Development of a Type IIs cloning strategy for the RUBY™ format
&
PCR optimization and development of a Type IIs cloning strategy for pool cloning

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Abstract
For Alligator Bioscience AB, a company developing immunotherapeutic antibodies, it is crucial to efficiently evaluate a large number of potential drug candidates at an early stage. The in-house developed RUBY™ concept, combining monoclonal antibodies into bispecific antibodies without lead optimization, requires implementation of high-throughput technologies. Restriction enzyme digestion, used for generating expression vectors through cloning of antibody fragments, is not suitable for high-throughput implementation. Instead, this thesis project established Type IIs cloning strategies for the RUBY™ format. Developed using a newly designed pcDNA3.4 expression vector, before utilizing the procedure for cloning of antibody fragments in pool. The pcDNA3.4 vector system was evaluated, against a previously used vector system, based on antibody production and protein characteristics. The pcDNA3.4 expression vector considerably improved antibody yield and decreased aggregation. Type IIs cloning was proven to be a viable and versatile strategy, as both PCR fragments and a donor vector could be used to achieve a high cloning efficiency. A donor vector will provide greater flexibility to future antibody development and does not require repeated amplification. The high cloning efficiency was maintained during pool cloning as well, illustrating that it is now possible to efficiently generate a greater number of antibodies in a desired format for future screenings.

Introduction

Cancer immunotherapy, once labelled as just promising, have in recent years proven to be a viable therapeutic option. A therapy based on utilizing the immune system of the host to counteract mechanisms crucial for cancer progress, either repressing inhibitory pathways or activating effector cells of the immune system. A great contribution to the field has been the development of monoclonal antibodies (mAbs), with successful anti-CTLA-4 antibody ipilimumab (Bristol-Myers Squibb) against melanoma paving the way in 2011 [1]. mAbs used in clinic have similar features as they all originate from immunoglobulin G (IgG) subclasses (Fig. 1A). They are characterized by two heavy (50 kD) and two light (25 kD) polypeptide chains, making up a total molecular weight of approximately 150 kD. Two constant regions (C_{H3} and C_{H2}) of the heavy chain constitutes the domain known as fragment crystallizable (Fc) which is able to interact with a wide range of cell surface receptors, from proteins of the complement system to Fcγ receptors [2]. Additional constant regions C_{H1} (heavy chain) and C_L (light chain) as well as variable regions V_H (heavy chain) and V_L (light chain), linked to the Fc domain by a hinge, are together referred to as fragment antigen binding (Fab). The Fab fragments dictates antigen specificity through hypervariable regions, located on V_H and V_L [3]. The hypervariable regions are further divided into six complementary-determining regions (CDRs), three situated on the heavy chain and three on the light chain [4].

Since 2011, several immunotherapeutic mAbs have been proven successful for treating a wide range of tumors. In addition to continuous development of mAbs the field has advanced towards generating bispecific antibodies, with the aim of achieving greater efficacy and safety compared to if two monospecific antibodies would be administered as a combined therapy [5]. The concept behind bispecific antibodies is to design one drug specific for two separate epitopes, located on one or two antigens [6]. A wide range of bispecific formats are under development, the majority of them as potential cancer treatments [7]. An example of a format already approved for clinical use, is the bispecific T cell engager antibody blinatumomab (Amgen). With a structure separating it from other bispecific formats, as it only consists of two single-chain variable fragments (scFv) linked together. Each scFv is constituted of a V_H and V_L brought together by a short linker, creating a single polypeptide. The drug utilizes the potential of bispecific antibodies by binding the CD3 receptor on CD8+ T-cells and surface protein CD19 on B-cells simultaneously, thereby increasing cytotoxicity [8]. However, each bispecific format with this type of design require individual construct optimization in order to be successful. It is therefore advantageous to generate bispecific antibodies with an Fc domain instead, preferably using a platform which combines mAb properties to generate bispecific antibodies [9]. The bispecific format RUBY™ (Alligator Bioscience) illustrated in Fig. 1B, intends to increase efficiency of bispecific antibody development by enabling the combination of two mAbs into an IgG bispecific antibody without lead optimization [10].

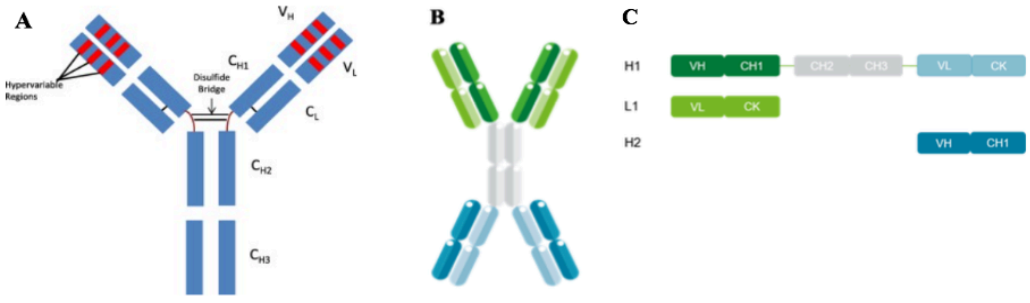

Figure 1. A representation of two antibody formats originating from the immunoglobulin G subclass. A) Representation of a monoclonal antibody [2]. B) The in-house designed bispecific antibody format RUBY™, a concept

utilized to combine properties of two monoclonal antibodies into a bispecific antibody. C) An illustration of the three different chains constituting the RUBY™ format, two heavy chains (H1 and H2) and a light chain L1 [10].

Efficient production of immunotherapeutic antibodies can be carried out using mammalian HEK293 cells, transiently transfected with expression vectors encoding antibody genes of interest. Still, generation of these vectors is limited by ineffective restriction enzyme cloning procedures that does not enable a high-throughput procedure [11]. An option to conventionally used cloning technologies is the Golden Gate cloning, a strategy which relies on Type IIs restriction enzymes instead of site-specific recombination (Fig. 2). It enables cloning of one or several DNA fragments from one vector to another, in a single-step reaction. It is made possible because Type IIs restriction enzymes are able to digest DNA four random nucleotides downstream the restriction site, represented by BsaI in Fig. 2. This can be utilized to design DNA containing flanking regions with the site which will be removed after digestion, allowing overhangs between fragments to be ligated without the risk of further cleavage [12].

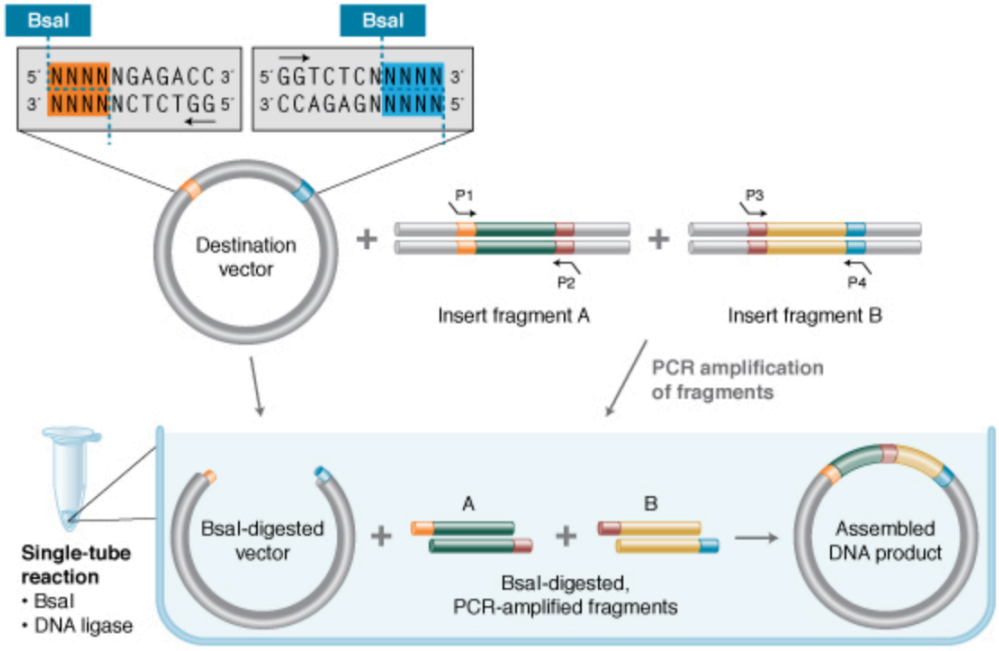

Figure 2. An overview of Golden Gate cloning. Type IIs restriction enzymes can be utilized in a single-tube reaction to clone multiple fragments into a destination vector [13].

A technological platform, effective in the production of bispecific antibodies, demands high-throughput technologies for screening of potential drug candidates. In order to generate a large number of clones in a given antibody format, it is of interest to improve both PCR amplification strategies of antibody fragments as well as cloning procedures. The first part of this project aimed towards establishing Type IIs cloning strategies for the in-house developed bispecific antibody format RUBY™, using a newly designed pcDNA3.4 expression vector. Previously used pAb944/945 expression system did not provide sufficient amounts of plasmid DNA or achieve a desired antibody production yield. The Type IIs cloning strategies included utilizing DNA fragments amplified by PCR, synthesized DNA and a donor vector during cloning. Following investigation of the new pcDNA3.4 vector system, a suitable antibody production protocol was implemented, and production evaluated against the pAb944/945 vector system. Once a cloning procedure and the pcDNA3.4 vector was system evaluated, allowing for a high-throughput format, the project continued with PCR optimization and Type IIs cloning in pool. With the aim of cloning

antibody fragments (V_H or V_L) from a pool obtained after initial screening of scFvs, into a mAb format.

Materials and methods

Vector systems used for cloning

Plasmids from the pAb944/945 vector system were used for PCR amplification of genes encoding L1, H2 or H1 (Fig. 1C), in order to assemble them via Type IIs cloning into the pcDNA3.4 TOPO vector. The pcDNA3.4 vector is a commercially available mammalian expression vector, with a CMV promoter, used to obtain high levels of protein expression [14]. At Alligator Bioscience AB, the pcDNA3.4 TOPO vector was designed to contain a multiple cloning site, chloramphenicol resistance, the *ccdB* gene and two AarI restriction sites for Type IIs cloning. Each plasmid in both vector systems, with a gene encoding an antibody chain, have been given a Plasmid ID listed in Table 1. Written in parenthesis is the bispecific antibody variant to which the antibody chain corresponds.

Table 1. An overview of the two vector systems used for cloning, pAb944/945 and pcDNA3.4. *Each plasmid containing a gene encoding an antibody chain have been given a Plasmid ID.*

The pAb944/945 vector system		**The pcDNA3.4 vector system**	
Plasmid ID	**Antibody chain**	**Plasmid ID**	**Antibody chain**
3269	L1 (v9, v10)	3596	L1 (v9, v10)
3310	H2 (v9)	3598	H2 (v9)
3312	H2 (v10)	3599	H2 (v10)
3356	H1 (v9)	3601	H1 (v9)
3358	H1 (v10)	3602	H1 (v10)

DNA preparation and purification

Bacterial cultures containing plasmids of the two vector systems were generated from *Escherichia coli* strains stored as glycerol stocks. Each clone was inoculated in 5 ml 1xLB media supplemented with 0.1 mg/ml carbenicillin, in 50 ml tubes. Tubes were incubated at 37 ºC, shaking at 225 rpm overnight.

Three different kinds of kits have been utilized to prepare DNA from the two vector systems at different occasions, the QIAprep Spin Miniprep Kit (#27106 QIAGEN), the NucleoSpin Plasmid Kit (#740499.250 Macherey Nagel) and the NucleoBond Xtra Midi Plus Kit (#740412.50 Macherey Nagel). Manufacturers protocols have been followed during DNA purification for all kits.

Preparation of DNA to be cloned in pool was performed by first culturing cells containing phagemid vectors with 32 scFv, from glycerol stocks, in a 96-well plate. Each well contained 1 ml 1xLB media, supplemented with 0.1 mg/ml of carbenicillin. Cells were cultured overnight at 37 ºC, shaking at 700 rpm and with a humidity of 80 %. Plasmid purification was carried out using the MagJET Plasmid DNA Kit (#K2792 Thermo Fisher Scientific), according to the manufacturer's protocol.

Quantification of DNA and protein

Concentration of purified DNA was measured using the Big Lunatic (Unchained Labs) set to quantify plasmid DNA through ultraviolet-visible spectroscopy or measuring dsDNA at A260 for fragments amplified by PCR. 2 µl of MilliQ water or elution buffer provided by the DNA purification kit was used as a blank, respectively.
Protein amount was quantified using ultraviolet-visible spectroscopy as well but set to measure total absorbance at 280 nm. For protein measurements, water was used as a blank.

Primers for amplification of genes encoding antibody fragments

Three different sets of primers were utilized during this project, designed at Alligator Bioscience AB via the GeneArt Primer and Construct Design Tool (Thermo Fisher Scientific) and ordered from Eurofins Genomics (Table 2). One set (H357, H358, H359 and H360) specific for PCR amplification of genes encoding L1, H2 or H1 from the pAb944/945 vector system. Another set (H362 and H365) designed to amplify the V_H-C_{H1} and V_L-Cκ regions of H1, to be assembled with Fc from a donor vector.

The final set (H285 and H286) utilized for amplification of 32 V_H fragments, each from a scFv encoded in a phagemid vector, to be used in pool cloning. All primers were designed to contain an AarI digestion site for the Type IIs restriction enzyme, located in a flanking region of the 5´end. Primers were resuspended in 10 mM Tris-HCl with a pH of 8.5, to a concentration of 100 µM and stored in -20 ºC. Before PCR, the stock solution was diluted to a 10 µM working solution using Nuclease-free Water.

Table 2. A representation of the primers used for PCR amplification of antibody fragments throughout the project*. L1/H2/H1 chains and V_H-C_{H1}/V_L-Cκ regions of H1 were amplified from the pAb944/945 vector system, while V_H fragments were amplified from scFvs.*

Forward primer	Reverse primer	Antibody fragment
H357	H359	L1 (3269)
H359	H360	H2 (3310)
H359	H360	H2 (3312)
H359	H358	H1 (3356)
H359	H358	H1 (3358)
H359	H362	V_H-C_{H1} of H1 (3356)
H365	H358	V_L-Cκ of H1 (3356)
H359	H362	V_H-C_{H1} of H1 (3358)
H365	H358	V_L-Cκ of H1 (3358)
H285	H286	V_H from scFv

PCR amplification of antibody fragments from the pAb944/945 vector system

Each PCR reaction consisted of 2.5 ng of DNA, 10 µl of 5x Phusion GC buffer, 2.5 µl of both forward and reverse primer (500 nM), 1 µl of dNTPs (10mM each), 1 µl of Phusion HT II High-Fidelity DNA polymerase (#F549S) and finally Nuclease-free Water to obtain a total reaction volume of 50 µl. In order to investigate the optimal temperature conditions for amplification of the

genes encoding L1/H2/H1, an annealing temperature gradient of 52-62 ºC was used. The cycling parameters were set to 95 ºC for 5 min, followed by 20 cycles of 95 ºC for 30 sec, 52-62 ºC for 30 sec and 72 ºC for 1 (<1000bp) or 2 min (>1000bp). The final extension step was set to 72 ºC for 7 min and the holding step to 4 ºC. An annealing temperature of 53.9 ºC was used for the PCR amplification using V_H-C_{H1} and VL-Cκ primers. Following PCR, amplified DNA fragments were purified using a QIAquick PCR Purification Kit (QIAGEN).
The size of the amplicons were analyzed on a 1% agarose gel in 1x Tris-acetate-EDTA buffer, run at 100V for 30 min. A 1 Kb Plus DNA Ladder (#10787018 Invitrogen) was used as a reference in order to evaluate size. The expected size of the amplified genes varies depending on the encoded chain, with L1 (3269, 3477) at 690 bp, H2 (3310, 3312, 3486) at 710 bp and H1 (3356, 3358, 3478) at 2100 bp.

PCR amplification of V_H fragments from scFvs in phagemid vectors

Developing strategies for PCR amplification of antibody fragments in pool (32 V_H fragments in total), involved three different alternatives: Option 1, Option 2 and Option 3 (Table 3). The first aimed towards amplifying 10 V_H fragments individually, from purified DNA. The second, to amplify the same DNA fragments as Option 1 but from a pool of purified DNA with 5 ng of each V_H fragment. The third alternative consisted of amplifying V_H from four pools of clones (2x10, 12 and 32 clones), generated by scraping glycerol stocks into 50 µl 10 mM Tris-buffer (pH 8.0) and denaturing the mixture at 95 ºC for 5 min. One pool with the 10 same clones as Option 1 and 2 (referred to as 3:1), another pool with 10 other clones (3:2), a third pool with 12 other clones (3:3) and at last a final pool with all 32 mentioned clones combined (3:4).

Table 3. V_H fragments were amplified from scFvs using three different alternatives (Option 1, Option 2 and Option 3). *Each alternative aimed towards amplifying a specific number of VH fragments, either from individually purified DNA (Option 1), from DNA pooled after purification (Option 2) or directly from glycerol stocks (Option 3).*

Alternative	Number of V_H fragments
Option 1	10
Option 2	10
Option 3:1	10
Option 3:2	10
Option 3:3	12
Option 3:4	32

1 ng of each individual V_H fragment was used for PCR amplification of Option 1, 1 ng of the pooled DNA for Option 2 and 2.5 µl of each alternative in Option 3. In addition to DNA, 10 µl of 5x Phusion GC buffer, 2.5 µl of both forward and reverse primer (500 nM), 1 µl of dNTPs (10mM each), 1 µl of Phusion HT II High-Fidelity DNA polymerase (#F549S) and Nuclease-free Water was added to obtain a total reaction volume of 50 µl. The cycling parameters were set to 98 ºC for 30 sec, followed by 25 cycles of 98 ºC for 10 sec, 70 ºC for 30 sec and 72 ºC for 30 sec. The final extension step was set to 72 ºC for 7 min and the holding step to 4 ºC. Following PCR, amplified DNA fragments were purified using a QIAquick PCR Purification Kit (QIAGEN). The size of the amplicons was analyzed on a 1% agarose gel in 1x Tris-acetate-EDTA buffer, run at 100V for 30

min, each V_H fragment expected to be 350 bp. A GeneRuler Low Range DNA Ladder (#SM1193 Thermo Fisher Scientific) was used as a reference in order to evaluate size.

Cloning of an Fc region into a donor vector

A variant of the Fc constant region, to be cloned into a donor vector (Plasmid ID 3614), was ordered as a synthesized fragment from GeneArt® Gene Synthesis service (Invitrogen). DNA was designed to contain an AarI Type IIs restriction site, to enable Type IIs cloning. The synthetic DNA fragment was diluted to a concentration of 20 ng/µl in 10 mM Tris-HCl, pH 8.5, prior to storage in -20 ºC.

In order to generate a donor vector with the synthesized Fc fragment as an insert (Plasmid ID 3614), cloning with the Zero Blunt TOPO PCR Cloning Kit (#K280020 Invitrogen) was performed. 60 ng of the synthesized DNA was added to 1 µl of pCR II-Blunt-TOPO, 1 µl of salt solution and 1 µl of autoclaved MilliQ water. After addition of all reagents, the mixture was incubated at RT for 15 minutes prior to transformation, colony picking and sequencing.

Type IIs cloning

Three different types of assembly reactions were carried out during the evaluation of Type IIs cloning strategies of the bispecific antibody format, each by using the GeneArt Type IIs Assembly Kit (#A15916 Invitrogen) with pcDNA3.4 as a destination vector. Regardless of cloning strategy, a 1:1 molar ratio between insert and vector was used with 1 kB as a reference for the insert (the amount of insert is adjusted based on the size of the vector). The first reaction was performed in order to assemble PCR amplified genes of L1, H2 and H1 into three separate vectors. The second, to assemble PCR amplified V_H-C_{H1} and V_L-Cκ DNA fragments together with the synthesized fragment. The third, an assembly reaction consisting of V_H-C_{H1} and V_L-Cκ DNA fragments in combination with donor vector 3614. For L1/H2/H1, 50/50/150 ng of PCR fragment was added to 75 ng of the pcDNA3.4 TOPO vector. While 50 ng for both V_H-C_{H1} and V_L-Cκ fragments together with string/donor vector (55/75 ng) was added to 75 ng of destination vector.

During Type IIs cloning of antibody fragments in pool, the same assembly kit and destination vector was utilized. Regarding Option 1, 2.5 ng of each individually amplified and purified fragment was pooled prior to cloning. As for Option 2 and 3, pools of DNA were generated before PCR. For each of the six assembly reactions (Option 1, Option 2, 3:1, 3:2, 3:3 and 3:4), 25 ng of purified PCR product was added to 75 ng of the pcDNA3.4 vector together with 75 ng of donor vector 3504 (with an Fc domain). In addition, a positive and negative control was included during all assembly reactions. A positive control based on 2 µl of pType IIs-CTRL vector together with 1 µl pType IIs destination vector and a negative control using autoclaved MilliQ water with 75 ng of pcDNA3.4.

Transformation

From each Type IIs assembly reaction or Zero Blunt TOPO cloning, 1 µl was added to 50 µl of One Shot TOP10 chemically competent *E. coli* (#C404006 Invitrogen). Cells were gently mixed and incubated on ice for 30 min prior to being heat-shocked at 42 ºC for 20 sec in a water bath. Cells were transferred back onto ice for a few minutes and 250 µl of S.O.C medium (#C404006 Invitrogen) was added prior to incubation at 37 ºC, shaking at 225 rpm for one hour. After incubation, each transformation reaction was plated on a LB agar plate and incubated at 37 ºC

overnight. Plates contained the appropriate antibiotics at a concentration of 0.1 mg/ml, either kanamycin for the Zero Blunt PCR cloning or carbenicillin for Type IIs cloning. As a positive control for the transformation, 1 µl of pUC19 (New England Biolabs) was added to competent cells, while 1 µl of autoclaved MilliQ water was added as a negative control.

Sequencing of colonies after transformation

After incubation, transformed colonies were counted and three colonies per transformation reaction picked for sequencing. For cloning strategies concerning the in-house developed bispecific antibody format, picked colonies were cultured in 5 ml of 1xLB media with 0.1 mg/ml of the specific antibiotic. Cultures were incubated overnight at 37 ºC and shaking at 225 rpm, followed by plasmid purification. Obtained DNA was diluted to a concentration of 100 ng/µl using autoclaved MilliQ water and 20 µl sent for sequencing at Eurofins Genomics. Sequencing results were analyzed using Lasergene's bioinformatics software (DNASTAR). Colonies confirmed by sequencing were saved as glycerol stocks by adding 500 µl of 50% glycerol to 500 µl of overnight culture and storing it at -80 ºC.

For cloning strategies developed for pool cloning, colonies were transferred to a 96-well PlateSeq plate (Eurofins Genomics). The plate was sent for plasmid extraction and sequencing at Eurofins Genomics. Retrieved sequencing data was analyzed in AnySpeeq and AbSpeeq (Bionamic AB).

Antibody production

Human embryonic kidney (HEK) 293 cells cultured in Expi293 Expression Medium (#1435102 Gibco) was utilized for production of bispecific antibody variants v9 and v10 by both vector systems, for evaluation of antibody production. 24 h before transfection, cells were counted using a Vi-CELL XR (Beckman Coulter) and diluted in fresh medium to a concentration of 3 million cells/ml for continuous growth overnight. 4.1 million cells/ml were seeded in 25.5 ml expression medium for each transfection (in 125 ml flasks), to which OptiPRO SFM Medium (#11530426 Gibco) containing expression vectors encoding H1, L1 and H2 was added (with a 1:2:1 ratio). In addition to the two bispecific antibodies, an isotype GFP mAb comprised of a heavy chain (Plasmid ID 3204) and a light chain (Plasmid ID 3205) was included as a control for the expression. Transfection was carried out by using an ExpiFectamine 293 Transfection Kit (#A14524 Gibco), containing Enhancer 1, Enhancer 2 and Expifectamine reagent. With the final addition of Enhancer 1 and 2 during transfection, a volume of 30 ml was obtained prior to incubation for four days at 120 rpm/ 36.5 ºC/ 8 % CO_2. Evaluation of the pcDNA3.4 vector system against pAb944/945, was carried out according to the transfection protocol described above (originally created for pAb944/945). In order to implement a suitable production protocol for the pcDNA3.4 vector system, the effect of adding Enhancer 1 and Enhancer 2 18-22 h after transfection was also investigated. Both when evaluating the pcDNA3.4 vector system and investigating the effects of enhancers, antibody production and following protein characterization was performed twice in order to replicate the results.

Protein purification

Supernatants were harvested following antibody production by centrifugation at 3500 rpm for 35 min and subsequent vacuum sterile filtration. Approximately 25 ml of supernatant was obtained from each sample, 1 ml was saved for analysis while remaining supernatant proceeded to antibody

purification. Automated purification was carried out by an NGC10 (Bio-Rad), a protein A chromatography system, using 1 ml MabSelect SuRe Affinity columns (GE Healthcare) and 5 ml HiTrap Desalt columns (GE Healthcare) in a tandem mode. 20 mM sodium phosphate (pH 7) was used as a binding buffer, 0.1 M citric acid (pH 3.5) as an elution buffer, PBS as a desalt buffer, 0.2 M NaOH as a wash buffer and 20% EtOH for column storage. Purified antibodies were eluted as fractions in a 96-well plate, thereafter manual pooling of eluted antibodies was performed and concentration measured using the Big Lunatic (Unchained Labs) prior to protein characterization.

Antibody quantification in supernatant

An OctetRED96 System (ForteBio) with Protein A biosensors was used to measure antibody concentration in supernatants. Samples were analyzed in a low sensitivity assay with the plate shaking at 400 rpm and a reading time of 2 min. Samples were analyzed both as undiluted and diluted 1:2 with Expi293 Expression Medium (Gibco). PBS with 1% BSA and 0.02% Tween 20 was used as a neutralization buffer and to hydrate biosensors before analysis. Regeneration buffer consisted of 10 mM Glycine with 0.02% Tween 20 at pH 1.1.

High-Performance Liquid Chromatography

Fractions of purified antibodies were analyzed by High-Performance Liquid Chromatography (HPLC) in order to investigate molecular weight and percental aggregation. It was carried out by Maria Mårtensson via a 1260 Infinity II LC System (Agilent Technologies). Approximately 10 µg of sample was analyzed on TSK gel Super SW mAB HTP 4.6x150 mm (Tosoh Bioscience) and TSK gel Super SW mAB HTTP Guard 3x20mm (Tosoh Bioscience) columns. 300 mM NaCl and 100 mM sodium phosphate, pH 6.8, was used as a mobile phase with a flow rate of 0.35 ml/min. The UV detector was set to identify analytes at a wavelength of 215 nm and 280 nm.

SDS-PAGE

Each reduced sample consisted of 3 µg of antibody together with autoclaved MilliQ water in order to obtain a volume of 19.5 µl. To each sample, 7.5 µl of 4xLaemmli sample buffer (#161-0747 Bio-Rad) and 3 µl of NuPAGE Sample Reducing Agent 10x (#NP0004 Invitrogen) was added prior to heating the mixture at 70 ºC for 10 min. The entire volume of 30 µl was loaded onto a Mini-7.5 % PROTEAN TGX Stain-Free Precast Gel (#4568023 Bio-Rad) placed in 1x Tris/Glycine/SDS Buffer (#1610732 Bio-Rad) running buffer. 5 µl of Precision Plus Protein Unstained Standard (#1610363 Bio-Rad) was finally added as a protein ladder before running the gel at 300V for 15 min.

For analysis of non-reduced antibodies, 3 µg of protein was added to autoclaved MilliQ water to achieve a sample volume of 19 µl. Same sample volume was used for supernatants, without any addition of water. 6 µl of NuPAGE LDS Sample Buffer (4X) (#NP0008 Invitrogen) was added, prior to heating at 70 ºC for 10 min. 25 µl was loaded onto a NuPAGE 7% Tris-Acetate Protein Gel (#EA0355BOX Invitrogen), placed in 1x NuPAGE Tris-Acetate SDS Running Buffer (#LA0041 Invitrogen). 7 µl Precision Plus Protein Dual Color Standard (#1610374 Bio-Rad) was loaded onto the gel as well, to be used as a ladder, prior to being run at 200V for approximately 50 min. Following electrophoresis, the gel was stained with InstantBlue (#ISB1L Expedeon) and destained with deionized water. Both reduced and non-reduced samples were analyzed with a ChemiDoc MP Imaging System (Bio-Rad).

LabChip analysis

An additional analysis of supernatants and purified antibodies were given by the LabChip GXII Touch instrument (PerkinElmer). Samples were analyzed both as reduced and non-reduced, supernatant only as non-reduced. The NuPAGE Sample Reducing Agent 10x (#NP0004 Invitrogen) was used to reduce antibodies. All practical procedures can be found in the Protein Express Assay User Guide (PerkingElmer).

UNcle analysis

In order to investigate thermal stability of the produced antibodies, an UNcle (Unchained Labs) was used to determine T_m. 9 µl of each antibody was applied to a Uni device and inserted into the instrument. 20 ºC was set as start temperature, with a thermal ramp rate of 0.4 ºC/min and a plate hold of 15 sec before reaching the end temperature of 95 ºC.

Results

PCR amplification of genes encoding antibody chains

The initial cloning procedure, assembling genes encoding antibody chains into pcDNA3.4 expression vectors, required PCR amplification of L1, H2 and H1 from the previously used pAb944/945 vector system (Primers found in Table 2). The amplification was investigated at four different annealing temperatures, ranging from 53.9 to 61.2 ºC, which all generated DNA fragments of expected sizes (Fig. 3A-D). Bands found at the bottom of each gel with a size below 75 bp, are to be considered primer dimers (Fig. 3A-D).

Evaluating Type IIs cloning strategies also included using a donor vector during cloning, encoding an Fc domain. The Fc domain can be combined with various Fab regions, depending on application, which provides the strategy with a greater flexibility. To enable this investigation, V_H-C_{H1} and V_L-C_κ regions of the H1 chains from the pAb944/945 vector system (Plasmid ID 3356 and 3358) was amplified by PCR. The amplification was analyzed using electrophoresis and evaluated against a DNA ladder. The analysis illustrates how DNA fragments of the correct sizes were amplified, indicated by bands correlating to their expected size (Fig. 3D). Each PCR reaction proceeded to PCR product purification, in order to continue with development of Type IIs cloning strategies.

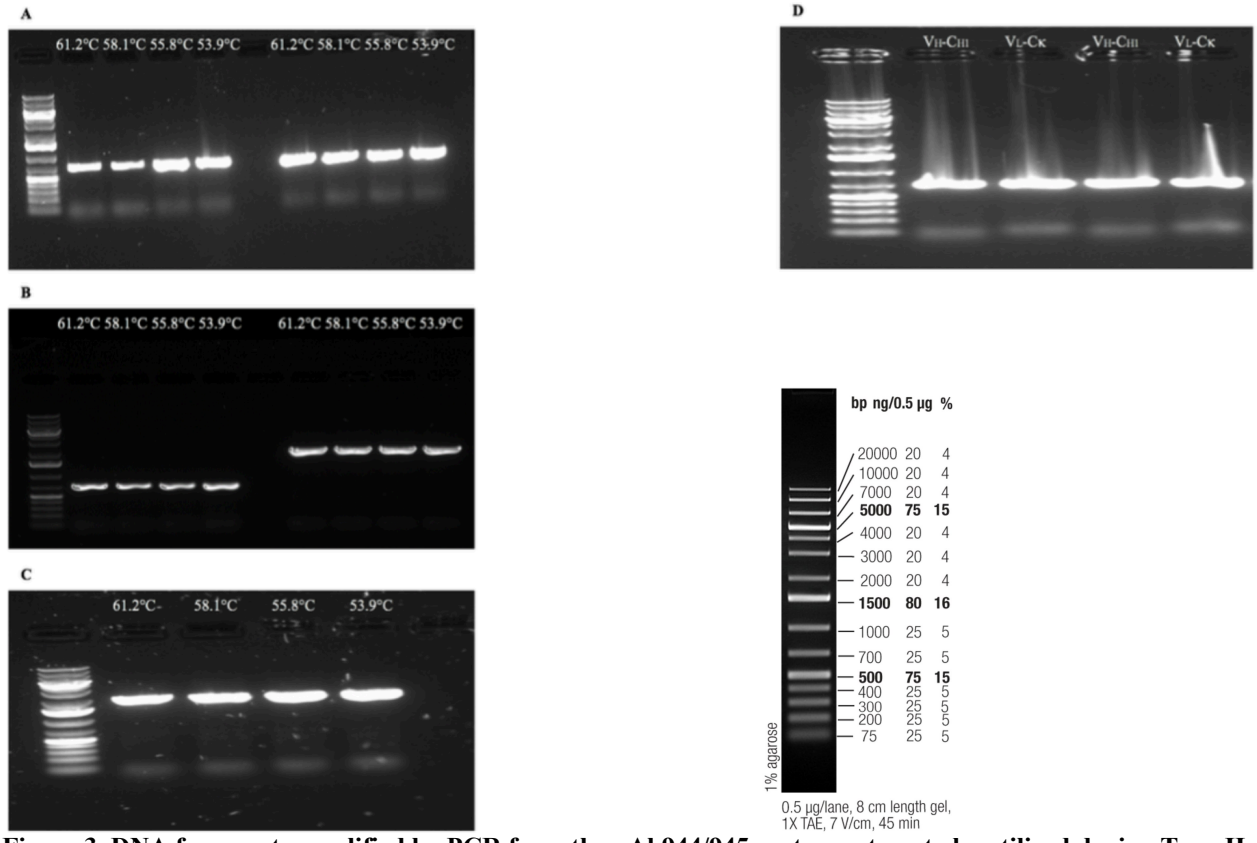

Figure 3. DNA fragments amplified by PCR from the pAb944/945 vector system, to be utilized during Type IIs cloning to generate pcDNA3.4 expression vectors. Amplification of L1/H1/H2 chains (A-C) was carried out using a temperature gradient, from 53.9 to 61.2 ºC, while amplification of V_H-C_{H1} and V_L-C_κ (D) was performed at 53.9 °C. Primers used for all PCR reactions can be found in Table 2. A) H2 (v9) amplified from Plasmid ID 3310 followed by L1 (v9) from Plasmid ID 3269. B) H2 (v10) amplified from Plasmid ID 3312 followed by H1 (v10) from Plasmid ID 3358. C) H1 (v9) amplified from Plasmid ID 3356. D) V_H-C_{H1} and V_L-C_κ regions of the H1 chains amplified from Plasmid ID 3356 and 3358 respectively. By comparing the displayed bands of each gel with the ladder (GeneRuler

1kb Plus DNA Ladder), it can be concluded that all amplifications generated DNA fragments equivalent to the expected size.

Type IIs cloning strategies for the in-house developed bispecific antibody format

The first aim of this project was to establish effective Type IIs cloning strategies for the bispecific antibody format RUBY™, using a pcDNA3.4 expression vector that would increase antibody production. Cloning of genes encoding the three bispecific antibody chains L1, H2 and H1 into the pcDNA3.4 vector was evaluated at first. Transformed colonies were sent for sequencing and retrieved data aligned against sequences originating from the pAb944/945 vector system. The results indicate that sending three colonies is sufficient to identify a correct clone (Table 4). In this thesis, cloning efficiency refers to the portion of correctly assembled clones out of the clones picked from selective medium and sent for sequencing. Only a few colonies were sequenced per cloning reaction and therefore an exact cloning efficiency could not be determined. With that in mind, no cloning reaction displays an efficiency below 75 %. Conclusively, it can be stated that a viable Type IIs cloning strategy was developed for generating clones in the RUBY™ format.

Table 4. Type IIs cloning of genes encoding the antibody chains L1, H2 and H1 into the pcDNA3.4 vector system. *The table includes the number of transformed colonies required to send for sequencing, in order to identify a correctly assembled clone.*

Plasmid ID	PCR fragment	Destination vector	Number sent for sequencing	Number sequenced	Number passed sequencing
3596	*L1 (*3269)	pcDNA3.4	3	3	3
3598	*H2 (*3310*)*	pcDNA3.4	3	3	2
3599	*H2 (*3312*)*	pcDNA3.4	3	3	3
3601	*H1 (*3356*)*	pcDNA3.4	3	2	2
3602	*H1 (*3358*)*	pcDNA3.4	3	3	2

Prior to Type IIs cloning using a donor vector (Plasmid ID 3614), the vector was generated through assembly of the synthesized Fc domain into a TOPO vector by Zero Blunt TOPO PCR Cloning. Transformed colonies were picked and sent for sequencing, which confirmed the assembly of a correct donor vector (data not shown). An alternative to using a donor vector during Type IIs cloning, would be to utilize the synthesized DNA fragment directly instead. Therefore, two kinds of assembly reactions were performed: one with the donor vector and one with synthesized DNA. After cloning, transformation and sequencing, retrieved data was aligned against H1 vectors of the pAb944/945 system. The results illustrate a cloning efficiency comparable to when using a single PCR fragment (Table 4) during Type IIs cloning, regarding both donor vector and synthesized DNA (Table 5). This suggest that a donor vector can be utilized in future cloning reactions, providing the cloning procedure with a greater flexibility.

Table 5. An overview of Type IIs cloning of the in-house developed bispecific format, using a donor vector and a synthesized DNA fragment. *The table illustrates how two PCR fragments and an Fc domain can be efficiently assembled in a single vector.*

Plasmid ID	PCR fragment 1	Fc domain	PCR fragment 2	Destination vector	Number sent for sequencing	Number sequenced	Number passed sequencing
3601	V_H-C_{H1} (3356)	3614	V_L-Cκ (3356)	pcDNA3.4	3	3	2
3602	V_H-C_{H1} (3358)	3614	V_L-Cκ (3358)	pcDNA3.4	3	3	3
3601	V_H-C_{H1} (3356)	DNA fragment	V_L-Cκ (3356)	pcDNA3.4	3	3	2
3602	V_H-C_{H1} (3358)	DNA fragment	V_L-Cκ (3358)	pcDNA3.4	3	3	3

Antibody production of the RUBY™ format cloned into the pcDNA3.4 vector system

Evaluating a new vector system, utilized for mono- and bispecific antibody production, is of importance to meet the high-throughput demands of a greater plasmid and antibody yield. Consequently, the pcDNA3.4 vector system was evaluated against the pAb944/945 system. Evaluation was performed by producing the two bispecific antibody variants v9 and v10, followed by a comparison of their production yield and protein characteristics.

An initial evaluation aimed to compare the total amount of each bispecific antibody variant produced by two systems, through analysis of harvested supernatants prior to antibody purification. It provides an initial approximation of antibody production and can be used to visualize a trend regarding how much of each antibody that is lost during purification.

A comparison of the antibody yield obtained for each of the two bispecific variants, when produced by pcDNA3.4 against pAb944/945, is presented in Fig. 4. The comparison illustrates how the pcDNA3.4 vector system increases the total production of the two bispecific antibody variants v9 and v10 with approximately 1.5-1.75 times.

Following analysis of the crude supernatant, it is of interest to establish the quantity of each antibody that can be obtained after protein purification. Host proteins produced by the HEK 293 cells must be removed in order to enable characterization of the produced antibodies. A ratio comparing purified amounts obtained from production with the pcDNA3.4 vector system against pAb944/945 indicate that utilizing the new vector for production of v9 and v10 increases antibody yield (Fig. 4).

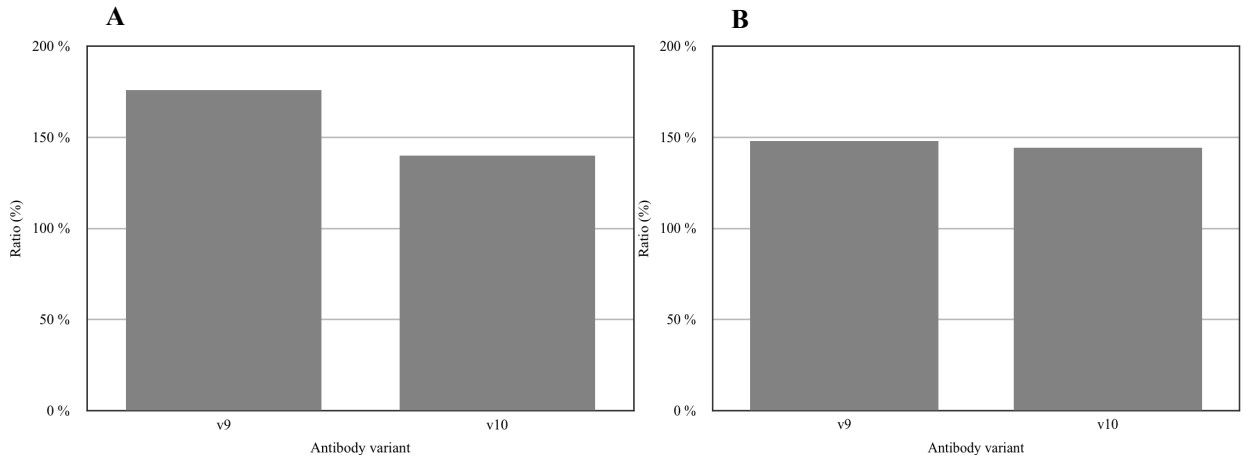

Figure 4. Analysis of bispecific antibody abundance in the supernatants as well as after purification using protein A chromatography. The presented ratio in both figures is an average calculated from two replicated experiments, comparing antibody production with the pcDNA3.4 vector against pAb944/945. A) Octete analysis of antibody abundance in the supernatants, using Protein A biosensors. B) Protein quantitation performed after NGC purification, measured at 280 nm using the Big Lunatic. Results suggest that production of antibodies in the RUBY™ format with the pcDNA3.4 vector system increases antibody yield, compared to using pAb944/945, seen both before and after protein purification.

Level of antibody aggregation after production with the pcDNA3.4 vector system

Apart from generating a sufficient DNA and antibody yield, it is of importance to evaluate antibody production with the pcDNA3.4 vector based on characteristics of the produced antibodies. Fraction analysis of purified antibodies was performed using HPLC, investigating antibody aggregation and protein purity. Antibody production of bispecific antibody variants v9 and v10 with the pcDNA3.4 vector result in a percental aggregation comparable to production with the pAb944/945 vector (Fig. 5). The pcDNA3.4 vector cause less aggregation in the case of variant v9, while the level of aggregation for v10 is similar regardless of vector. If taking both antibody yield and protein purity in consideration, these results suggest that using the pcDNA3.4 vector for production of bispecific antibodies is beneficial for production of antibodies in the RUBY™ format.

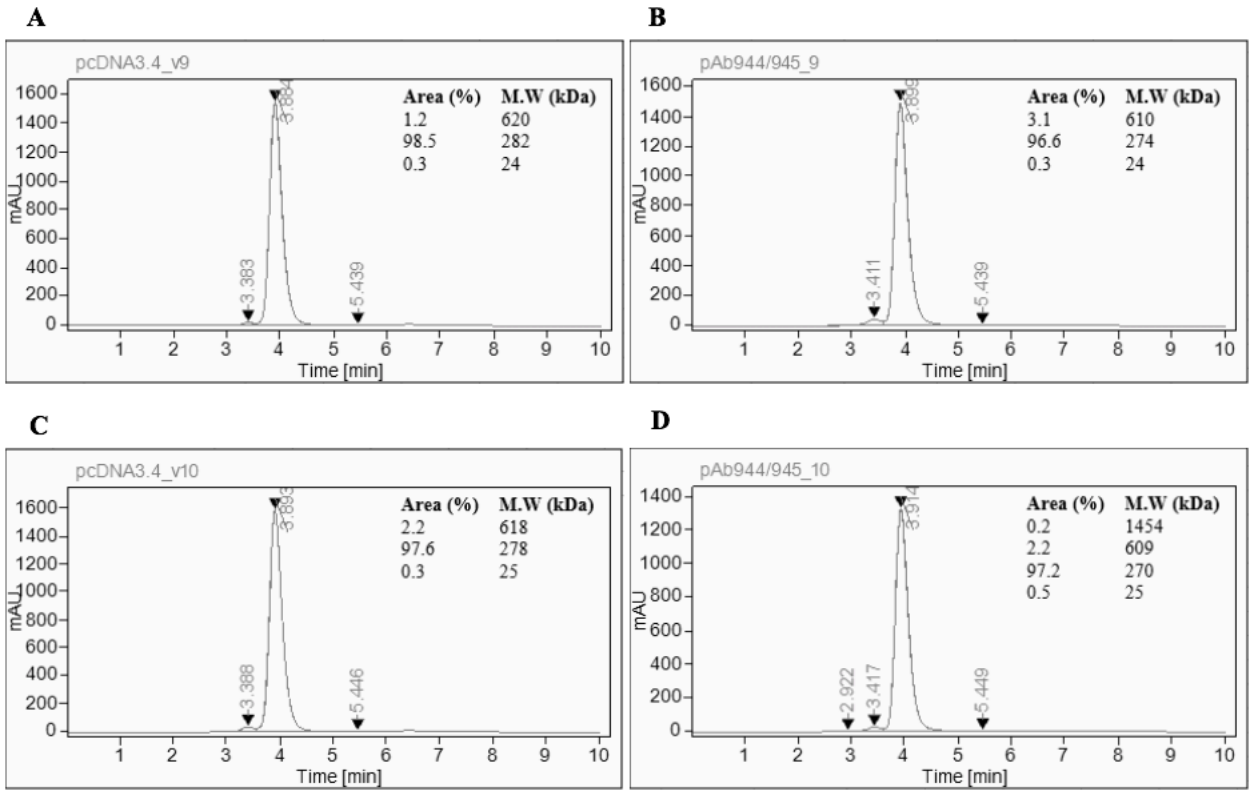

Figure 5. Investigation of purity and aggregation, using HPLC, of two bispecific antibody variants produced by the two vector systems pcDNA3.4 and pAb944/945. Data illustrated was selected from one round of antibody production but is representative of both. A), C) Fractions detected in purified samples of v9 and v10 respectively when produced by the pcDNA3.4 vector. B), D) Fractions detected in purified samples of v9 and v10 respectively when produced by the pAb944/945 vector. Data indicates that bispecific antibody production with the pcDNA3.4 vector system does not have a negative impact on antibody production, illustrated by the peaks present before the large sample peak.

Characterization of bispecific antibodies using SDS-PAGE

During evaluation of the two vector systems, pcDNA3.4 and pAb944/945, it is essential to establish that the evaluated bispecific antibody variants have been assembled and produced correctly. Supernatants, non-reduced purified antibodies and reduced purified antibodies can all be analyzed in order to determine the size of protein fragments present prior to and after purification. It illustrates the variety of host cell proteins removed during purification as well as the size of each antibody chain.
An initial SDS-PAGE analysis after purification indicated that regardless of vector system utilized for antibody production, v9 and v10 appears to have the expected size of approximately 250 kD (Fig. 6A). A protein with the same size can be found in the analyzed supernatants as well, together with a wide range of host cell proteins which have been removed after purification (Fig. 6B). In addition, analysis of reduced antibodies illustrate that the variants are comprised of antibody chains with the correct sizes (Fig. 6C).

In order to confirm the observed results, analysis proceeded by using a LabChip GXII Touch instrument. An instrument generating an additional molecular weight sizing, both as a virtual gel and an electropherogram. If considering the gel, results confirm the observations made after SDS-PAGE for the bispecific antibody variants v9 and v10 (Appendix Fig. 1). The electropherogram of

reduced antibodies demonstrate similar results, assigning each specific antibody chain of both variants produced by the pcDNA3.4 vector system a more precise molecular weight (Appendix Fig. 2A-B). A weight which is identical to when the two variants v9 and v10 are produced by the pAb944/945 vector system (Appendix Fig. 3A-B). These data confirm the previous results from HPLC and SDS-PAGE, that a pcDNA3.4 vector system can be used to produce correct bispecific antibodies of the RUBY™ format. In addition, the result also indicates the importance of protein purification in order to obtain only the desired antibody.

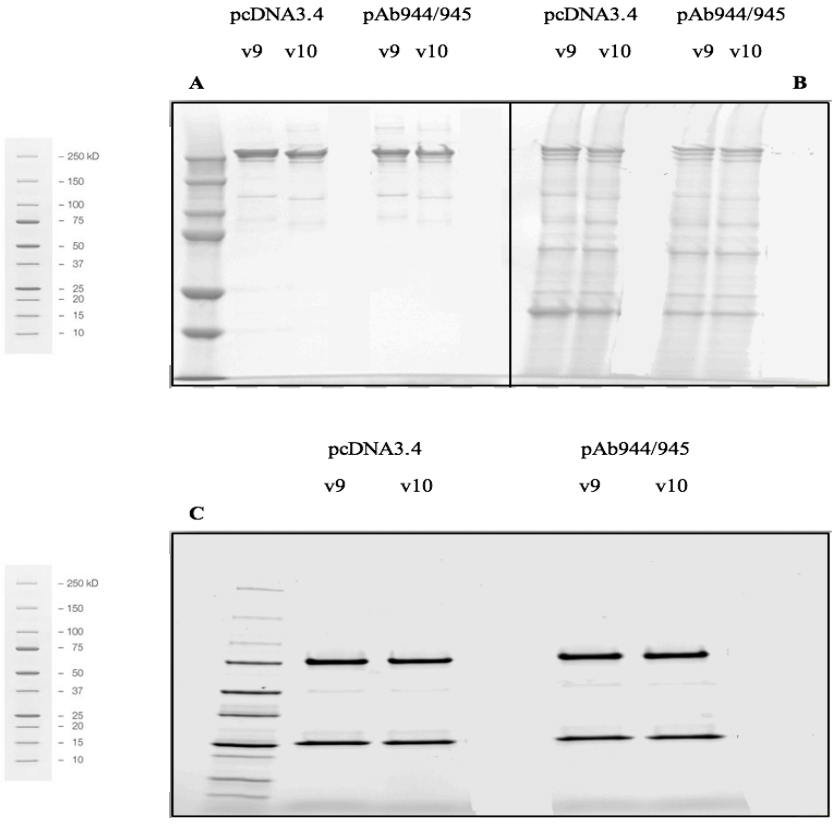

Figure 6. The produced bispecific antibody variants v9 and v10 was characterized by SDS-PAGE, evaluating assembly and production. Data illustrated have been selected from one round of antibody expression but is representative of both. A) Analysis of non-reduced purified antibodies. B) Analysis of non-reduced supernatants. C) Analysis of reduced purified antibodies. Illustrated results indicate that antibodies in the RUBY™ format can be correctly produced by the pcDNA3.4 vector system.

Thermal stability

In order to investigate if antibody production by the pcDNA3.4 vector system generates bispecific antibodies with a different thermal stability, compared to pAb944/945, a T_m analysis of antibody variants v9 and v10 was executed. By exposing the purified antibodies to an increasing temperature, the protein will unfold and the barycentric mean (BCM) will increase. The analysis indicates that utilizing a pcDNA3.4 vector system to produce bispecific antibody variants, does not affect thermal stability of the antibodies (Fig. 7A-B). Illustrated by an almost identical T_m, regardless of vector system used for antibody production.

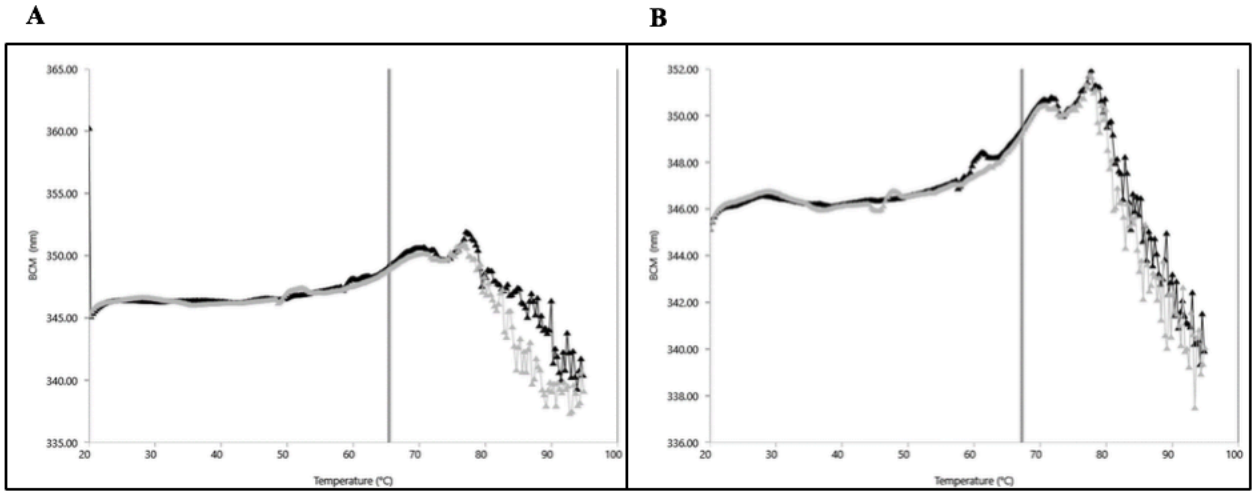

Figure 7. The two bispecific antibody variants v9 and v10 were analyzed based on T_m, in order to investigate if antibody production with the pcDNA3.4 vector system would affect thermal stability. Data illustrated have been selected from one round of antibody expression but is representative of both. The graph illustrates how the barycentric mean (BCM) increase with temperature, as the protein unfolds, during T_m measurements with an UNcle instrument. A) v9 produced by pcDNA3.4 (grey) and pAb944/945 (black). B) v10 produced by pcDNA3.4 (grey) and pAb944/945 (black). Data suggest that bispecific antibody production with the pcDNA3.4 vector system does not compromise thermal stability.

Optimization of bispecific antibody production using the pcDNA3.4 vector system

Previous results from mAbs and other bispecific formats, indicate that the pcDNA3.4 vector system is superior to pAb944/945 regarding antibody production (Fredrika Carlsson, personal communication). Therefore, it is of interest to optimize the original antibody production protocol for bispecific antibody production in the RUBY™ format. This optimization involved investigating the effects of Enhancer 1 and Enhancer 2 on production of bispecific antibody variants v9, v10 and the additional variant v19. An analysis was enabled by adding Enhancer 1 and 2 to identical batches of transfected cells but at two different time points: either during transfection or 18-22 h after.

Adding enhancers (Enhancer 1 and Enhancer 2) at the time of transfection results in a greater total antibody production by the pcDNA3.4 vector system compared to when added the day after (including the mAb control), illustrated in Fig. 8A. As a consequence of a higher antibody concentration in the supernatant, a greater protein yield is obtained after automated purification using NGC as well (Fig. 8B).

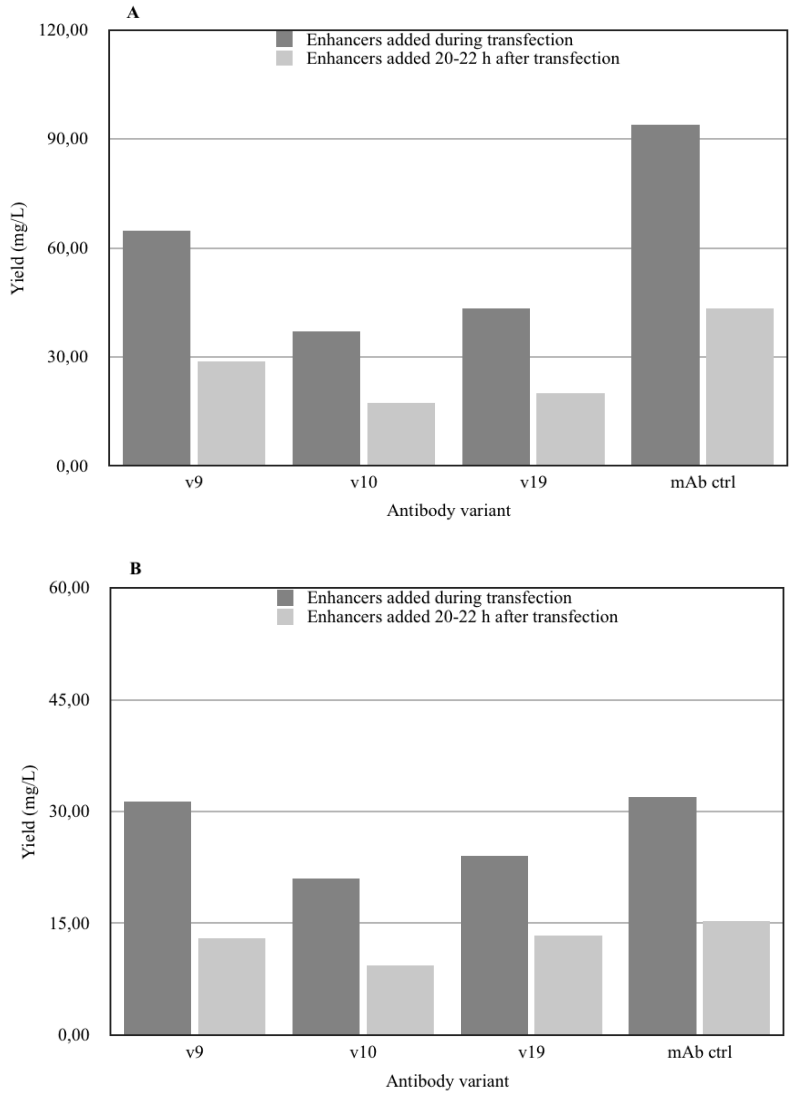

Figure 8. A comparison of antibody production, adding enhancer either during transfection or 18-22 h after. A) Antibody concentration measured in crude supernatants using Protein A biosensors during Octete analysis. B) Antibody concentration measured after NGC purification with the Big Lunatic. Data illustrated have been selected from one round of antibody production with the pcDNA3.4 vector system but is representative of both. The two graphs indicate that adding enhancers during transfection results in the greatest antibody yield.

The effects of adding enhancers post transfection on protein aggregation

Previously displayed data (Fig. 8) suggests that enhancers are important factors during protein production, since they have a great impact on antibody yield. In order to establish their impact on protein purity, the three produced bispecific antibody variants v9, v10 and v19 were analyzed using HPLC. Results indicate that adding enhancers 18-22 h after transfection has no detectable effect on antibody aggregation (Fig. 9). An observation which also applies to production of a mAb, as the control remained unaffected by adding enhancers post transfection.

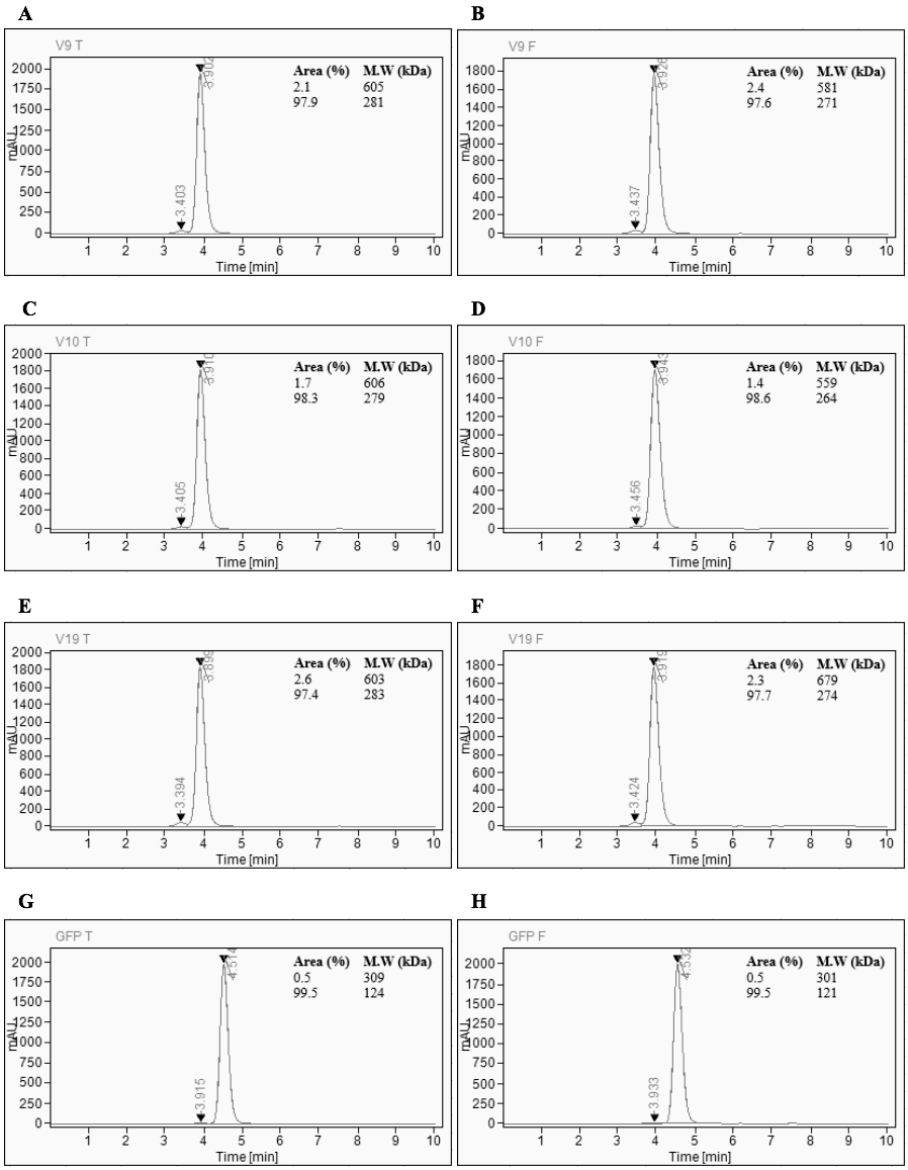

Figure 9. HPLC analysis of the three bispecific antibody variants v9, v10 and v19 produced with the pcDNA3.4 vector system, adding enhancer either during transfection or 18-22 h after. A), C), E), G) Representing v9, v10, v19 and the mAb control respectively, produced with enhancers added at the time of transfection. B), D), F), H) Representing v9, v10, v19 and the mAb control respectively, produced with enhancers added post transfection. Data illustrated have been selected from one round of antibody production with the pcDNA3.4 vector system but is representative of both. The fraction analysis suggests that adding enhancers 18-22 h after transfection does not have an effect on protein aggregation.

Characterization of antibody production with enhancers added post transfection

By analyzing supernatants, non-reduced antibodies and reduced antibodies it is possible to characterize the proteins both before and after purification. Prior to purification, there was a wide range of host cell proteins present in the supernatant of each bispecific antibody variant (Fig. 10A). After NGC purification using Protein A columns, the majority of the proteins present consisted of bispecific antibodies (Fig. 10B). By reducing them, each antibody chain could be distinguished, and their molecular weight illustrated (Fig. 10C). The SDS-PAGE indicates that all three bispecific antibody variants in the RUBY™ format and the mAb control, have been correctly produced in

both cases. This data suggests that adding enhancers 18-22 h after transfection have no impact on antibody assembly. Results which was confirmed by LabChip analysis, both as a virtual gel and an electropherogram (results not shown).

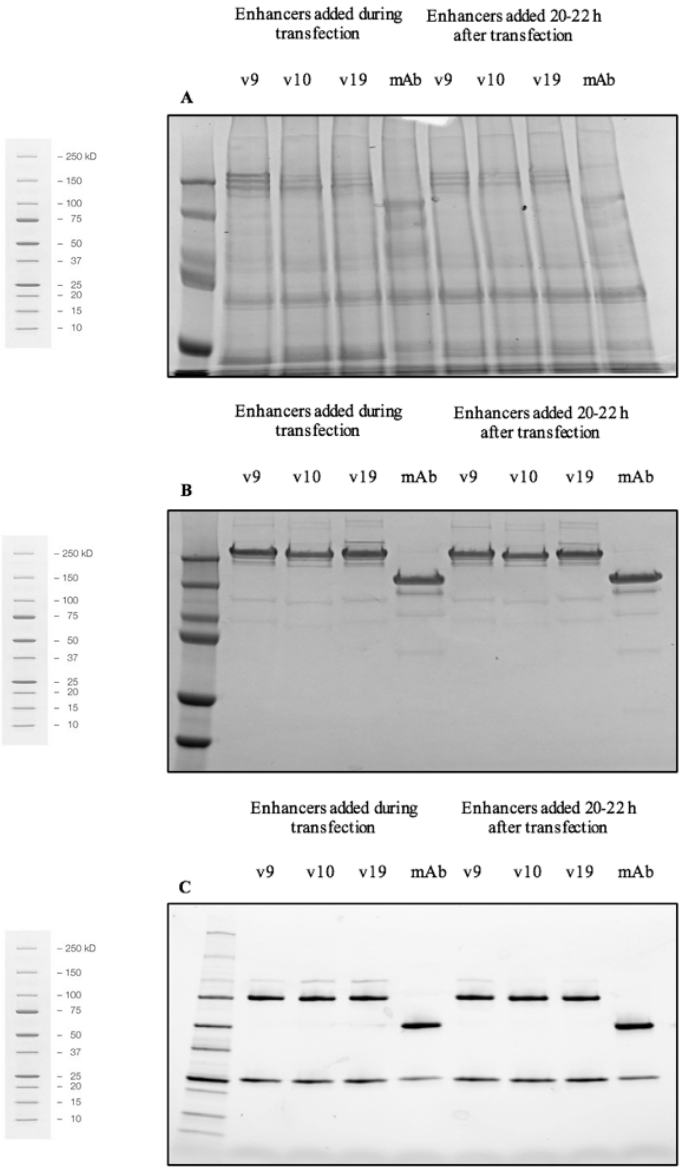

Figure 10. Molecular weight sizing of bispecific antibody variants v9, v10 and v19, produced either with enhancers added during transfection or 18-22 h after. A) Analysis of non-reduced supernatants prior to purification using SDS-PAGE. B) Analysis of non-reduced purified antibodies using SDS-PAGE. C) Analysis of reduced purified antibodies using SDS-PAGE. Data illustrated have been selected from one round of protein characterization, using SDS-PAGE, but is representative of both. The results indicate that adding enhancers 18-22 h after transfection do not affect the production of a correct protein.

Impact of enhancers on thermal stability

Effects of adding enhancers post transfection on thermal stability of the three bispecific variants v9, v10 and v19 was investigated by a T_m analysis. As illustrated in Fig. 11A-D, thermal stability remains unaffected by including enhancers after transfection since the melting temperature is

almost identical between antibody variants (including the mAb control). Concluding that adding enhancers 18-22 h after transfection during production of bispecific antibodies in the RUBY™ format, does not compromise their thermal stability.

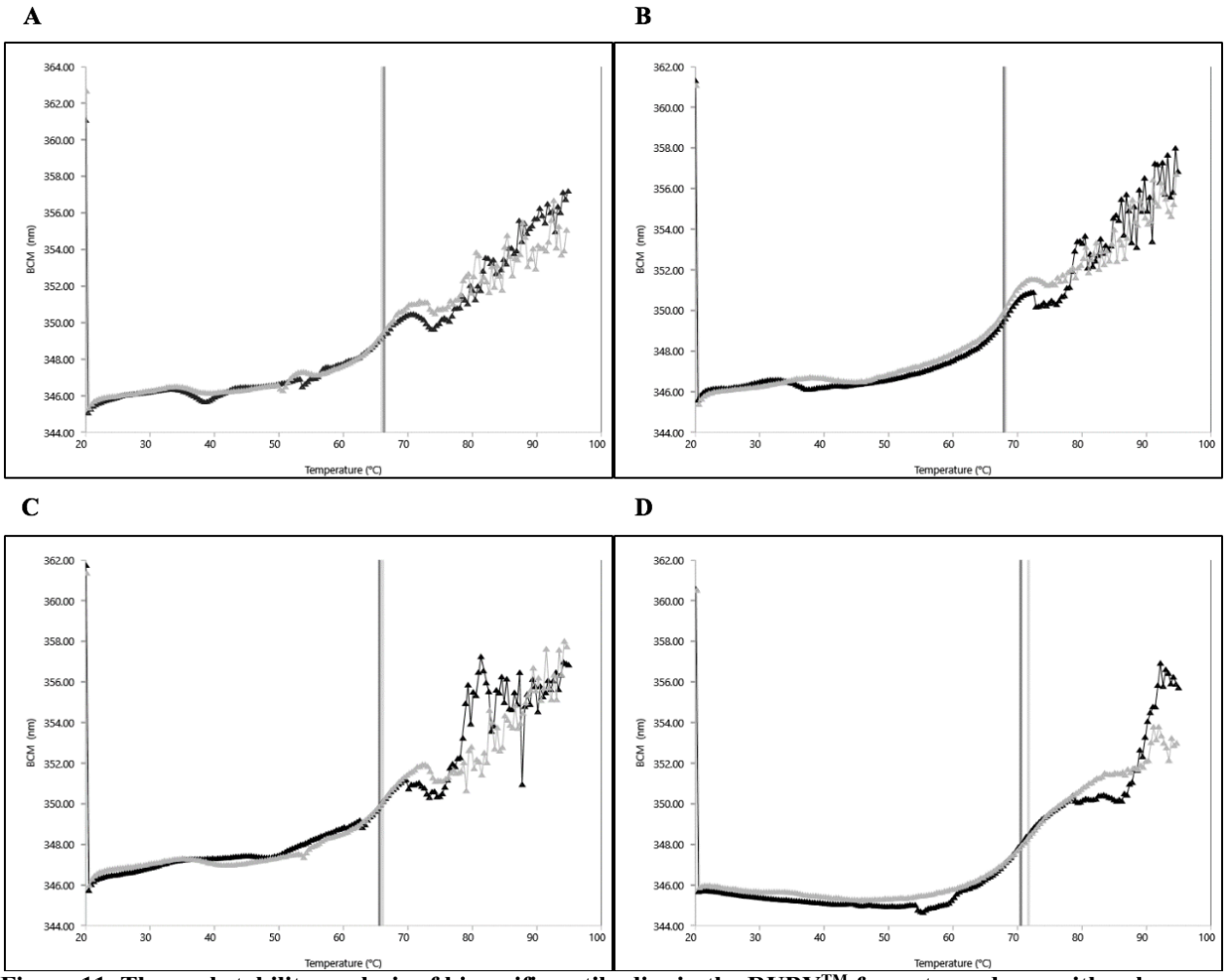

Figure 11. Thermal stability analysis of bispecific antibodies in the RUBY™ format, produces with enhancers added either during or 22-20 h after transfection. A) v9 produced with enhancers added during transfection (black), compared against v9 produced with enhancers added 18-22 h after transfection (grey). B) v10 produced with enhancers added during transfection (black), compared against v10 produced with enhancers added 18-22 h after transfection (grey). C) v19 produced with enhancers added during transfection (black), compared against v19 produced with enhancers added 18-22 h after transfection (grey). D) mAb ctrl produced with enhancers added during transfection (black), compared against mAb ctrl produced with enhancers added 18-22 h after transfection (grey). These results suggest that adding enhancers post transfections has no impact on the thermal stability of the produced bispecific antibody variants. Data illustrated have been selected from one round of antibody production but is representative of both.

Type IIs cloning strategy for cloning in pool

After a Type IIs cloning strategy for the RUBY™ format had been established, using an expression vector that improved antibody production, it was of interest to implement a high-throughput procedure for generating a large quantity of clones in a mAb format. A strategy which can be applied to bispecific antibody production in the future as well, increasing screening efficiency. The procedure involved optimizing a PCR protocol for pool amplification of 32 different V_H fragments

from scFvs, encoded by phagemid vectors, followed by assembly with an Fc region in pool. In total, three different alternatives that generated a large number of clones in the RUBY^{TM} format were investigated: Option 1, Option 2 and Option 3 (Table 3) Option 1 can be considered as a control group, as DNA of 10 V_H fragments were individually purified and amplified by PCR before pool cloning. Option 2 intended to individually purify DNA of the same fragments as in Option 1, but to pool DNA prior to PCR and Type IIs cloning. Option 3 represents the most high-throughput procedure, it was based on pooling glycerol stocks of phagemid vectors followed directly by PCR and cloning in pool. Option 3:1 corresponds to pooling the same 10 phagemid vectors as utilized in Option 1 and 2, while 3:2 refers to a pool of 10 other vectors, 3:3 to 12 other vectors and 3:4 to all 32 vectors combined.

An optimized PCR protocol would be able to amplify V_H fragments both from purified DNA and glycerol stocks, without unspecific amplification. After performing PCR with several different template concentrations as well as cycling parameters, ideal settings were identified. Denaturing was performed at 98 °C for 30 sec, followed by 25 annealing cycles (98 °C for 10 sec, 70 °C for 30 sec and 72 °C for 30 sec) and extension at 72 °C for 7 min. A PCR program which is less time-consuming compared to previously utilized settings. As a result, V_H fragments originating from all three alternatives could be amplified (illustrated in Fig. 12A-B). This indicates that it is now possible to generate V_H fragments from pooled DNA in several different ways, the most high-throughput method being directly from glycerol stocks of 32 different scFv clones.

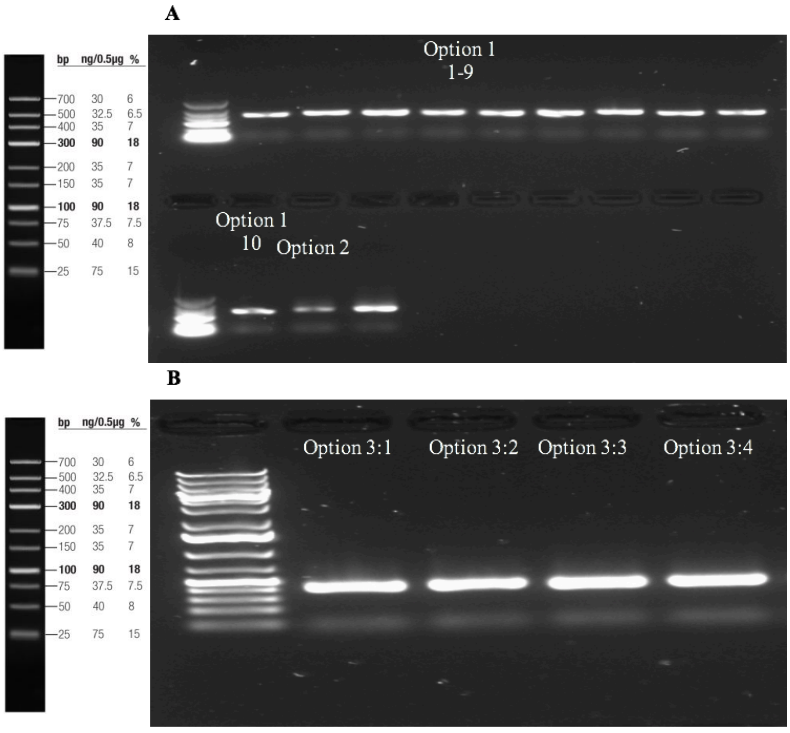

Figure 12. V_H fragments amplified by PCR from scFvs, encoded by phagemid vectors, in order to optimize a PCR protocol for pool amplification. Amplification of three different alternatives were investigated; Option 1, individual DNA purification and amplification; Option 2, individual DNA purification but pooling of fragments before PCR; Option 3, pooling of glycerol stocks followed directly by PCR amplification. A) Representing amplification of Option 1 and Option 2. B) Representing amplification of the four different subcategories of Option 3. Results indicate that it is possible to generate VH fragments from individual clones using all three alternatives, with Option 3 being the most important in order to achieve a high-throughput format.

Type IIs cloning of V_H fragments in pool was performed using a donor vector encoding an Fc region (Plasmid ID 3504), assembling both fragments into the pcDNA3.4 destination vector. In order to evaluate alternatives for efficiently generating clones in the desired format, produced by a vector system that has been shown in this thesis to be beneficial for antibody production. For each alternative, three transformed colonies for each Type IIs cloning reaction were picked and sent for sequencing. The retrieved sequencing data was processed in Anyspeeq and Abspeeq, performing an automated quality control by removing all duplicates and sequences of poor quality, before aligning it against the sequenced glycerol stock from which all V_H fragments were obtained (data not shown). For all options, sending three colonies per expected cloning reaction was enough to identify a majority of the unique clones (Table 3). As for Option 3:4, the most high-throughput procedure, 23 out of 32 V_H fragments pooled from a glycerol stock was successfully assembled. This indicates a high cloning efficiency for all alternatives, even when antibody fragments are generated directly from glycerol stocks. These results indicate that it is now possible to generate a large quantity of clones in the RUBY^{TM} format via Type IIs cloning, for efficient screening of future drug candidates.

Table 6. A summary of Type IIs cloning of antibody fragments in pool, used to create clones in a mAb format. *The table illustrates the expected number of cloning reactions for each option and how many transformed colonies that requires sequencing in order to identify a majority of unique clones. Illustrating that Type IIs pool cloning can be used to efficiently generate clones in the desired format, enabling a high-throughput method for screening future drug candidates.*

Alternative	Type IIs cloning reactions	Transformed colonies sent for sequencing	Unique clones identified
Option 1	10	30	8
Option 2	10	30	9
Option 3:1	10	30	8
Option 3:2	10	30	7
Option 3:3	12	36	10
Option 3:4	32	96	23

Discussion

If considering this project in a broad view, the aim with improving a technological platform for antibody development is to reduce the time it takes from drug discovery to drug approval. Shukla et al. explains how one reason behind the successful development of mAbs during the recent years, is the use of technological platforms. Platforms using stable expression systems based on mammalian cells, with optimized expression vectors and purification processes such as Protein A affinity chromatography [13]. An approach which have been adapted for bispecific antibody development at Alligator Bioscience, presented and utilized throughout this project, strengthened by the new RUBY^{TM} concept. An in-house developed concept, that can be utilized to generate bispecific antibodies from mAbs (or others such as Fabs or scFvs) and allowing for screening of drug candidates in their final format.

In order to utilize the full potential of the RUBY^{TM} format, cloning techniques have to be efficient and high-throughput compatible. Previously used cloning procedures involved restriction enzyme digestion in order to generate expression vectors, a method which is not compatible with a high-throughput workflow and does not achieve a high cloning efficiency. Therefore, it was of interest to implement Type IIs cloning strategies for generation of bispecific antibodies in the RUBY^{TM} format. Strategies involved utilizing both DNA fragments as well as a donor vector, together with a newly designed pcDNA3.4 vector system in order to increase antibody production. The pcDNA3.4 vector system was evaluated against the previously used pAb944/945 system and a bispecific antibody production protocol was implemented.

This project has illustrated that Type IIs cloning is a fast and simple way of assembling DNA fragments into a destination vector. Previously utilized cloning techniques at Alligator Bioscience have required sequencing around a dozen of transformed colonies per clone to achieve an acceptable cloning efficiency. In comparison, sending three colonies per clone after Type IIs cloning was sufficient. Even if an exact cloning efficiency was not established during this project, as sending only three colonies gives a skewed result, the trend indicates that a desirable efficiency of 80 % could be achieved after more extensive evaluation. In addition, Type IIs cloning strategies have the advantage of being compatible with a donor vector. As explained by Festa [14], a donor vector removes the need for continuous PCR amplification of a gene since it only has to be constructed once. Further, a donor vector provides flexibility when evaluating antibodies in the discovery/development phase. For instance, Fc isotypes can be cloned from various donor vectors into a destination vector to evaluate bispecific or monoclonal antibodies with different properties. In a similar fashion, this project combined an Fc region with V_H-C_{H1} and V_L-Cκ fragments in order to assemble a H1 fragment of the RUBY^{TM} format. The same theory applies to synthesized DNA fragments, however it has the major drawback of being depleted over time.

Production of bispecific antibody variants in the RUBY^{TM} format using the pcDNA3.4 vector system, resulted in a greater yield compared to using pAb944/945. Investigating the effects of enhancers on antibody yield was an attempt to further optimize protein production. In the user guide provided by Thermo Fisher Scientific for Expi293 Expression Systems, it is stated that enhancers should be added 18-22 h after transfection in order to obtain the greatest antibody yield [15]. However, results presented during this project did not indicate any improvements compared when adding them at the time of transfection. Instead, adding enhancers post transfection gave a considerably lower yield compared to addition at the time of transfection. An additional aspect that should be considered during future evaluations of bispecific antibody production in the RUBY^{TM} format, is the antibody chain ratios applied during transfection. The current protocol utilizes a 1:2:1

ratio for L1:H2:H1, which is based on antibody production with the pAb944/945 vector system. Previous experiments at Alligator Bioscience have illustrated that the ratio between heavy and light chains have can have an impact on antibody yield and aggregation. Taken together, this indicates that the currently utilized expression method requires further investigation and optimization.

Apart from evaluating the pcDNA3.4 vector based on antibody yield, it was of importance to consider characteristics of the produced proteins. For example, a well-established problem seen during antibody development, is the occurrence of protein aggregation. It affects antibody efficacy, quality and safety, and can be reduced by selecting a beneficial expression system [16]. The previously used pAb944/945 vector system achieved an acceptable level of aggregation, with monomeric fractions above the threshold of 95 %. As presented in this thesis, the level of aggregation remained similar after antibody production with the pcDNA3.4 vector system as well. Another desirable trait when designing production systems for antibodies, is to generate proteins with a high thermostability, as it improves both manufacturing and storage [17]. pAb944/945 produced thermally stable antibodies but pcDNA3.4 had to be evaluated in order to establish that the trait was not jeopardized. The results emphasize the total benefit of utilizing the pcDNA3.4 vector system for production of bispecific antibodies in the RUBY™ format, instead of pAb944/945, as aggregation decreased while a high thermal stability was maintained.

In summary, it can be concluded that 1) Type IIs cloning can be utilized to effectively generate clones in the RUBY™ format; 2) DNA fragments amplified by PCR, synthesized DNA and a donor can all be utilized during Type IIs cloning; 3) The pcDNA3.4 vector system is beneficial when it comes to antibody production of the in-house developed bispecific antibody format, compared to the pAb944/945 vector system, increasing protein yield and decreasing aggregation; 4) Enhancer 1 and Enhancer 2 should preferably be added during transfection, in order to obtain the greatest antibody yield; 5) Antibody fragments can be amplified by PCR directly from pooled glycerol stocks of scFvs; 6) Type IIs cloning in pool can be used to generate a large number of clones in a mAb format, allowing for future high-throughput screening of potential drug candidates.

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Assets%2FLSG%2Fmanuals%2FMAN0007814_Expi293ExpressionSystem_UG_2018.pdf&title=VXNlciBHdWlkZTogRXhwaTI5MyBFeHByZXNzaW9uIFN5c3RlbQ== (accessed May 31 2019).
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Appendix

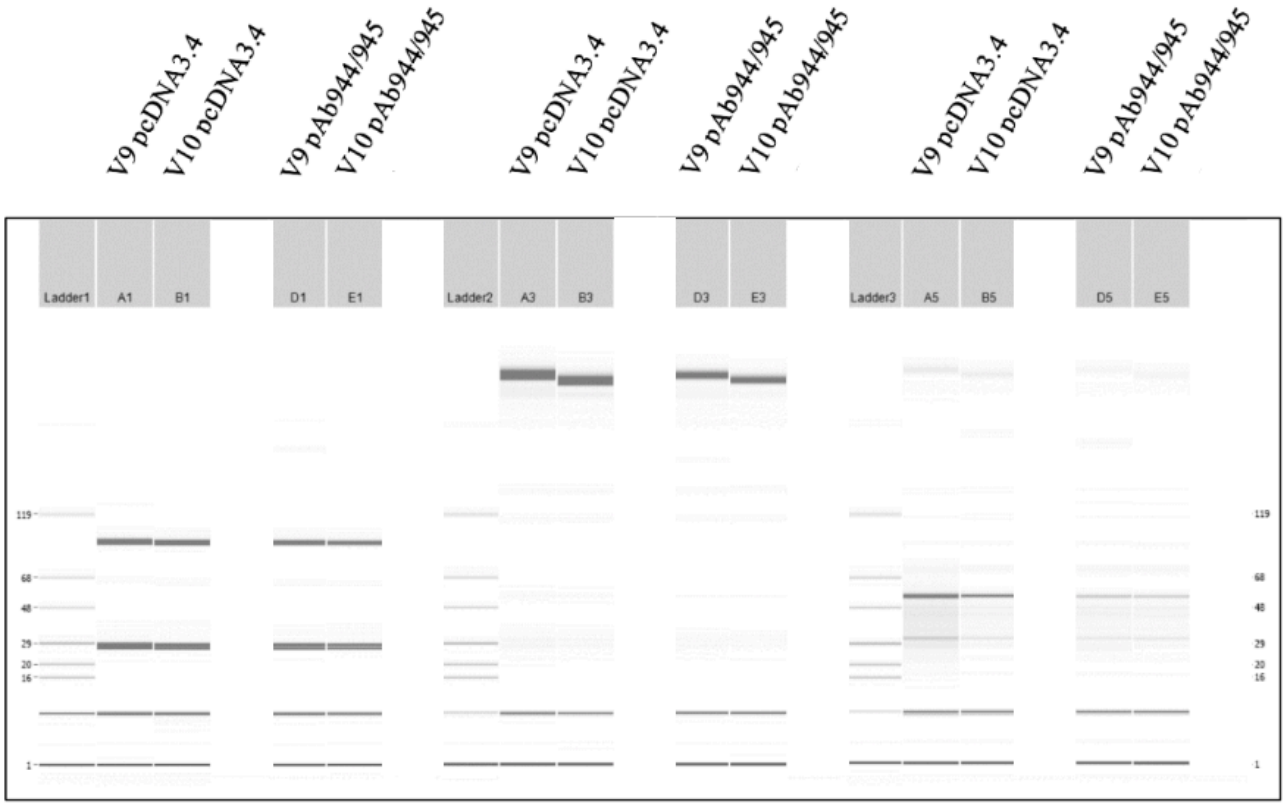
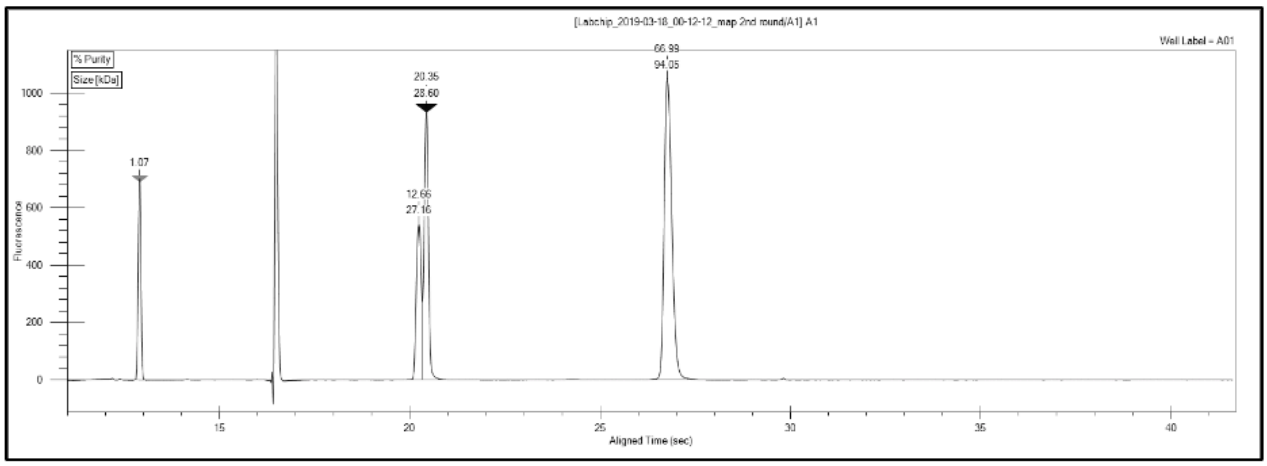


Figure 1. Protein characterization of bispecific antibodies produced by the two vector systems pcDNA3.4 and pAb944/945, through LabChip GXII analysis. Data illustrated have been selected from one round of antibody expression but is representative of both. Reduced purified antibodies, non-reduced purified antibodies and non-reduced supernatants were analyzed, displayed on the virtual gel in said order. The bispecific antibody variants v9 and v10 were correctly produced by both vector systems, as both intact proteins and individual antibody chains correspond to the expected size.

A



B

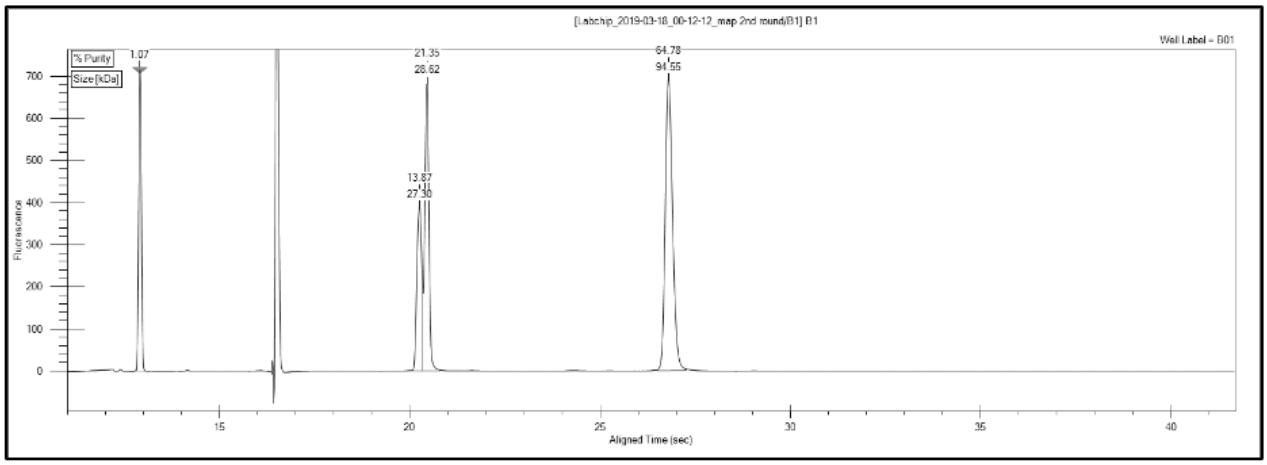


Figure 2. Molecular weight sizing of reduced bispecific antibody variants v9, and v10 produced by the pcDNA3.4 vector system, using a LabChip GXII Touch instrument. Data illustrated have been selected from one round of antibody production but is representative of both. The first peaks represent a low marker and the second a system peak, followed by the sample peak. A) Analysis of v9, the three peaks representing L1, H2 and H1 respectively. B) Analysis of v10, the three peaks representing L1, H2 and H1 respectively. Results indicating that the pcDNA3.4 vector system have produced the two bispecific antibody variants correctly.

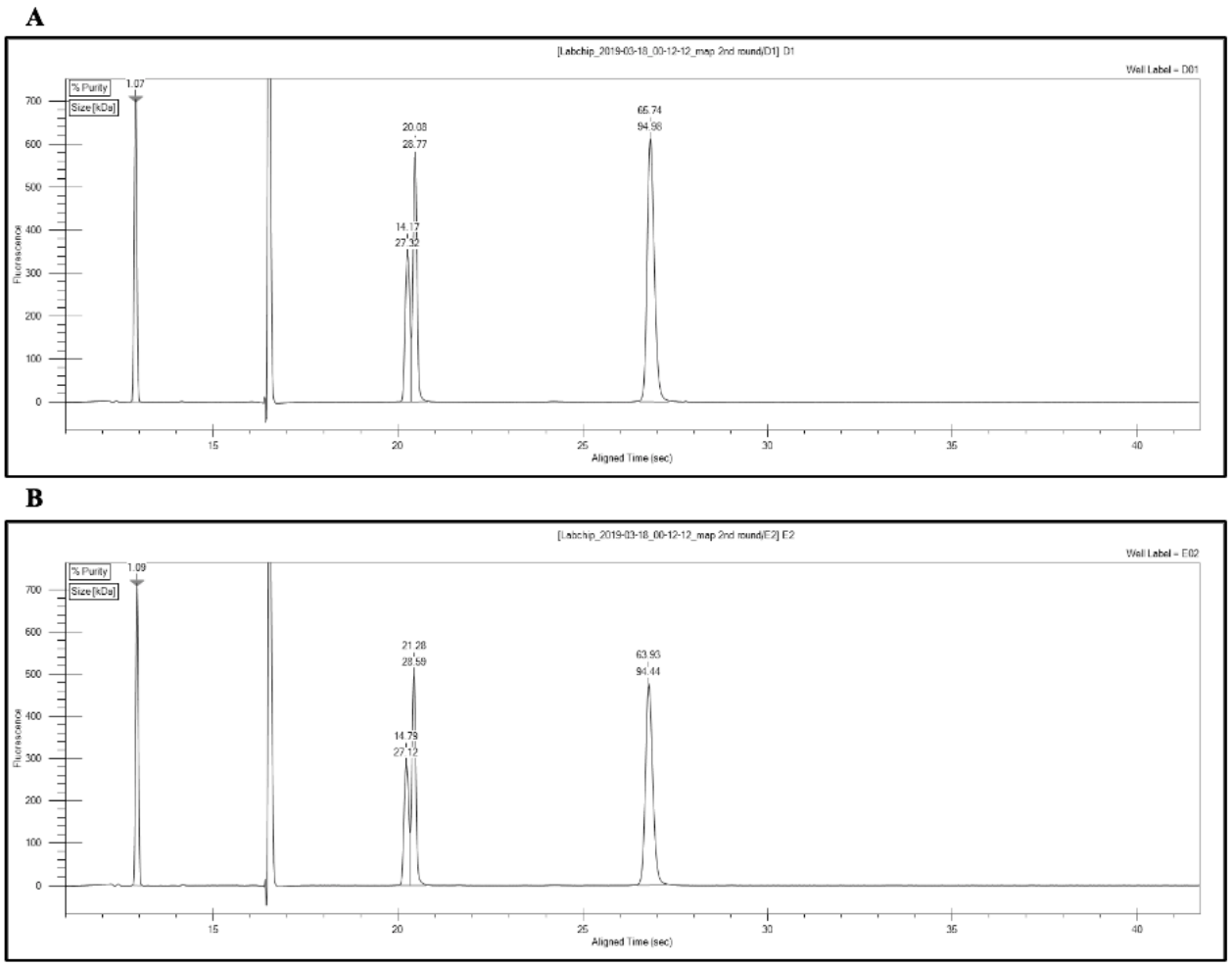


Figure 3. Molecular weight sizing of reduced bispecific antibody variants v9 and v10, produced by the pAb944/945 vector system, using a LabChip GXII Touch instrument. Data illustrated have been selected from one round of antibody production but is representative of both. The first peaks represent a low marker and the second a system peak, followed by the sample peak. A) Analysis of v9, the three peaks representing L1, H2 and H1 respectively. B) Analysis of v10, the three peaks representing L1, H2 and H1 respectively. All three antibody variants have been produced correctly by the pAb944/945 vector system.