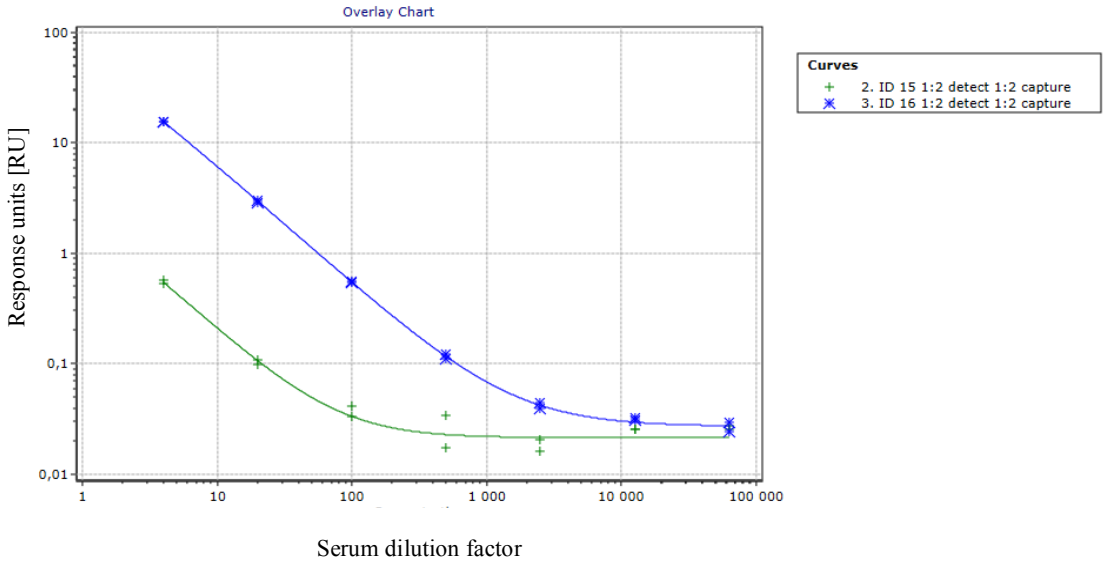

Figure 16. Low positive sera titre 1:4→1:62500 in optimal conditions; capture b-AAV2 and detect a-AAV2 diluted 1:2. Highest S/B ratio for both ID 15 and 16 were obtained at the lower serum dilution 1:4.

Using the optimal conditions, individuals with low immune responses were detectable in the bridging assay format. Highest signal was obtained at the lowest serum dilution, 1:4, for both ID 15 and ID 16. In Figure 15 high positive serum ID 13, had highest S/B at 1:4, but saturation occurred. Therefore, the sera will be diluted 1:4 in the screening on this assay format with the knowledge that high positive sera are not numerically comparable.

3.2.3 Cut off determination and screen for TAb immune response

Human serum from 31 individuals were screened for TAbs against AAV2 capsids. For all individuals, the measurement was also performed without the capture b-AAV2 molecule to obtain the background. The results from the screen can be seen in Figure 17, note the log-scale on the y-axis.

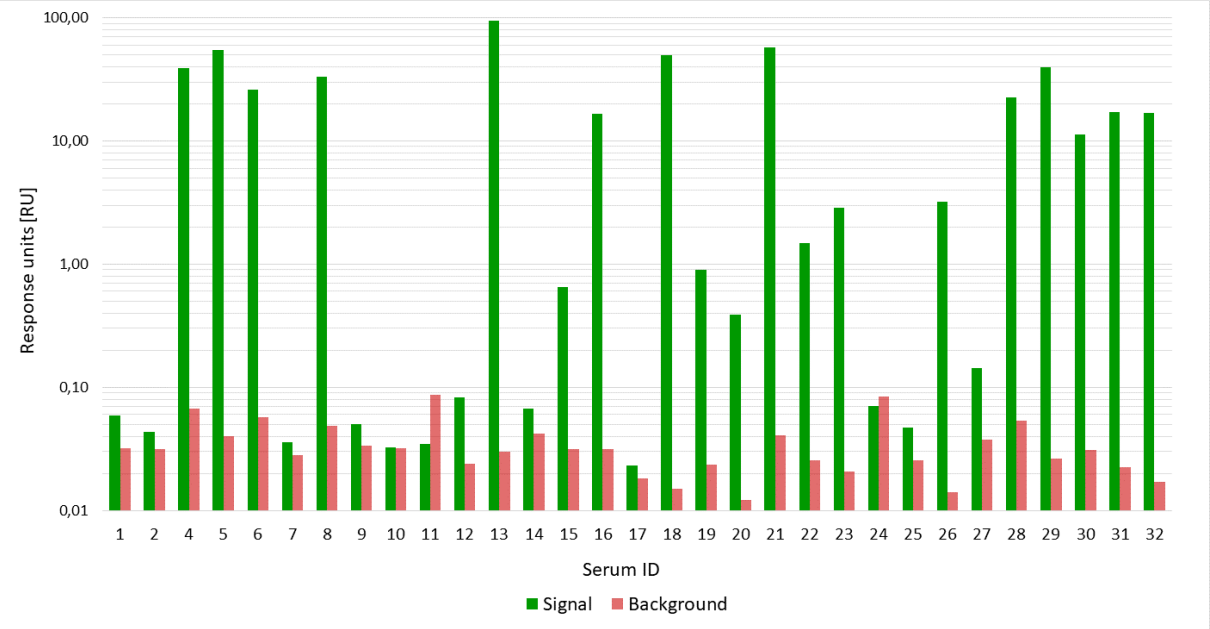

Figure 17. Sera from 31 individuals were screened for TAbs against AAV2. The sera were diluted to 25% and the capture molecule b-AAV2 was diluted 1:2 and detect a-AAV2 diluted 1:2.

The results from the screen on the bridging assay format were that low backgrounds are obtained, independent of individual screened. Individual ID 3 is excluded from the screen since it is a serum pool. Since the background is similar between all individuals, a common screening cut off was set. The sera with a mean signal lower than the mean background+3SD are considered negative controls (NC), marked with red triangles in Figure 18.

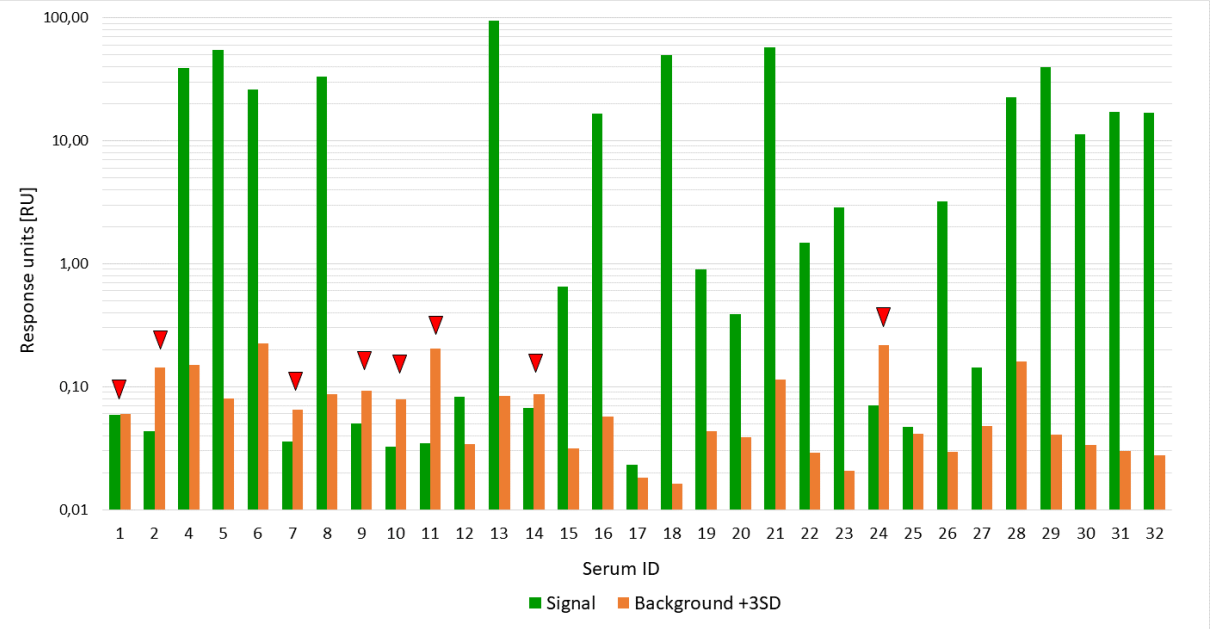

Figure 18. Sera from 31 individuals were screened for TAbs against AAV2 capsids with mean background +3SD in orange. The individuals considered as negative controls are marked with red triangles.

The NCs mean signals, found in Table 6, was then used to calculate the cut off with 99% confidence level using equation 1. A matlab-script was used to calculate the function, found in Appendix 2.

Table 6. The individuals considered as negative controls and the corresponding mean signals

Serum ID NC	Mean signal [RU]
ID 1	0.059
ID 2	0.043
ID 7	0.036
ID 9	0.051
ID 10	0.032
ID 11	0.035
ID 14	0.067
ID 24	0.070

$$Cut\ off_{99\%\ confidence\ level} = \bar{X} + SDt\sqrt{1 + (\frac{1}{n})} = 0.0995$$

where $t = 3.180$

In Figure 19, the signals from the screen are plotted with the calculated screening cut off. Individuals with signal higher than the cut off are classified as positive and marked with blue triangles.

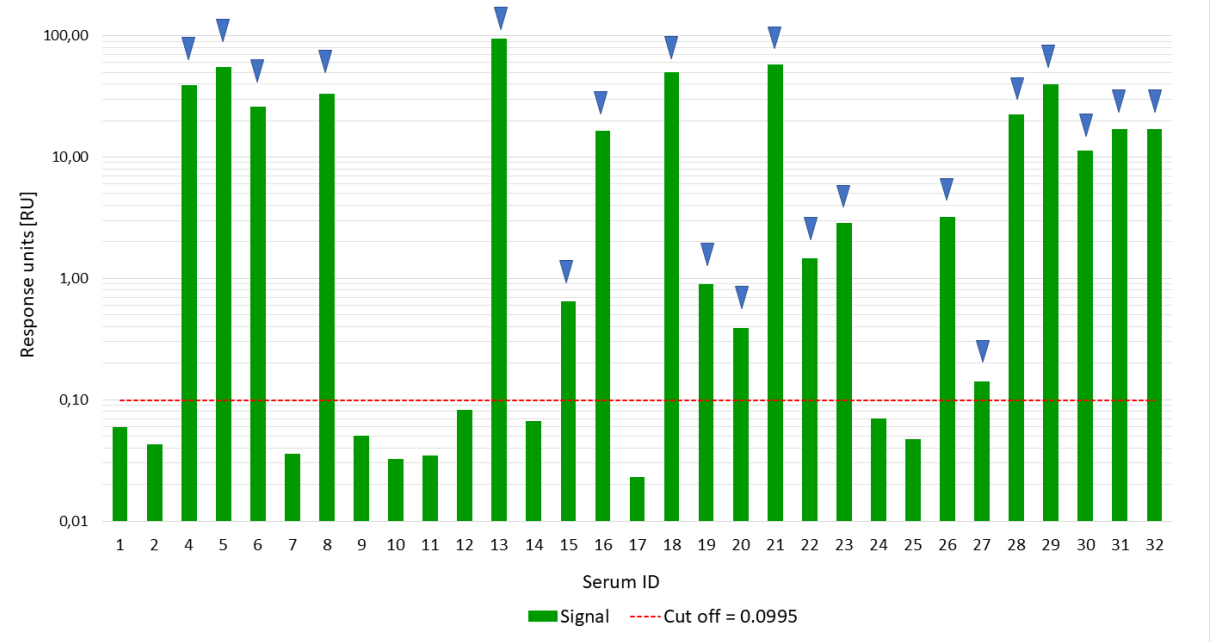

Figure 19. Sera from 31 individuals were screened for TAbs against AAV2 capsids with the calculated screening cut off = 0.0995. Positive individuals are marked with a blue triangle.

The result from the screen was that 20/31 (65%) are positive for AAV2 immune response using the calculated common screening cut off. If the screening cut off instead was mean background +3SD as for the indirect assay format, 74% (23/31) of the individuals would screen as positive, seen in Figure 18.

3.3 Generic anti-AAV adsorption assay

The last immune assay format evaluated was a Generic Anti-AAV Adsorption assay (GAAA). As capture component, a CaptureSelect Biotin Anti-AAVX Conjugate (biotinylated camelid antibody fragment) with specificity to most serotypes of adeno-associated viruses, was used. As analyte, empty AAV2 capsids and serum were mixed and the IgGs bound to the capsids were detected by the same molecule as in the indirect assay format, Alexa labelled mouse-anti human Fc. A model of the GAAA format is shown in Figure 20. Initially the Bioaffy 1000 CD was used, before introducing the Gyrolab Mixing CD 96.

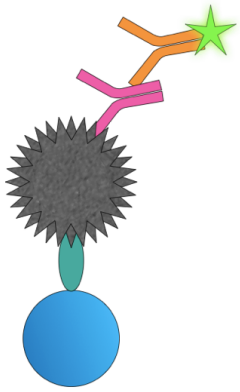

Figure 20. Schematic model of the GAAA format, where the blue circle represents the streptavidin-coated particles on the solid phase, the turquoise is the biotinylated anti-AAVX, grey circles represent empty AAV2 capsids, pink represent the captured analyte antibody and orange is detection antibody and green star represents fluorescent tag.

3.3.1 Model system

As a first evaluation of GAAA format, a mix of 0.17-20 000 pM monoclonal mouse anti-AAV2 and 500, 250, 125 and 50 pM AAV2 capsids was used as analyte, shown in Figure 21. The capsid-antibody complexes were captured on the solid phase by CaptureSelect anti-AAVX and detected with an Alexa labelled rabbit anti-mouse F(ab')2 fragment. The Bioaffy 1000 CD was used.

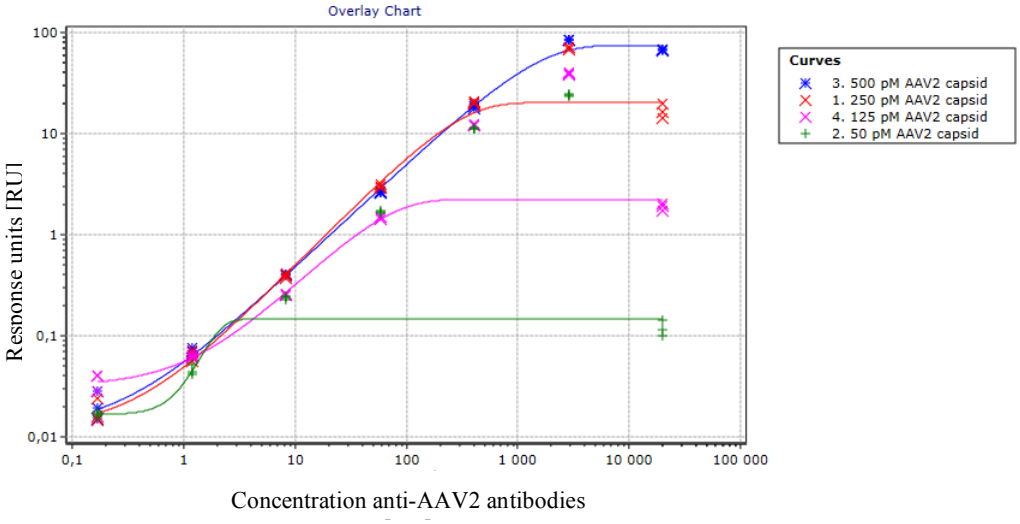

Figure 21. Model system with titre of monoclonal mouse anti-AAV2 antibodies 0,17→20 000 pM as analyte, mixed with 500, 250, 125 and 50 pM empty AAV2 capsids. The capsid-antibody complexes were captured on the solid phase by 100 µg/ml CaptureSelect anti-AAVX and detected with 25 nM Alexa labelled rabbit anti-mouse 2F(ab') fragment.

Highest responses are obtained when using 500 pM AAV2 capsids. For high concentrations of the monoclonal anti-AAV2 antibody, the signal decreases. For all capsid concentrations, the highest response is achieved at 2857 pM monoclonal antibody concentration.

3.3.2 Optimizing the Generic Anti-AAV Adsorption assay with human sera

Two individuals that had previously showed positive responses, ID 4 and 5, were diluted to 5% and 1% and mixed with a dilution series of AAV2 capsids, to investigate what serum-capsid ratio gives robust measurements using serum, see Figure 22.

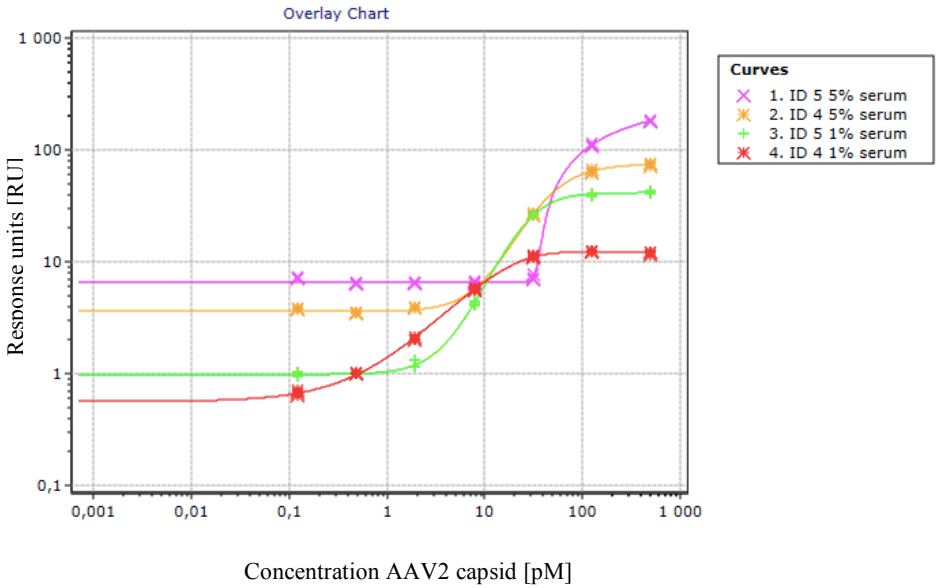

Figure 22. Individual serum ID 4 and 5 were diluted to 1% and 5% mixed with a titre of AAV2 capsids (0.12-500 pM). The concentration of the capture element anti-AAVX was 100 µg/ml and 25 nM detection molecule, Alexa labelled mouse-anti human IgG Fc.

It was possible to detect anti-AAV2 immune response in human sera using assay format 1, since the response increases with increasing concentration of capsids. The background is higher for serum ID 5 than for serum ID 4, but also has a higher overall response. The background seems serum-dose dependet since the background is higher for 5% sera than 1% sera for both ID 4 and 5. When comparing 1% and 5% serum, using 1% sera provides linearity at lower concentration of capsid. This means that lower anti-AAV2 IgG concentrations can be measured when using 1% serum. Highest S/B ratio is obtained at 500 pM capsids.

The same experiment was performed for ten additional individuals, diluted to 1%. In Figure 23 the individuals that are positive for immune response against AAV2 are assembled.

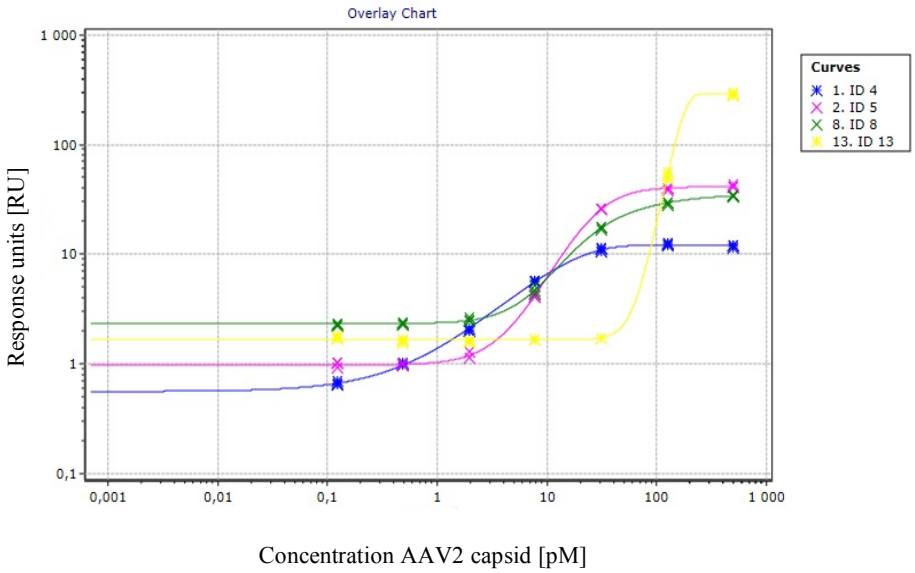

Figure 23. Assemble of positive human serum diluted 1%, mixed with a titre of AAV2 capsids (0.12-500 pM). The concentration of the capture element anti-AAVX was 100 µg/ml and 25 nM detection molecule, Alexa labelled mouse-anti human IgG Fc.

In Figure 24 the individuals with constant responses, independent of capsid concentration, are assembled. These are considered negative.

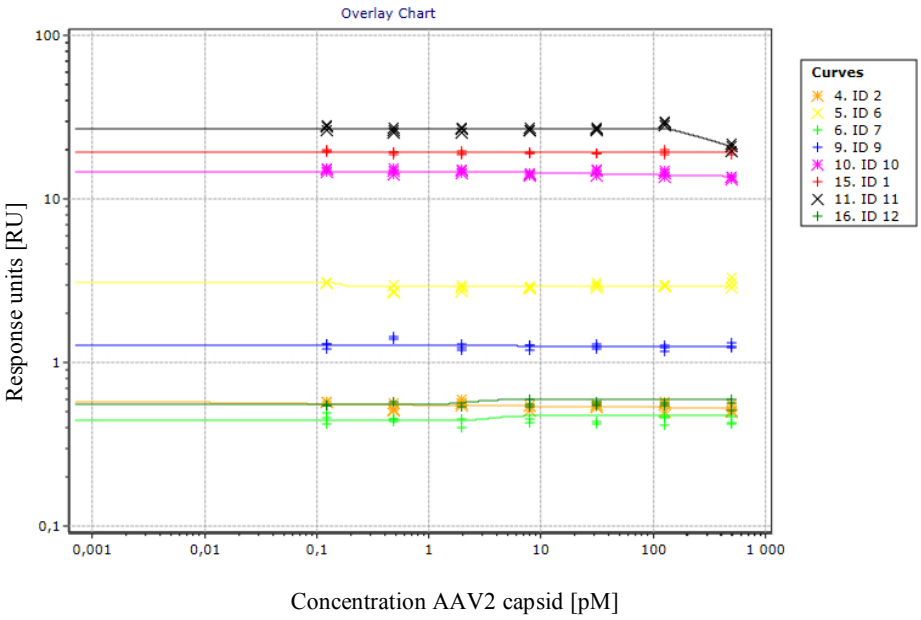

Figure 24. Assemble of negative human serum diluted 1%, mixed with a titre of AAV2 capsids (0.12-500 pM). The concentration of the capture element anti-AAVX was 100 µg/ml and 25 nM detection molecule, Alexa labelled mouse-anti human IgG Fc.

Previous screens have shown ID 6 to be positive, but was classifies as negative using this assay format. The background levels shown in Figure 24 are higher than seen on the two previous formats evaluated.

3.3.3 Evaluate the cause of the high background

As a first experiment to find the cause of the high background, capsids without serum present was run as a control. The results were blank, meaning that without serum no high background responses are obtained (data not shown). As a second control, Rexxip F buffer was used as analyte, meaning no serum or no capsids as analyte. This also gave blank responses (data not shown). This indicates that the high background might instead be due to an interaction between Abs in some serum and the capture element, CaptureSelect biotinylated anti-AAVX.

To examine if the high background was due to an interaction with the capture element, 1% sera were run either without capsid, or without both capsid and capture element. The blue bars in Figure 25 shows the responses when no capsid is used and orange bars when no capsid and no capture is used.

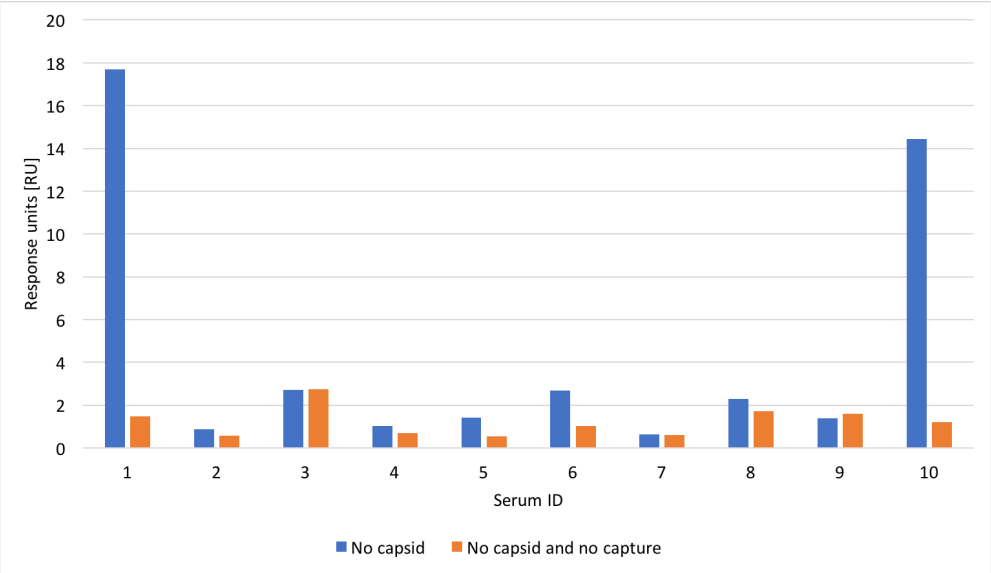

Figure 25. Comparing two background by screening ten individual human sera diluted 1%. Blue bars: 100 µg/ml capture anti-AAVX, 1% serum and 25 nM Alexa labelled mouse anti-human as detect. Orange bars: 1% serum with 25 nM Alexa labelled mouse anti-human as detect.

As visualised in Figure 25, the background response without the capture present is generally somewhat lower compared to when it is present. However, for ID 1 and ID 10, the background is dramatically reduced without the capture ligand indicating an immune response to the anti-AAVX ligand. Both ID 1 and ID 10 was classified as negative for immune response against AAV2 in previous assay formats. These two individuals might as well have immune response, but could be falsely classified as negative due to the interaction with the capture element.

3.3.4 Integrating the incubation step using a Gyrolab Mixing CD

Up to this point, assay format 1 has been performed using the Bioaffy 1000 CD using 1% PMT, where serum and capsids were manually pre-mixed before added to the micro titre plate. To save hands-on time and have a controlled incubation time for the interaction between the anti-AAV2 antibodies in the sera and the AAV2 capsids, Bioaffy 96 mixing CD was used. On the Bioaffy 96 mixing CD the capsids and the sera are mixed automatically on the CD, with adjustable and controlled incubation time. In the experimental set up used, the reagent to analyte volume ratio was 2.5 times higher for the mixing CD. When using the mixing CD, 5% PMT is used.

To perform a first anti-AAV2 quantification on the new CD type, 1% positive ID 6 serum was run with a capsid titre and with 2.5 lower concentration of capture and detect than used for the Bioaffy 1000 CD in Figure 23 and Figure 24 to adjust for the larger volume applied.

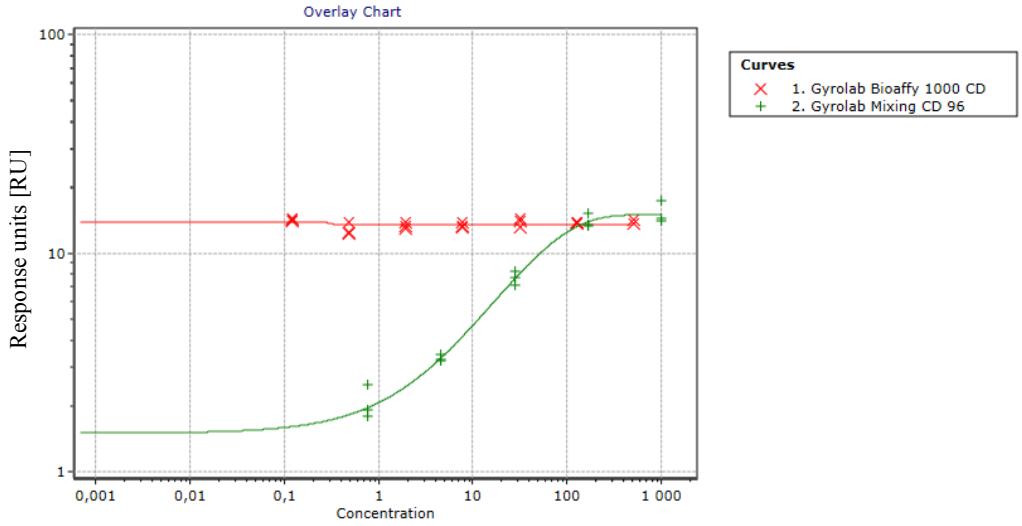

Figure 26. To perform a first anti-AAV2 quantification on the new CD type, 1% positive ID 6 serum was run with a capsid titre of 0.17-1000 pM with 2.5 lower concentration of capture and detect than used for the Bioaffy 1000 CD.

The red curve is the same as plotted in Figure 24, but with 5% PMT to compare with green curve from the mixing CD. When using the Mixing CD, the background was greatly lowered. A big difference between the two CD types is that on the mixing CD, capsid and serum are mixed automatically in the CD structures with 15 minutes controlled incubation time. When using the mixing CD, ID 6 was positively classified for immune response against AAV2.

In Figure 27, two different incubation times were evaluated on the Mixing CD, 15 and 45 minutes, using 2.5% ID 6 serum.

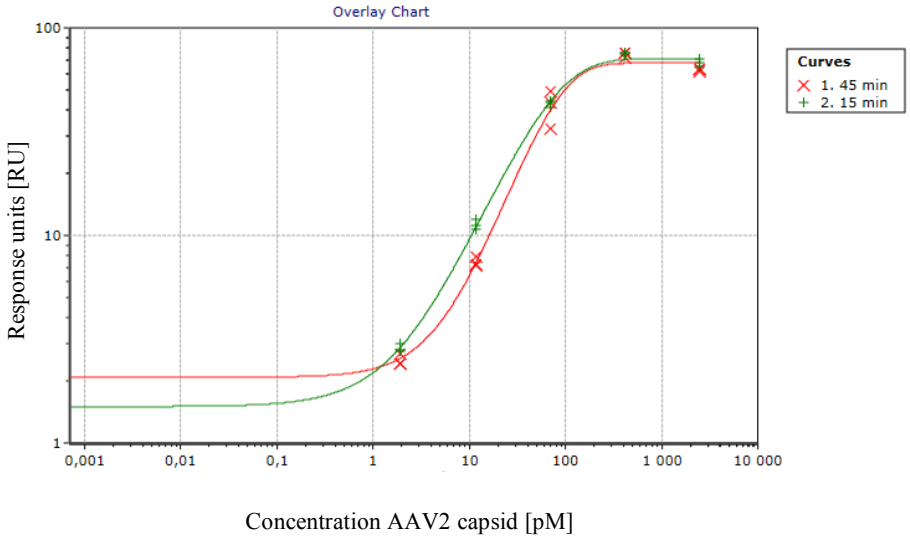

Figure 27. Positive serum ID 6 was diluted 2.5% and run with 15 and 45 minutes incubation time on the Bioaffy Mixing CD 96.

The longer incubation time did not affect the performance remarkably. The background is slightly lower when using 15 minutes incubation time.

3.3.5 Optimize analyte and reagent concentration

To optimize the concentration of analyte and reagent, two sera that has shown positive responses, ID 5 and 15, were run together with capsid titre 5000-0.32 pM AAV2 capsids. ID 5 has previously shown high positive responses and ID 15 has previously shown low positive responses. The aim was to get as high S/B for low positive serum since this results in a more sensitive assay. The signal to background ratio is calculated by dividing the response at 500 pM with the background, 0 pM capsid. The different combinations of serum concentration and detection concentration with the resulting S/B is presented in Table 7 for the serum ID 15 and Table 8 for ID 5.

Table 7. The run concentrations for serum ID 15 and detect, with the resulting signal at 5000 pM capsids divided by the signal at 0 pM capsid (S/B). The highest S/B is underlined.

Serum	Serum concentration	Detection concentration	S/B
Low positive ID 15	5%	25 nM	**5.2**
Low positive ID 15	5%	10 nM	**6.9**
Low positive ID 15	10%	25 nM	**10.8**
Low positive ID 15	10%	15 nM	**8.9**
Low positive ID 15	10%	10 nM	**11.9**
Low positive ID 15	10%	5 nM	**12.5**

Table 8. The run concentrations for serum ID 5 and detect, with the resulting signal at 5000 pM capsids divided by the signal at 0 pM capsid (S/B). The highest S/B is underlined.

Serum	Serum concentration	Detection concentration	S/B
High positive ID 5	5%	25 nM	**47**
High positive ID 5	5%	10 nM	**73**
High positive ID 5	10%	25 nM	**55**
High positive ID 5	10%	15 nM	**50.8**
High positive ID 5	10%	10 nM	**60.8**
High positive ID 5	10%	5 nM	**81.2**

Highest S/B ratio was achieved using 10% serum with 5 nM Alexa labelled mouse anti-human detection molecule for both individuals.

In Figure 28 the capsid titre curves in the optimal conditions, 10% serum with 5 nM detect, is plotted.

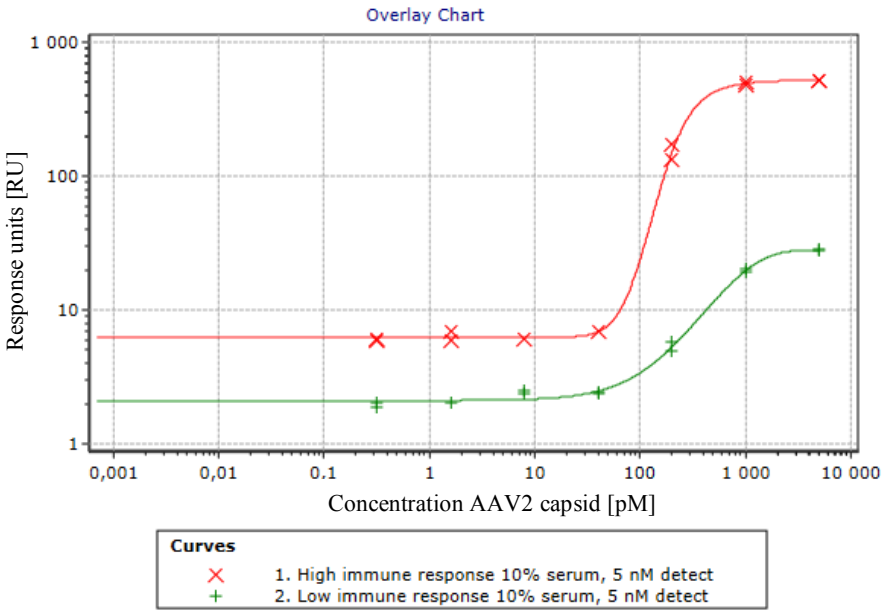

Figure 28. The full capsid titre curves for the conditions that gave highest S/B in Table 7 and Table 8 are showed, where the sera are diluted to 10% with 5 nM detect mouse anti-human IgG Fc and 100 µg/ml capture anti-AAVX.

For serum ID 5 with a high immune response, saturation begins at capsid concentration around 200 pM. For the serum with low immune response, ID 15, saturation begins at 1000 pM. Saturation does not affect the screening negatively but to save reagent, excess use of capsids is unnecessary. Therefore 2500 pM are considered fit for purpose to perform the screen on the GAAA format.

3.3.6 Screen serum for pre-existing AAV2 immune response

Human sera from 31 individuals were screened for anti-AAV2 IgGs on the GAAA format. The background for each individual serum is serum without capsid, seen in red.

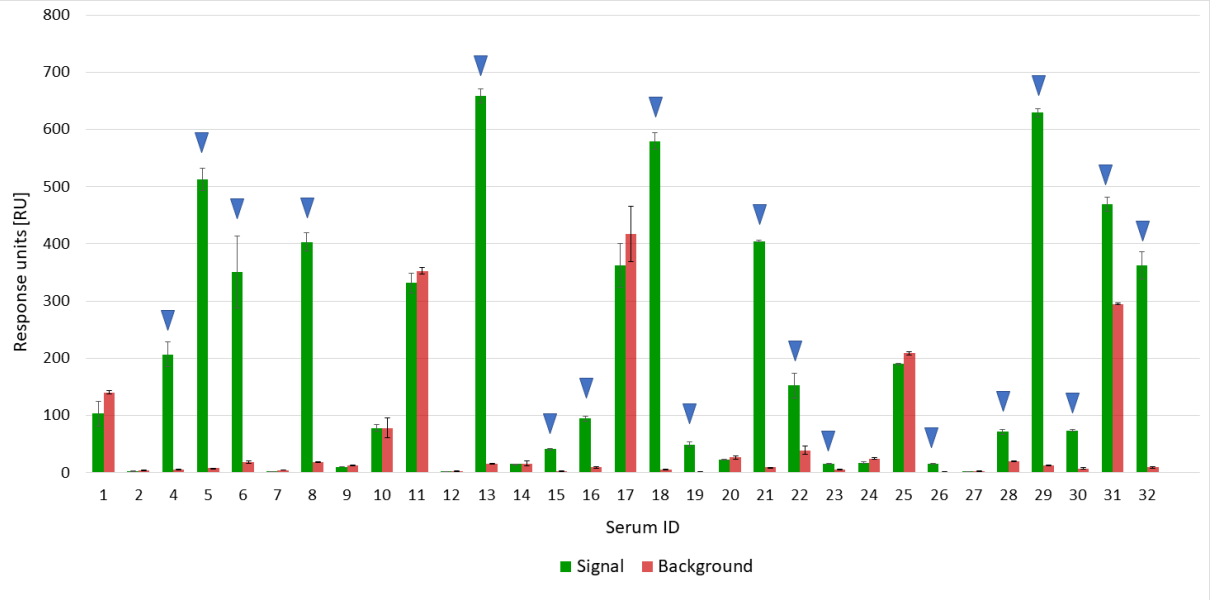

Figure 29. Human sera from 31 individuals were screened for anti-AAV2 IgGs on the GAAA with analyte concentrations 10% serum and 2500 pM AAV2 capsids. Reagent concentrations used were 5 nM detect and 100 µg/ml capture. The background for each individual serum is 10% serum without capsid, seen in red. ID 3 is not included since it was a serum pool. The data is visualized as mean response ± SD.

Individuals with higher signal than background were classified as positive, marked with blue triangles in Figure 29. The background is varying a lot between individuals, and is especially high for individual 1, 10, 11, 17, 25 and 31.

3.3.7 Screening cut of determination

Since no negative controls are evaluable and the background is varying between individuals, an individual screening cut off of mean background ± 3SD is set. The screen results with cut offs are shown in Figure 30.

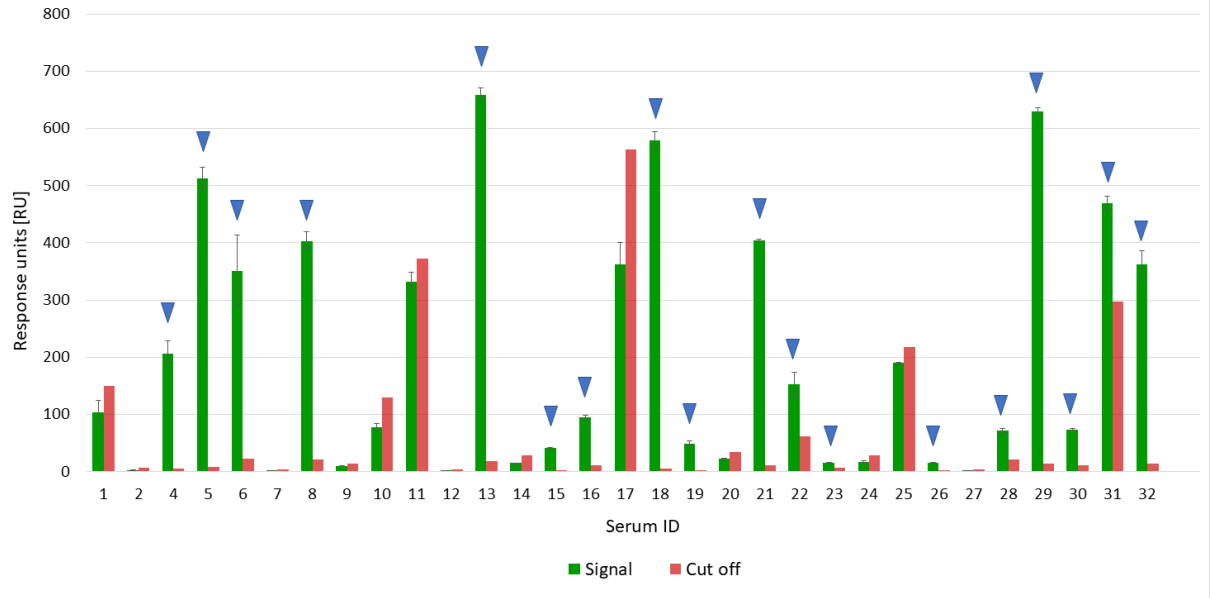

Figure 30. Full screen with screening cut off. The data is visualized as mean response ± SD.

Using the cut off as classifier for immune response to AAV2, did not change the results of the screen. The positive individuals are again marked with blue triangles in Figure 30.

3.3.8 Improve the signal to background ratio using POROS™ CaptureSelect™ AAVX Affinity Resin

It was previously shown that one explanation for the high background on assay format 1 is that some components in the sera from certain individuals are interacting with the capture element, CaptureSelect anti-AAVX. The hypothesis was that adsorbing the sera with the same molecule as the capture molecule, could remove the interacting parts in the sera and possibly increase the signal to background ratio. It was concluded that adsorp the serum in 20% (v/v) POROS™ CaptureSelect™ AAVX Affinity Resin increased the signal to cut off ratio for most sera evaluated. The experiments where resin% is optimized and evaluated can be find in appendix 3.

The 31 individual sera were purified in 20% (v/v) resin and the results from the screen are shown in Figure 31.

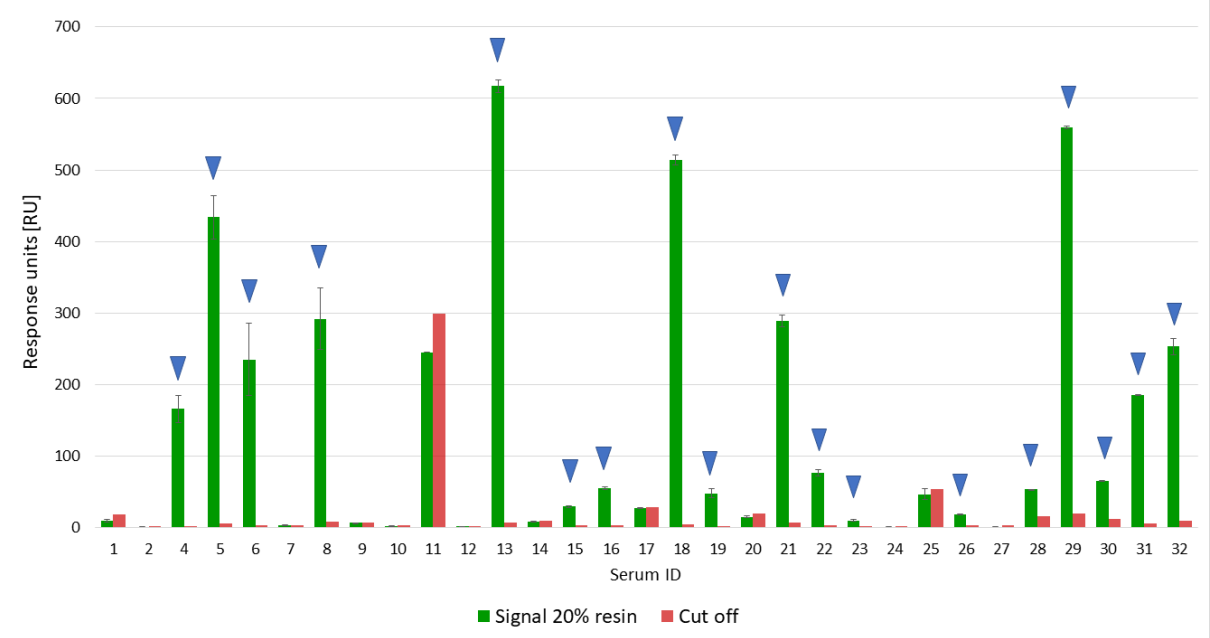

Figure 31. Purified human sera from 31 individuals were screened for anti-AAV2 IgGs on the GAAA with analyte concentrations 10% purified serum and 2500 pM AAV2 capsids. Reagent concentrations used were 5 nM detect and 100 µg/ml capture. The background for each individual purified serum is 10% serum without capsid, seen in red. ID 3 is missing since it is a serum pool. The data is visualized as mean response ± SD.

When screening with adsorbed serum, the same individuals are classified as positive as when screening non-purified serum. 58% (18/31) of the individuals were classified as positive. ID 11, but also ID 25, have significantly higher background than the other individuals. ID 3 is missing since it is a serum pool. Since a sensitive assay is of essence, the signal to cut off ratio was compared in Figure 32 between the two screens; non-purified sera (screen shown in Figure 30) and sera purified with 20% resin (Figure 31).

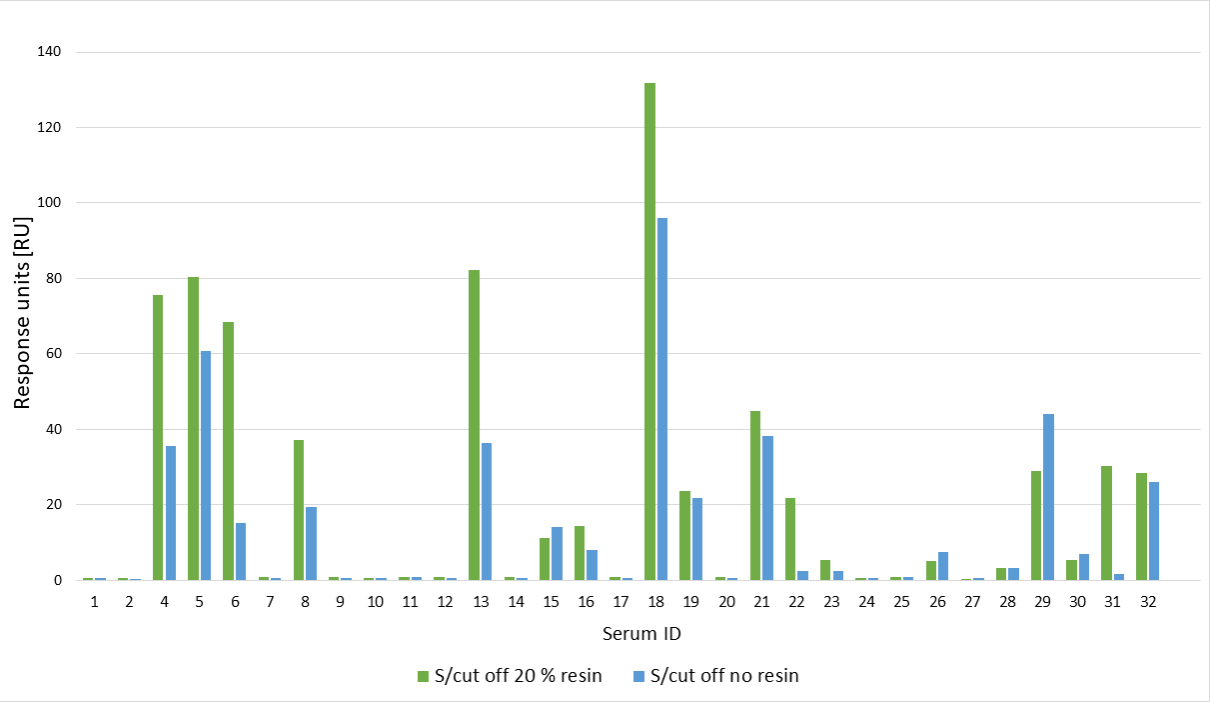

Figure 32. Comparison between full screen with non-purified and purified serum by plotting the S/cut off ratio for both screens.

When purifying the sera in 20% (v/v) POROS™ CaptureSelect™ AAVX Affinity Resin, the signal to cut off ratio increases for all individuals expect ID 1, 11, 15, 24, 25, 26, 27, 29 and 30. This means that 71% (22/31) of the screened individuals gets a higher S/cut off when purified with resin. The biggest increase in S/cut off is for ID 6, 22 and 31, which are all classified as positive.

The individuals with highest background on the GAAA format would benefit most from the resin purification, but they might not have any immune response to AAV2. This means that the signal and the background will decrease equally and the S/cut off ratio will not increase using resin. If the individuals with highest background would have been positive, an increase in s/cut off would probably be seen. ID 31 is positive and had a high background using non-purified serum. When ID 31 was purified with resin the background has a large decrease.

3.3.9 Screen for immune response against multiple AAV serotypes

Since the capture molecule used in the GAAA format has affinity towards several AAV serotypes, five individuals with low positive immune response against AAV2 were screened for immune response against AAV1, AAV3, AAV5, AAV8 and AAV10 empty capsids. The results can be seen in Figure 33, where the AAV2 response and cut off are from previous screen seen in Figure 31.

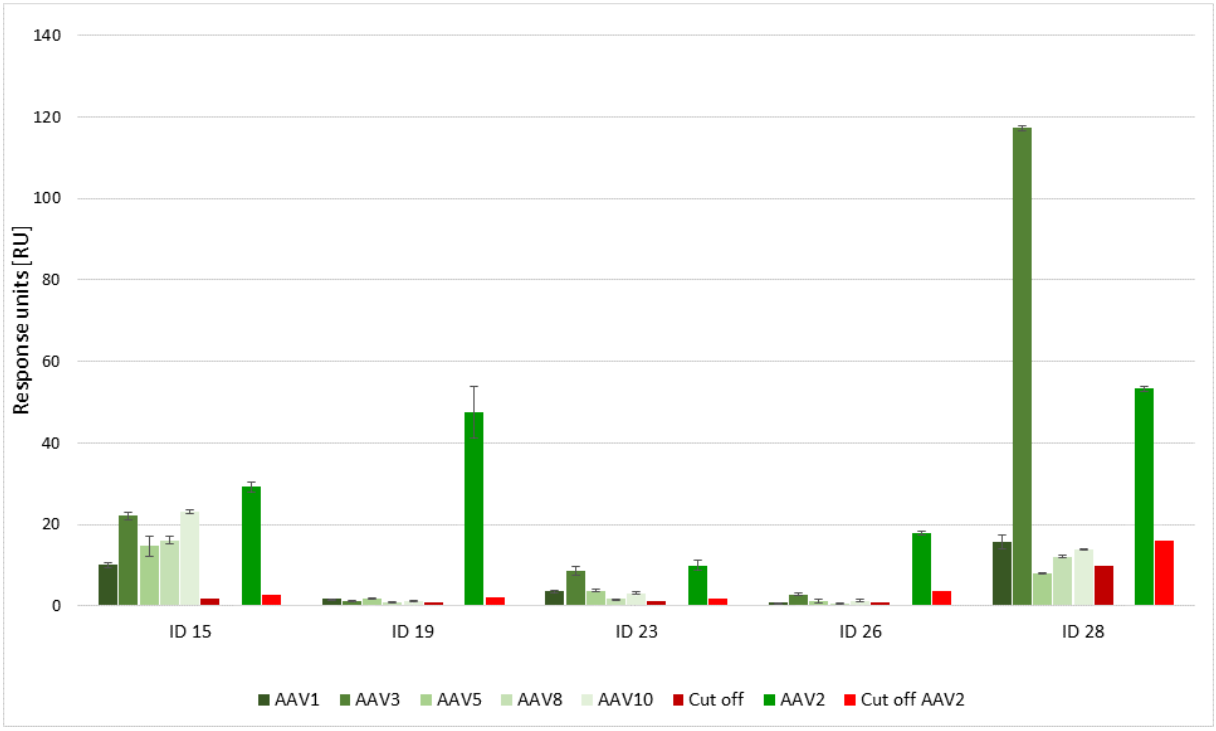

Figure 33. Five individuals with low positive immune response against AAV2 were screened for immune response against AAV1, AAV3, AAV5, AAV8 and AAV10 empty capsids. The sera were purified with 20% (v/v) resin and diluted to 10% purified serum. Just like previous screens 100 µg/ml anti-AAVX, 5 nM detect and 2500 pM capsids was used. The data is visualized as mean response ± SD.

The results visualise the genericity of the assay format where several serotypes can be evaluated in a single run. For ID 15, 19, 23 and 26, AAV2 immune response is higher than AAV1,3,5,6 and 10. For ID 28 the immune response is higher against AAV3. In Table 9 the results from the screen is summed.

Table 9. The screening results of the five individuals for immune response against AAV1, AAV2, AAV5, AAV8 and AAV10.

	AAV1	AAV2	AAV5	AAV8	AAV10
ID 15	+	+	+	+	+
ID 19	+	+	+	+	+
ID 23	+	+	+	+	+
ID 26	+	+	+	-	+
ID 28	+	+	+	+	+

Using the cut off mean background +3SD, all individuals were classified as positive for all serotypes, except serum ID 26 to AAV8.

3.4 Summary of results: Comparing the three assay formats

In Table 10 the results from the three anti-AAV2 immune response screens are assembled. The screening results from the indirect assay format and the GAAA format with sera treated with resin, were that the same 58% (18/31) individuals were classified as positive. The individual screening cut offs was determined to mean background +3SD. When screening

using the bridging assay format, two additional individuals are classified as positive, ID 20 and 27, making 64.5% (20/31) of the individuals classified as positive. On the bridging assay format a common cut off described in section 2.5.3 was used. If the screening cut off mean + 3SD would have been applied for the bridging assay as well, 74% of the 31 individuals would be classified as positive.

Table 10. Screen results from the indirect assay, bridging assay and GAAA format, where the individuals that have a signal higher than the cut off were classified as positive.

Serum ID	Indirect assay format	Bridging assay format	Generic Anti-AAV2 Adsorption Assay format
1	-	-	-
2	-	-	-
4	+	+	+
5	+	+	+
6	+	+	+
7	-	-	-
8	+	+	+
9	-	-	-
10	-	-	-
11	-	-	-
12	-	-	-
13	+	+	+
14	-	-	-
15	+	+	+
16	+	+	+
17	-	-	-
18	+	+	+
19	+	+	+
20	-	+	-
21	+	+	+
22	+	+	+
23	+	+	+
24	-	-	-
25	-	-	-
26	+	+	+
27	-	+	-
28	+	+	+
29	+	+	+
30	+	+	+
31	+	+	+
32	+	+	+

To compare the sensitivity between the three assay formats, the signal to background ratio from the final screens in Figure 12, Figure 19 and Figure 31 are plotted in Figure 34.

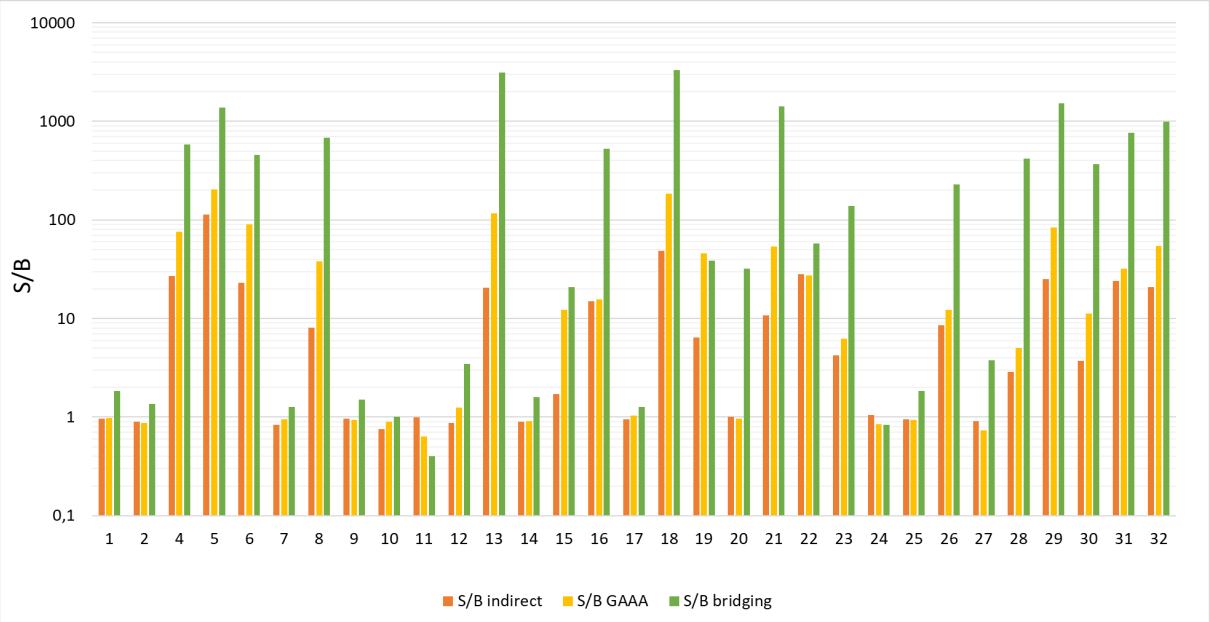

Figure 34. The signal to background ratio for the indirect assay, GAAA and bridging assay format screens, with log-scale on the x-axis.

For 29/31 of the individuals screened, the S/B is significantly higher for the bridging assay than for the indirect assay and the GAAA format. This can be explained by the low backgrounds obtained when screening using the bridging assay format. The indirect assay and GAAA format resulted in the results from the screen, but the S/B was overall greater (19/31) for the GAAA format than for the indirect assay format, due to lower backgrounds in the GAAA format.

To summarise the strengths and weaknesses of the three assay formats, parameters like consumption of the most expensive reagent (AAV2 capsids), labellings (Biotin och Alexa Fluor) required and run time is compared in Table 11.

Table 11. Summary of the strengths and weaknesses of the three assay formats

	Capsid reagent consumption for 1 CD (16 individuals in triplicated)	Capsid labellings required	Generic	Sample treatment required	Run time for 1 CD	Sensitivity S/B	Background	Screens for
Indirect	13 µl	1	No	No	~1 hr	Least sensitive	Individual	IgGs
GAAA	16 µl	0	Yes	Yes, resin purification	~2 hrs	Second most sensitive	Individual	IgGs
Bridging	26 µl	2	No	No	~1 hr	Most sensitive	Low common background	All capsids binding antibodies

Two individuals stood out in the screens performed using the indirect assay and GAAA format, ID 11 and 25. They had unusually high responses for both signal and background. When screening on the bridging assay, the high signal and background did not appear and the

individuals could be classified as negative. If ID 11 or 25 were to be positive, the high background could have caused a false negative classification in the indirect assay and GAAA format. The results from the screens for ID 11 and 25 are plotted in Figure 35, for easier comparison.

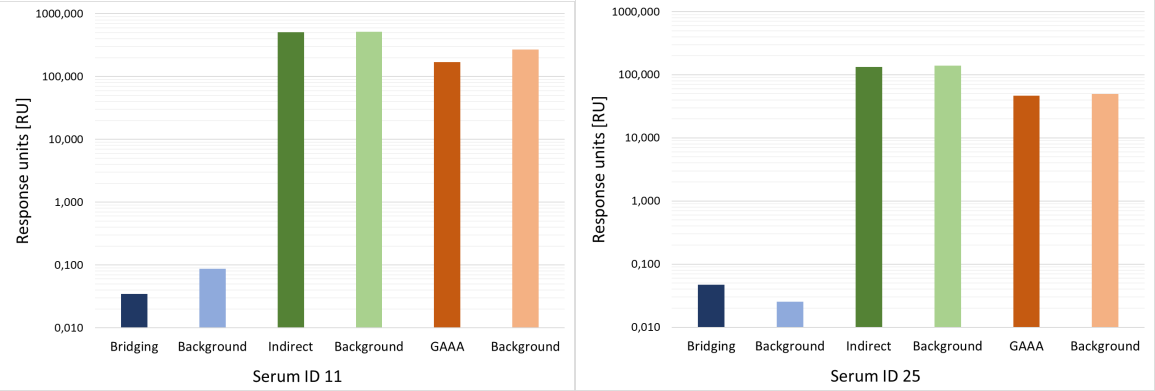

Figure 35. The screening results for serum ID 11 and 25 using the three assay formats.

4 Discussion

The three assay formats evaluated can all be used as a screening tool for pre-existing anti-AAV2 antibodies in human serum, with different strengths and weaknesses. Using the indirect and bridging assay format, 16 individuals can be screened in triplicates using one CD in 1 hour. The Gyrolab platform allows 5 CDs in one run, making it a high throughput screen. Using the GAAA format, 16 individuals can be screened in 2 hours and eliminates the need to label the capsids which could be valuable in early development where a large number of modified capsids can be screened for immune responses.

4.1 Comparing the different formats and applications

Using the indirect assay format and the GAAA format, the same 18 individuals were classified as positive. The indirect assay format and the GAAA format have the detection molecule and the individual screening cut off determination in common. From experience at Gyros Protein Technologies, the detection molecule used has in some cases shown higher backgrounds than other detection reagents. The higher and variable background obtained using the indirect assay format and GAAA format is most likely due to smeared serum residues on the solid phase being detected which have been observed previously at Gyros Protein Technologies in similar assay formats with anti-human IgG as detect. The bridging assay format does not have the background issues and has background levels close to the instrumental background. By using a common screening cut off based on the work of Frey (1998), 20 individuals were classified as positive on the bridging assay format. The two individuals classified as positive on the bridging assay format, but not on the indirect or GAAA format, was ID 20 and 27. The same two individuals are the ones with lowest positive responses the bridging assay. Whether the screening result differs between the formats due to

the indirect and GAAA formats having such high backgrounds that the individuals are falsely classified as negative, or the cut off for the bridging assay being too low, is hard to say. Although, the signal for ID 20 and 27 are 32 and 4 times higher respectively, than the background on the bridging assay format, making it likely that the bridging assay is just more sensitive and a better option when sensitivity is of essence. For example, the TAb assays developed in this study, together with a Nab titre assay could be used to evaluate the immune response against AAV2 in a patient.

In this study 58% of the 31 individuals screened were classified as positive for anti-AAV2 IgG immunity using the indirect assay and GAAA format and 65% of the 31 individuals screened were classifies as positive using the bridging assay format. In previous total IgG screens using an indirect Enzyme-Linked Immunosorbent Assay (ELISA), 72% of the 202 serum samples were classified as positive (Boutin *et al.* 2010), resulting in a slightly higher prevalence than obtained in this study.

One advantage using the indirect assay formats is that it only requires one capsid labelling, compared to the bridging assay requiring two. The GAAA format requires no capsid labelling, making it generic and easy to screen for several serotypes. This is useful when for example screening the immune response for a large number of recombinant AAVs during the search for a gene and cell therapy vector. For the GAAA format, on the other hand, a resin purification to increase the sensitivity seems necessary which is an extra step. The GAAA format is also performed on the Gyrolab Mixing CD which integrates the capsid adsorption process in the run, but in the same time making the run time twice as long as for the indirect and bridging assay formats.

Both the indirect assay and the GAAA format uses a mouse anti-human with specificity to the Fc region on human antibodies as detection molecule, quantifying anti-AAV2 IgGs. Using the bridging assay format all capsid binding antibodies are detected, even though most anti-AAV2 antibodies are IgGs (Ronzitti *et al.* 2020).

4.2 Screening cut off determination

To obtain a statistically defined screening cut off there is a need for large number of negative controls. Due to the lack of negative controls, and since the background was individual for the indirect assay and GAAA format, a different strategy had to be used. In many studies, twice to three times the mean background is used as a threshold for when an individual in considered positive (Frey *et al.* 1998). This strategy is easy to use, but lack statistical relevance. A possible future development of this project would be to screen more individuals and form a more sophisticated cut off. Since the background for the bridging assay was similar between individuals, the model from the work of Frey *et al.* (1998) was applied. In their studies, a large number of negative controls were available, and the model was based on results from ELISA experiments. Still, statically relevance is provided for the screening cut off, taking in consideration the number of independent negative controls available.

A possible development of this project would be to apply the immune-inhibition approach to obtain negative controls (Schneider *et al.* 2016). Then, excess AAV2 capsids would be added to serum samples inhibiting the pre-existing immune responses. Using the negative controls obtained, a screening cut point would be assessed, possibly resulting in a lower cut point and therefore a lower false negative rate. In this study, measurement without the capture element was used as background. If using the immune-inhibition approach this can be avoided.

4.3 ID 11 and 25

Two individuals, ID 11 and 25, stood out in the screens performed using the indirect assay and GAAA format. They had unusually high responses for both signal and background. When screening using the bridging assay, the high signal and background did not appear and the individuals could be classified as negative. If ID 11 or 25 were to be positive, the high background could have caused a false negative classification. To further evaluate the cause was not a priority in this project, but there are multiple possible explanations. Antibodies in some specific sera might interact with the solid phase, being detected by the mouse anti-human IgG Fc using in the indirect assay and GAAA format but not by the Alexa labelled AVV2 capsids using in the bridging assay. Another possible explanation is that antibody complexes are smeared on the solid phase and detected by the mouse anti-human IgG Fc.

The responses for signal and background for ID 11 and 25 are lower on the GAAA assay than the indirect assay. If the high responses are due to an interaction with the solid phase, this can be explained by the GAAA format having a fully saturated solid phase with the small anti-AAVX fragment, limiting the binding sites for interactions with the solid phase. It was shown in section 3.1.1 that the biotinyled capsids was not saturated on the indirect assay format.

5 Conclusion

In this study, it is shown that all three immunoassay formats can be used to screen for TAbs against adeno-associated virus serotype 2 capsids. All three immunoassay formats performed well and depending on in which context the application will be used, the different formats offers different advantages. In this study 58% of the 31 individuals screened were classified as positive for anti-AAV2 IgG immunity using the indirect assay and GAAA format and 65% of the 31 individuals screened were classifies as positive using the bridging assay format. The generic anti-AAV adsorption assay offers the ability to easily screen for several viral serotypes without having to label the capsid, and the bridging assay provides high sensitivity. Provided, is automated and high throughout immunoassays where 16 individuals can be screened in one-two hours, even though further optimization, cut off development and a larger data set is needed to obtain a fully sophisticated screening tool.

6 Acknowlegdements

First, I would like to thank my supervisor Daniel Forsström for being so involved in my project from start to finish. You always took time to answer my questions and discussing my results with me. I could not have asked for a better supervisor.

I would also like to thank my subject reviewer Staffan Svärd for your expertize in the area and your comments.

Lastly, I would like to thank all my colleagues at Gyros Protein Technology for being always being helpful, making my entire stay a positive experience.

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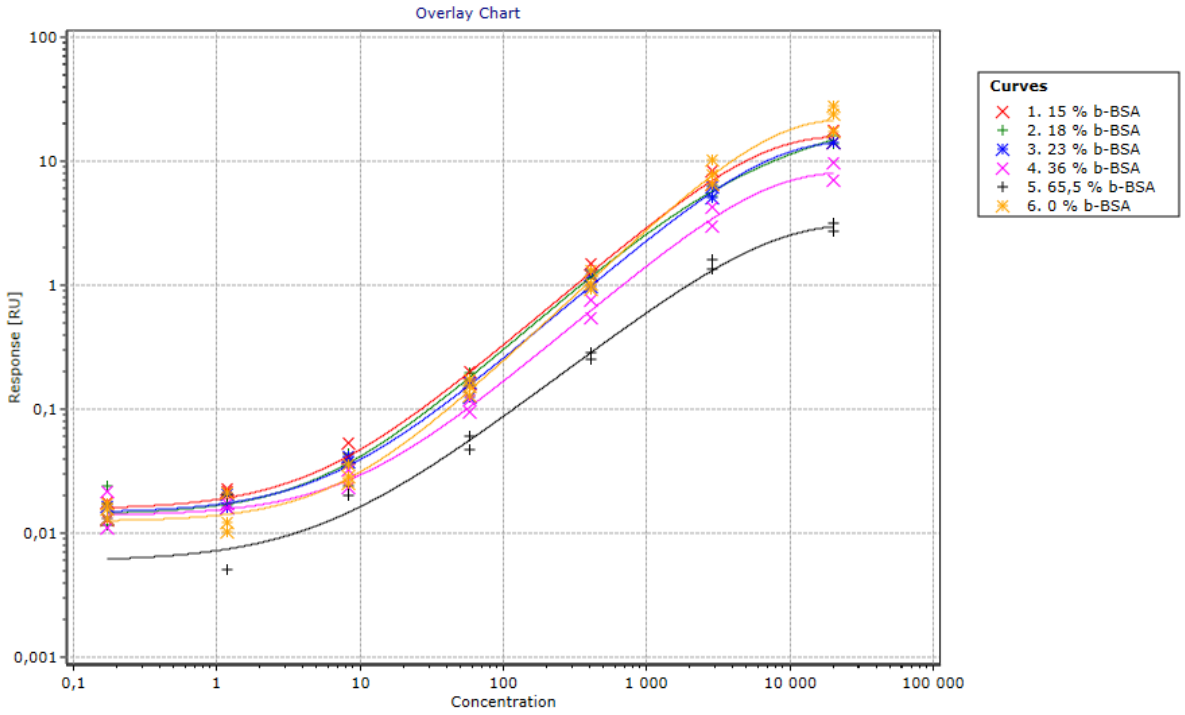
Appendix 1 – Lower the capture element density using b-BSA on the bridging assay format

When using a bridging assay, the Fab regions of the analyte antibody is bound to the capture and detection molecule. When using a high density solid phase in an immunoassay there is a risk that both Fab-regions of the antibody binds to the capture elements, blocking the binding site for the detecting molecule. A possible solution is to lower the density of the capture molecule by mixing it with a molecule that does not bind the analyte. The effect of lower the density of the capture molecule b-AAV2 was investigated by mixing it with 65, 36, 23, 18 and 15% b-BSA.



The response decreases with more b-BSA, meaning that spacing out the capture element has no effect, more than diluting it. If both Fab-regions would bind to the capture molecules on the bridging assay, the signal would initially increase since more binding sites for the detection molecule would be available, then decrease.

Appendix 2 – Matlab function

```
function [cutoff] = cutoff_func(x)
%The parameter t is selected from the table in the paper ”A
statistically
%defined endpoint titer determination method for immunoassays”
(Frey et al. 1998)
t=3.18;
cutoff=mean(x)+std(x)*t*sqrt(1+(1/max(size(x))));

end
```

Appendix 3 – Purifying sera using POROS™ CaptureSelect™ AAVX Affinity Resin on the GAAA format

To further confirm that the high background on the GAAA format is due to antibodies in specific sera interacting with the capture element, CaptureSelect Biotin Anti-AAVX Conjugate, eight individuals were purified with 10, 20 and 30% (v/v) POROS™ CaptureSelect™ AAVX Affinity Resin, before addition to the microtiter plate, see Figure 1. To obtain background values, all measurements were performed with serum diluted with 10, 20 and 30% Rexxip F without mixing with AAV2 capsids. The hypothesis was that purifying the sera with the same molecule as the capture molecule, could remove the interacting antibodies in the sera and possibly increase the signal to background ratio.

The eight individual sera run showed different behaviours on the GAAA screen without resin purification in Figure 1, to investigate how all kinds of serum would be effected by resin addition.

- ID 18, 29: Positives with high S/B
- ID 1, 10, 25: High backgrounds (happens to be negative)
- ID 27: Low background and low signal, negative control.
- ID 31, 22: Positives with low S/B

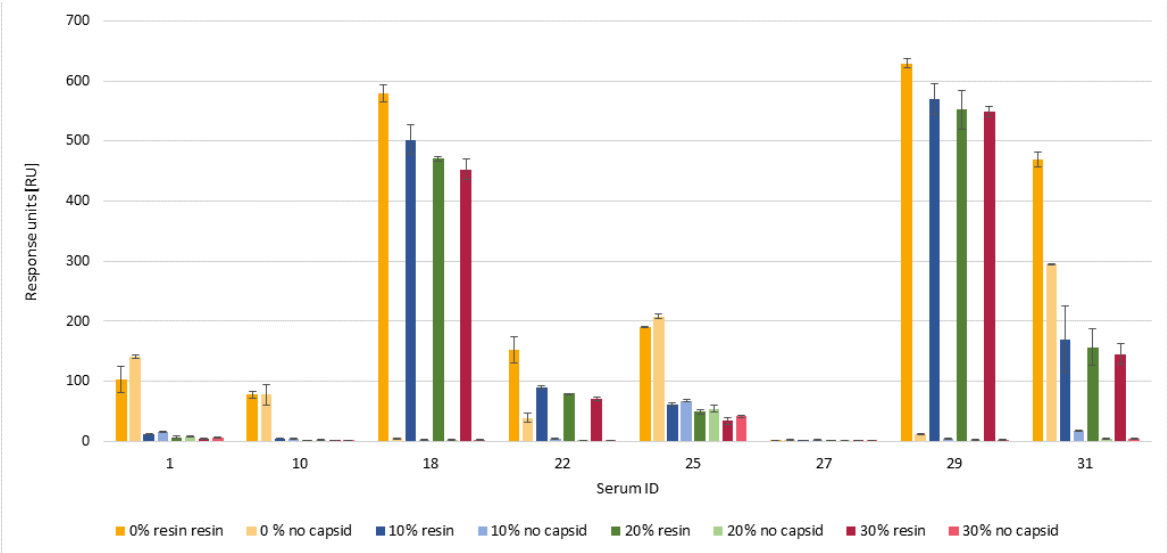


Figure 1. Human serum from eight individuals were purified with 0, 10, 20 and 30% resin.

All individuals except the negative control ID 27 get lower background when purified in resin. When screening on the GAAA format the cut off is Mean background +3SD. To easier analyse the effect of the resin purification and since the sera purified in higher percent resin also get more diluted, the signal to cut off ratio was calculated, shown in Table 1.

Table 1. The signal to cut off ratio for the different resin% used

Serum	S/cut off no resin	S/cut off 10% resin	S/cut off 20% resin	S/cut off 30% resin
1	**0,921**	0,752	0,709	0,589
10	**0,605**	0,576	0,656	0,971
18	**96,141**	89,958	83,016	129,193
22	**2,479**	15,582	26,988	38,404
25	**0,874**	0,829	0,700	0,709
27	**0,633**	0,320	1,075	0,412
29	**44,066**	68,695	149,753	96,097
31	**1,575**	7,731	24,308	19,543

For ID 18 and 29, positives with high S/B on the screen, the S/cut off was not negatively affected by using resin. Lower responses are obtained both with and without capsid, but equally, most likely due to the serum dilution. For ID 1 and 10, sera with high background on the screen, both signal and background decreased more than the resin diluted them and S/cut off stay somewhat constant. If these sera would have been positive the S/cut off might increase. For high background ID 25, the background stays high. For the negative control, ID 27, all values were close to blank. For ID 31 and 22 the S/cut off increased when purified with more resin. The signal decreases a bit, and the background decreased a lot. On these individuals, the resin has a large effect.

In conclusion, 20% resin will be used for the screen since both positives with high background, ID 31 and 22 got higher S/cut off. The positives with low background does not seem to get decreased signals more than dilution with resin.