Development of a high-throughput antibody engineering and expression profiling platform – Cellective BioTherapy case study

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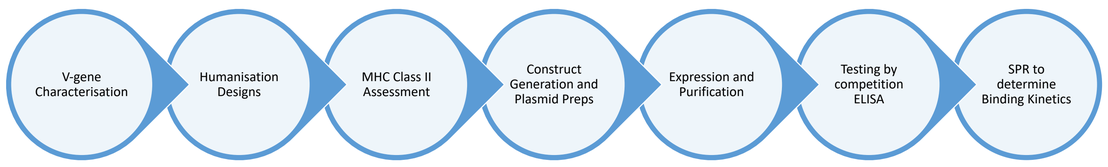
Introduction

A broad range of Antibody Discovery methodologies are employed to establish novel therapeutic biomolecules – while next generation technologies such as yeast or mammalian display are becoming increasingly widespread, more established methods including phage display and mouse-immunization remain common. In the case of the latter approaches antibodies are discovered with favourable target binding properties, but are 'non-human' by nature, and therefore are optimised by subcloning into pre-validated mAb frameworks, 'humanization' and immunogenicity assessment. This process is carried out using a mammalian expression system to ensure associated validation is as consistent as possible with the final manufacturing platform.

Here, we present data on Oxford Genetics proprietary expression system, comprising optimised mAb constant region vectors, automated HEK-based expression platform, as well as a custom-developed humanization algorithm. The below data is presented in the context of a case-study carried out with Cellective BioTherapy, who are establishing B-cell targeting antibodies.



Variable Region Sequence Confirmation

Reverse transcription and PCR from first strand synthesis were performed using total hybridoma RNA from Cellective. Amplicons were generated using multiple degenerate primers designed against different murine signal peptides and constant domains. The amplicons corresponding in size to the murine variable domains were submitted for Sanger Sequencing (Fig. 1), the traces from which confirmed the sequence identity of the variable domains as previously characterised by Cellective.

Figure 1. Sequence trace data for portion of CBT-103 kappa variable domain aligned against the anticipated sequence template.

Humanization using Oxford Genetics Proprietary Algorithm

Oxford Genetics proprietary algorithm was used to identify the CDRs (Fig. 2) and select human framework regions (FRs) closely resembling the murine frameworks in question. The algorithm replaces in silico murine FR amino acids sequences with closely related human FRs. Vernier Zone residues and those flanking the CDRs are selectively backmutated to the original murine residues. Between 4 and 6 of the top scoring variable regions sequences were picked for each light and heavy chain V region.

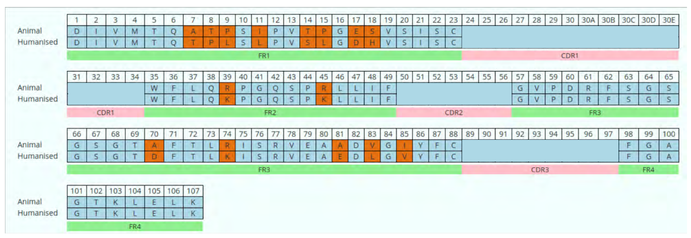


Figure 2. Oxford Genetic's proprietary humanization algorithm. The algorithm predicts the CDR definitions from an amino acid sequence and finds human FR regions of close similarity to murine the murine FRs.

Plasmid design for transient antibody expression in HEK293-EBNA cell line

Oxford Genetics have created a comprehensive standardised vector toolkit for appending antibody variable regions to any chosen constant region (Fig. 3) The vectors facilitate transient expression in Oxford Genetics HEK293-EBNA cell line. Variable regions from Cellective were synthesised and subcloned into vectors for the addition of the constant domain of human IgG4, with hinge stabilising mutations to the murine heavy chain variable regions, and the addition of the constant domain of human IgK to the murine light chain variable regions (Fig. 4). Plasmids were prepared for transfections in a high-throughput parallel manner at MIDI scale using a Wizard® MagneSil® Plasmid Purification System on the Hamilton Star robot (Fig. 5).

	IgG1(CH)	IgG2(CH)	Ig2a(CH)	Ig2b(CH)	Ig2c(CH)	IgG3(CH)	IgG4(CH)	IgA1(CH)	IgA2(CH)	IgE(CH)	IgM(CH)	IgK(CL)	Igl(CL)
Human constant region	P01857	P01859				P01860	P01861	P01876	P01877	P01854	P01871	P01834	P0CG04
Mouse constant region	P01868		P01863	P01867-2	NG_005838.1 GeneID404712	P03987-2		P01878		P06336	P01872	P01837	P01843
Rabbit constant region	P01870										P03988	DQ402473.1	P01847
Rat constant region	P20759.1		P20760	P20761	P20762							P01836	P20766
Macaque constant region	AF045537.1											AF050635.1	HQ214073.1
Sheep constant region	X69797.1											AY734683.1	AY734681.1
Goat constant region													AY835588

Figure 4. Plasmid map for Cellective CBT-103 variable region appended to human constant regions for IgG4 with hinge stabilising amino acid substitutions.

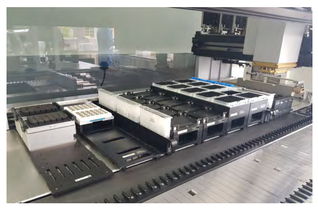


Figure 5. High Throughput MIDI prep platform using Magnetic beads on Hamilton Star Robot.

Transient Expression of Chimeric and Humanized Antibody Variants

Pairs of heavy and light humanized constructs were used to transfect 50 mL cultures of HEK293-EBNA cells using FectoPro and grown in BalanCD media. The Epstein Barr virus EBNA protein, stably expressed by HEK293-EBNA cells associates with EBV OriP elements present in the vector backbones, facilitating effective partitioning of the plasmid in daughter cells following cell division, enabling longer expression times and higher antibody titres from transient expressions. Figure 6 shows the antibody titres in raw culture at day 7 post transfection. Transient expression enables rapid parallel production of multiple antibody variants in sufficient quantities to perform subsequent binding analysis.

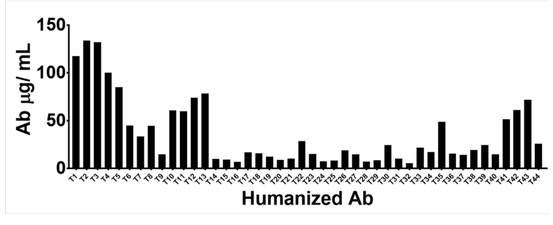


Figure 6. Expression Levels tested by HPLC or Quantitative ELISA for all 44 humanized antibody variants.

High Throughput Purification of Variants with Magne™ Protein A Beads

In total, 44 humanized antibody variants were purified directly from unclarified culture supernatant in a high-throughput manner using Promega Magne™ Protein A Beads in the HSM 2.0 Instrument (Fig. 7). Antibody concentrations were determined by A_{280} nm (Fig. 8). Lead candidates were purified using an AKTA Pure with a 1 mL HiTrap Mab Select SuRe column. The purified antibodies were eluted using 200 mM Sodium Glycine and were buffer exchanged into PBS pH 7.4 using ThermoFisher Slide-A-Lyzer™ Dialysis Cassettes or Zeba™ Spin Desalting Columns. The lead candidates were analysed by SDS PAGE (Fig. 9).

Figure 7. Heater Shaker Magnet (HSM)

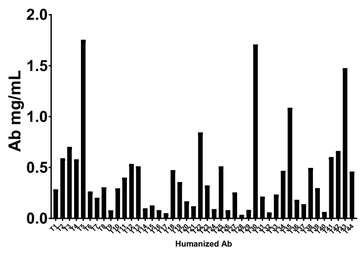


Figure 8. Antibody Yields Following HSM Purification and Buffer Exchange

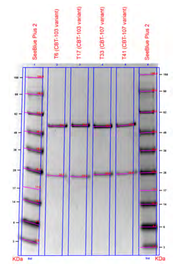


Figure 9. SDS-PAGE Gels for lead candidates

Screening by Competition ELISA

The humanized antibody variants were tested for their ability to compete for binding of the antigen against their biotinylated murine equivalents in a competition ELISA (Fig. 10). Alkaline Phospatase conjugated streptavidin followed by P_NPP treatment enabled detected of bound biotinylated antibody by A_{405} nm on a BMG Fluroscan plate reader. A reduction in signal was seen at higher concentrations of test antibodies able to compete for antigen binding. IC_{50} values were calculated from the straight line portion of each plot and relative binding efficiencies were calculated with respect to the chimeric or murine equivalents.

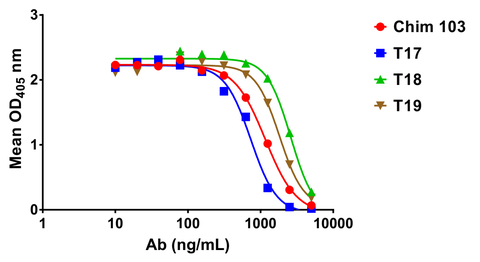


Figure 10. 4-parameter fit for 3 test antibodies (blue, brown and green) versus chimeric antibody (red)

MHC Class II Binding Prediction

Oxford Genetics used matrices described by Sturniolo et al. (1999) to generate software enabling the identification of MHC Class II HLA-DRB1 allele binding nanomers (overlapping 9-amino acid peptides constituting binding epitopes) within each humanized antibody variable domain. The software excluded nanomers corresponding to human Ab germline sequences, and enabled the prediction of promiscuity (the number of alleles binding each nanomer). DRB1 proteins display the peptides on the cell surface forming CD4+ T-cell epitopes, so promiscuous nanomers are considered particularly immunogenic. Humanization of Cellective CBT-103 and CBT-107 resulted in fewer immunogenic nanomers, as (see Fig. 11) showing the predicted nanomer positions and promiscuities for murine CBT-103 V_H and one of the humanized CBT-103 V_H sequences.

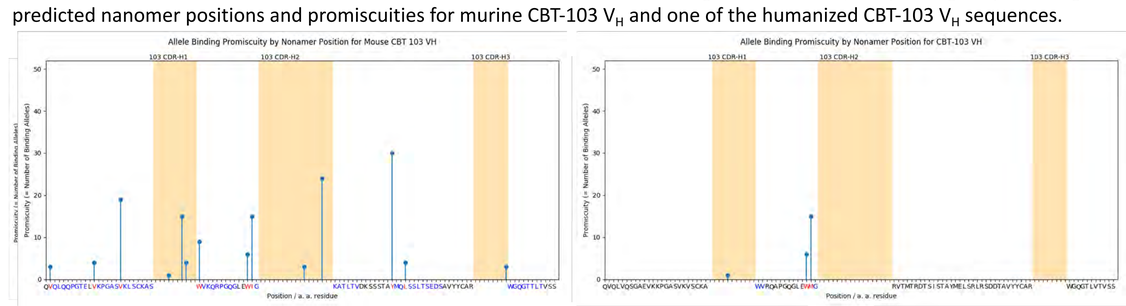


Figure 11. Nanomer binding position (by first amino acid (red) in each nanomer (blue)) is plotted on the X-axis, and the promiscuity of each nanomer for DRB1 alleles is plotted on the Y-axis. Murine CBT-103 V_H (left) is seen to possess greater numbers of nanomers than humanized CBT-103 V_H (right) indicating that humanization has significantly reduced the potential immunogenicity of this variable region.

SPR for Lead Candidates

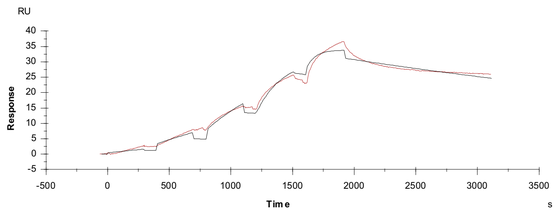


Figure 12. Sensogram for variant T17 humanized Ab binding

	ka (1/Ms)	kd (1/s)	KD (M)
Antibody	Mean	Mean	Mean
T17	1.54E+05	1.84E-04	1.21E-09
T33	9.57E+03	1.75E-04	1.83E-08
T41	9.68E+03	1.63E-04	1.69E-08
T6	2.13E+04	6.31E-04	2.95E-08

Figure 13. Kinetic constants for the lead antibody candidates

Surface Plasmon Resonance (SPR) was performed for the four lead Ab candidates based on binding data from the competition ELISA assays, and upon the MHC Class II Assessment for immunogenicity. The candidate antibodies were immobilised to the Biacore chip with the target antigen as the analyte flowed over the chip. An SPR Sensogram for T17, one of the lead humanized antibody candidates, is shown in figure 12. The coloured plot represents the measured data in RU (Response Units) indicating antibody-antigen complex formation and dissociation with time. The black plot is the predicted binding model with respect to the data. In this example, the data is a good fit for a 1-to-1 antibody-antigen binding model. Figure 13 shows the measured kinetic constants for the four lead antibodies, which all have KD values in the low nM range. Additionally, all the lead antibodies display low (Kd) dissociation rates, suggesting that they bind tightly to the target antigen. Some antibodies displayed biphasic binding, best described by a 2-state binding model, which could potentially be explained by the presence of a secondary binding site within the antigen. T17 was the preferred candidate, with the lowest KD, the fastest Ka (association rate), and a good fit for a 1-to-1 binding model.

Summary and Outlook

- Oxford Genetics has utilised its proprietary humanization algorithm to design a series of 44 humanized antibodies for Cellective's CBT-103 and CBT-107 mouse antibodies.
- Antibodies were cloned, expressed and purified on Oxford Genetics unique High Throughput Antibody production platform and tested for their ability to bind the Antigen in competition ELISA assays. Lead candidates were selected for both parent antibodies, based upon binding data from the ELISA assays and by using Oxford Genetics proprietary MHC Class II assessment software to scan the antibodies for CD4+ T-Cell epitopes and provide a measure of their likely immunogenicity.
- Lead humanized antibody candidates with comparable IC_{50} values to the parent antibodies were purified on the AKTA Pure and were tested by SPR to determine the kinetic constants for these antibodies. SPR revealed that all four lead antibodies possess KD (dissociation constant) values in the low nM range with low Kd (dissociation rate), indicating that they all bind tightly to the antigen.
- Oxford Genetics is further automating its expression platform to increase its automated production and characterisation capacity to >96 mAbs/week.