

Thesis for the Master's degree in Molecular Biosciences

Affinity maturation of a T cell receptor by use of phage display

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60 study points



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Abbreviations

Amp	Ampicillin
APC	Antigen presenting cell
BCR	B cell receptor
BSA	Bovine serum albumin
C	Constant
CD	Celiac disease
CDR	Complementarity determining region
Cfu	Colony forming unit
DC	Dendritic cell
DNA	Deoxyribonucleic acid
E. coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
EF	Enrichment factor
ELISA	Enzyme linked immunosorbent assay
Fab	Fragment antigen binding
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
Ig	Immunoglobulin
Kan	Kanamycin
MHC	Major histocompatibility complex
MOI	Multiplicity of infection
NA	NeutrAvidin
NIP	5-iodo-4-hydroxy-3-nitro-phenacety
OD	Optical density
ON	Over night
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
pL	Protein L
pMHC	Peptide MHC
POI	Protein of interest
Rpm	Rounds per minute

RT	Room temperature
scTCR	Single chain TCR
scFv	Single chain fragment variable
ssDNA	Single-stranded DNA
TAG	Tetracycline/Ampicillin/Glucose
TEA	Triethylamine
Tet	Tetracycline
TCR	T cell receptor
TG2	Transglutaminase2
TMB	3,3',5,5'-Tetramethylbenzidine
V	Variable
Wt	Wild type

Abstract

Celiac disease (CD) is characterized by an abnormal immune response that affects the small intestine of humans. While the cause and origin of the disease is unknown, the onset of the pathology is clearly associated with ingestion of wheat gluten, barley and rye, and susceptibility is strongly associated with certain human leukocyte antigen (HLA) molecules. More than 90% of the patients express HLA-DQ2.5 and most of the remaining express HLA-DQ8. Furthermore, gluten-specific CD4⁺ T cells are mainly reactive to either of two immunodominant gluten peptides, gliadin- α 1a or gliadin- α 2, in the context of HLA-DQ2.5. The ability to detect and characterize this dominant peptide-major histocompatibility complex (pMHC) presentation on cells would allow for studies of cause and origin of the disease. The aim of this thesis has therefore been to develop a detection reagent potentially serving this purpose. The T cell receptor (TCR) has evolved to recognize pMHC complexes specifically and thus represents the ideal agent for their targeting. In order to employ TCRs as trackers of antigen presentation, however, the affinity has to be improved.

As starting point, a stabilized single chain TCR (scTCR) composed of the variable domains of a HLA-DQ2.5-gliadin- α 1a specific TCR was displayed on filamentous phage as a fusion to the phage coat protein pIX. The complementarity determining region (CDR)3 loops of the two variable domains were then randomized to create phage libraries. Furthermore, the resulting phage libraries were selected on recombinant HLA-DQ2.5-gliadin- α 1a, through four rounds, to enrich for pMHC specific clones. Since a naïve single chain fragment variable (scFv) phage library had been panned against the same target in the laboratory, the selection was continuously monitored for presences of scFv phage contamination as well as for scTCR clones using PCR. scTCR phage was detected throughout the selection rounds, and as of the second round, scFv phage was detected in some of the phage libraries. In the third and second selection rounds, the phage libraries were pre-selected on protein L (pL) to counteract scFv phage propagation.

Following selection, the phage libraries were analyzed in ELISA for binding to HLA-DQ2.5-gliadin- α 1a. When phage packaging was performed with M13K07 helper phage rescue, 14 of 22 libraries showed reactivity. Subsequent single clone phage screening did not identify any binders and sequencing proved that a substantial part of the library comprised scFv clones.

Further investigation revealed that the scFv phage was inefficiently produced upon DeltaPhage rescue. This was exploited to knock down the ELISA signal caused by the scFv clone. Indeed, when phage library rescue was performed with the DeltaPhage, only three of the 22 libraries showed reactivity. Single clone phage screening of these libraries identified two candidate clones, which turned out to be scFv clones after all.

1. Introduction

1.1 Phage display technology

1.1.1 Phage display

Phage display was first described by Smith [4] and has proven to be a powerful method for selection of peptides and folded domains with desired binding specificities [5]. Phage display is a term that translates into display of foreign peptides or polypeptides on the surface of a phage particle. The filamentous phage M13 consists of five structural proteins that coat a single-stranded DNA (ssDNA) molecule. The major coat protein pVIII is the most abundant, covering the length of the phage particle. Hence, the number of units of pVIII in a virion is dependent on genome size. The phage particle is capped at one end by 3-5 copies of the minor coat proteins pIII and pVI, and at the other end by pVII and pIX (Figure 1).

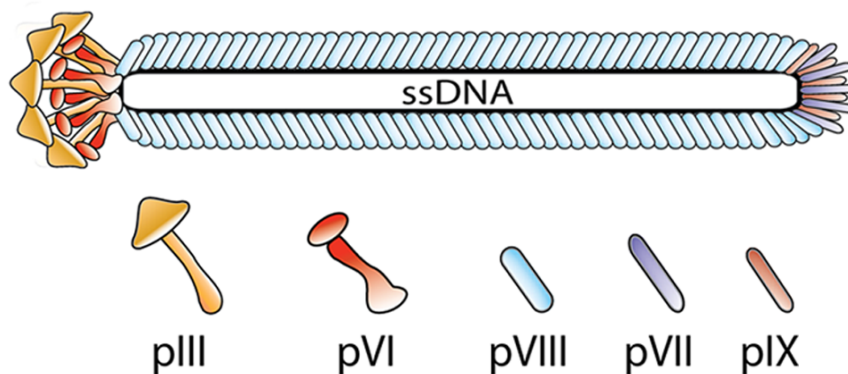


Figure 1: Schematic presentation of the filamentous phage. Five structural proteins coat the ssDNA genome. The figure is adapted from [6].

The filamentous phage can infect *Escherichia coli* (*E. coli*) bacteria that express surface F pili. Phage infection occurs through binding of the pIII on the phage to the F pilus on the bacteria and translocation through the TolA receptor complex in the bacterial membrane [7, 8]. The filamentous phages replicate and assemble with a non-lytic life cycle [9]. All capsid proteins are located in the inner membrane prior to virion assembly [10], but only pIII, pVI and pVIII are synthesized as precursors with classical N-terminal signal sequences. pVII and pIX appear to be synthesized without such signal peptides and their mechanism of translocation to the membrane is still incompletely understood, but at least pIX may depend on the YidC translocon as recently described [11-13].

Phage display can be accomplished by e.g. subcloning the gene encoding a protein of interest (POI) in-frame to the gene encoding a capsid protein. The POI displayed on the phage is thus

encoded in the ssDNA genome harbored by the same phage particle. This provides a physical link between the phenotype and genotype, which is a key feature of the selection platform. pIII and pVIII display are referred to as the conventional phage display formats, being the most commonly used display capsids. However, POI have been fused to and displayed on all five capsid proteins and the use of pVII and pIX display have lately been shown to be particularly efficient in affinity selection [6].

A phage display library is a collection of billions of unique clones that encode and display different fusion proteins. Such libraries may be composed of peptides, protein variants and gene fragment- or cDNA-encoded proteins and may hold more than 10^{12} individual members [14]. Library members exhibiting desired phenotypes are selected and enriched for in a selection process. Selection is carried out by incubating the phage library with a target of interest that is often immobilized on a solid phase, removal of nonbinding phages by washing, and release of the binding phages by changing the pH, competitive elution or by protease digestion. The eluted phages are used to infect *E. coli* for amplification, prior to the next round of selection. This process is normally repeated for several rounds, before individual clones are analyzed, and this is usually required to isolate high-affinity binders [9].

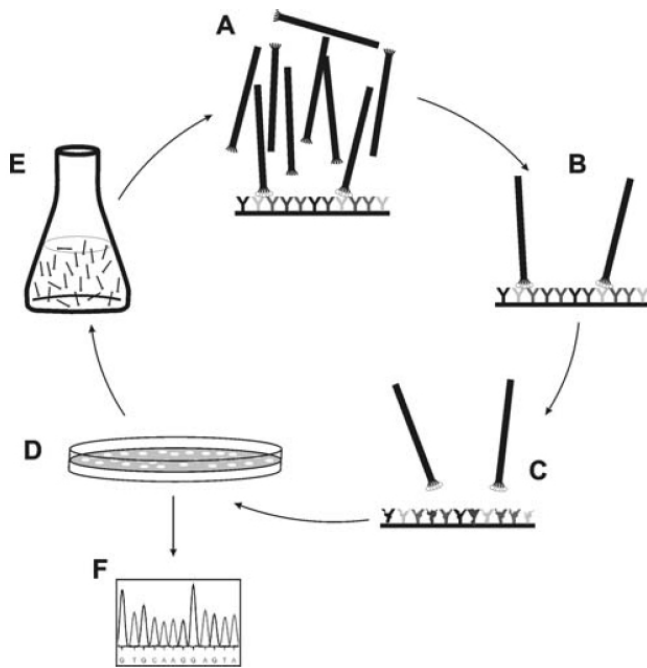


Figure 2: General overview of phage display selection. Library phages are incubated with target (A). Nonbinding phages are washed away (B), and binding phages are eluted (C). Eluted phages are introduced into *E. coli* for amplification on solid (D) and/or in liquid growth medium (E). Individual clones are available for analysis after each selection round (F). The figure is adapted from [15].

1.1.2 Display formats

POI-coat protein fusions can be encoded in a phage vector or in a phagemid vector [9]. Phage vectors are derived from the genome of filamentous phage and encode all the proteins required for replication and virion assembly. Classical phage vector system yields high valence display of the POI, because the fusion protein will be the only source available of the coat protein to which the POI-encoding gene is fused [16].

Phagemids are plasmids that usually encode for one POI-capsid fusion, and in addition carry two origins of replication, one phage-derived and one the plasmid-derived, and a packaging signal of filamentous phage [17]. Since the phagemid contain the gene encoding for one coat protein only, a helper phage is used to provide the genetic material encoding the remaining coat proteins and also other proteins required for phage production. This process, by which phage production is achieved by helper phage supplementation, is called “phage rescue”. Both phagemid-carrying phages and helper phage genome-carrying phages are produced by phage rescue [9]. In this system, the display coat protein is provided by two sources and both phagemid-derived fusion protein and wt coat protein encoded by the helper phage genome, are incorporated into the rescued virions yielding low valence display.

There are advantages and drawbacks with both systems. The phage vector system always ensures genotype-phenotype coupling, while in the phagemid vector system phage displaying fusion proteins will have one of two genotypes. This may lead to loss of selected clones during selection if the phagemid is not preferentially packaged upon phage rescue [9]. The phagemid vector is more efficiently transformed due to the small size compared with phage vectors [17], and this is especially important when wanting to generate high diversity libraries. Whereas the phage genome is very compact and sensitive to genetic alternations, the phagemids are genetically more stable and often constructed to facilitate quick exchange of the POI.

Low valence display is favorable for applications such as high affinity selection where high monomeric affinity is the aim. However, when standard phagemid systems are used, only a few percent of phages actually carry the fusion protein [18]. Hence, when highly diverse libraries are used in the initial round of selection, and each clone is present in limited numbers (<1000), the risk of losing desired clones at random is high. High valence display is more efficient in retrieving specific clones. This has been demonstrated by comparing selections conducted with phage libraries and phagemid libraries, where a greater number of specificities were retrieved from the phage libraries [19].

High valence display using phagemid vectors can be achieved by using modified helper phages that fail to encode a functional version of the display coat protein. Such systems have been described for both pIII and pIX coat proteins [20, 21]. By exploiting this, initial rounds of selection may be performed with increased efficiency in retrieval of specific clones. By switching to a classical helper phage, subsequent selection rounds may be performed with low valence display to select for affinity among the specific binders isolated in the initial selection. In this way beneficial properties of the phage vector and phagemid vector systems are combined.

1.1.3 Applications of phage display

Many different proteins have been displayed on the phage and the technology has been used for various applications, including epitope mapping of antibodies [22], screening for receptor ligands [23], *in vitro* evolution of proteins [24], discovery of enzyme substrates [25], analysis of protein-protein interactions [26] and a lot more. Isolation of monoclonal antibodies from

large antibody libraries is by far the most successful use of phage display. The fragment antigen binding (Fab) and the scFv formats are the two most popular formats for antibody display. The scFv consist of the variable domains of the heavy and light chains that are connected by a flexible peptide linker, and thus comprises the antigen binding site [27].

1.2 Antigen binding receptors of adaptive immunity

1.2.1 Lymphocytes and their role in the immune system

T lymphocytes (T cells) and B lymphocytes (B cells) are important components of the adaptive immune system. The antigen binding receptor of the B cells recognize structures on the surface of antigens, such as those secreted by or expressed on surface of pathogens. Naïve B cells express cell surface membrane-bound B cell receptors (BCRs). Upon exposure and BCR ligandation of antigen the B cell may proliferate and differentiate into memory cells or plasma cells that secrete antibodies, a soluble form of the BCR. The antibody molecule mediates its effect by neutralization or by engaging other immune cells. The cell surface TCRs of T cells recognize foreign antigen as peptides bound to MHC on the surface of antigen presenting cells (APCs). Two major T cell subsets exist, CD4⁺ and CD8⁺ T cells, which are different in the co-receptor they express, CD4 and CD8, respectively, and in their ligand binding properties. CD4⁺ T cells recognize endosome-derived antigenic peptides in complex with MHC class II molecules on professional APCs. CD8⁺ T cells recognize cytosol derived antigenic peptides in complex with MHC class I molecules, that are presented on nucleated cells. Upon activation, the naïve T cells proliferate and differentiate into effector and memory cells. The CD4⁺ T cells develop into T helper cells and the CD8⁺ T cells develop into cytotoxic T cells. In this thesis the main focus will be on the TCR.

1.2.2 The TCR molecule

The TCR molecule is a membrane-bound heterodimeric glycoprotein composed of two polypeptide chains, either α and β in $\alpha\beta$ T cells or γ and δ in $\gamma\delta$ T cells. The complete structure of a heterodimeric T cell receptor was first proposed in 1984 [28]. Each chain consists of an intracellular segment, a single transmembrane α -helix and two extracellular domains, one constant (C) and one variable (V) located proximal and distal to the membrane, respectively. An inter-chain disulfide bridge in the membrane proximal part of the molecule covalently couples the two chains [28]. The TCR molecule resembles the fragment antigen

binding (Fab) part of an antibody and, along with antibodies and MHC molecules it belongs to the immunoglobulin superfamily. The extracellular domains of the TCR adopt an immunoglobulin (Ig) fold comprising two closely packed anti-parallel β -sheets connected by a conserved disulfide bridge [29, 30]. The β -strands are connected by flexible loops, and in the V domains, three of these loops, are particularly diverse and position to contact the antigen. The highly diverse loops are called hypervariable loops, or more commonly the CDRs, as the surface they form is complementary to the antigen they bind.

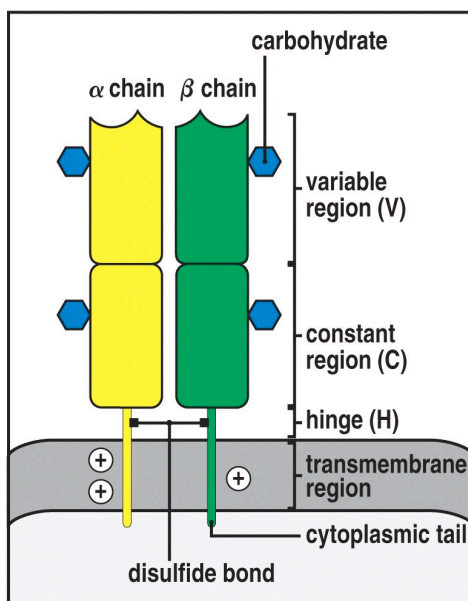


Figure 3: Schematic presentation of a $\alpha\beta$ TCR. The TCR is composed of two transmembrane glycoprotein chains, α and β . The figure is adapted from Immunobiology 8th edition [3].

1.2.3 Antigen recognition by TCRs

The TCR is restricted to recognize its antigen presented in the context of a MHC molecule [31]. Since the first crystal structure of intact TCR bound to pMHC was determined in 1996 [32, 33], dozens of unique TCR-pMHC complexes have been reported. These crystallographic data have given insight into the interaction between the TCR and pMHC and some general features are observed. The TCR bind in a roughly diagonal orientation relative to the long axis of the MHC peptide-binding groove, with the $V\alpha$ and $V\beta$ domain positioned above the N- and C-terminal halves of the peptide, respectively. This generally places the germline-encoded CDR1 and CDR2 loops in contact with conserved helical residues of the MHC, while the CDR3 loops, which exhibit the greatest degree of sequence diversity, primarily contact the peptide [2]. Kinetic analyses suggest that the TCR first make the appropriate contacts with the MHC to orient itself into the correct position for recognition of the peptide [34]. Thus, TCR-pMHC specificity is ensured by a restricted docking mode that involves TCR recognition of

both the peptide and the MHC molecule. Structural comparisons of TCRs in free and antigen bound forms have revealed that conformational changes, mainly in the CDR3 loop region, occur upon binding [35]. Flexibility of the contact surface might enable the TCR to adapt to various pMHC ligands, resulting in some degree of promiscuity [36].

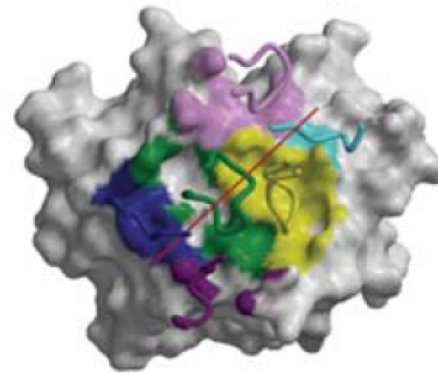
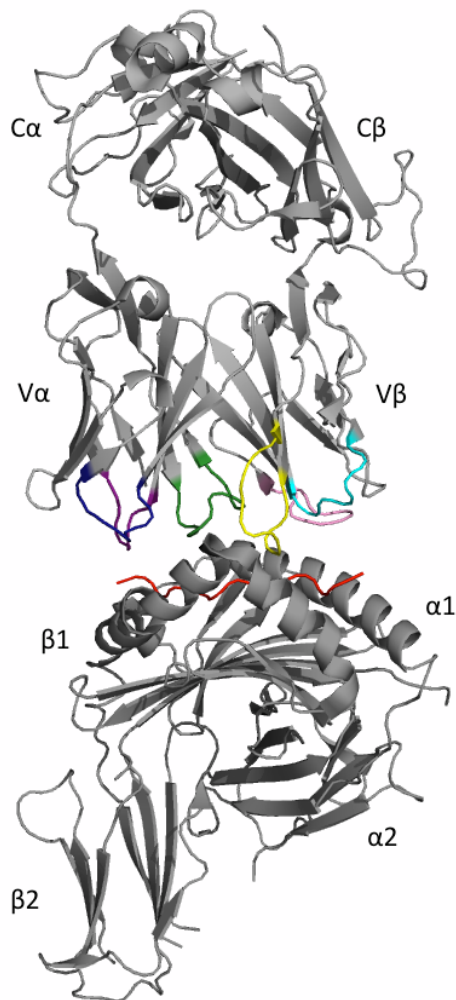


Figure 4: (Left) Structure of a TCR-pMHC class II complex. The CDR loops of the TCR are indicated in color: CDR1 β cyan, CDR2 β pink, CDR3 β yellow, CDR1 α dark blue, CDR2 α magenta and CDR3 α green. The β chain HV4 loop is shown in orange. The red line is the bound peptide. (Above) Footprint of a TCR/pMHC class II complex. The top surface of a MHC is shown and the TCR contact area is colored by the CDR loops. The figure to the left was prepared using PyMol v1.3 (Schrodinger LLC, NY, USA) from PDB ID: 1fyt, and the above figure was adapted from [2].

Like for antibodies, TCR diversity is created through a rearrangement of germline gene segments that also involve deletion and insertion of random nucleotides, to generate a huge collection of specificities against potential antigens. During development in the thymus, T cells whose receptors recognize self-peptides in the context of self MHC too strongly are eliminated [37]. Unlike antibodies, TCRs do not undergo somatic hypermutation and affinity maturation upon activation to produce high affinity clones. Consequently, native monovalent $\alpha\beta$ TCRs have low affinities. The intrinsic affinity of TCRs for ligand has been measured, mostly by SPR-based approaches, to be in the range of 1-100 μ M, and relatively slow association and very fast dissociation kinetics characterize the interaction [38, 39]. This is in

contrast to the kinetics reported for antibody-antigen interactions. Weak affinity has proved to apply for many interactions occurring between adjacent lipid bilayers of interacting cells. While soluble antibodies and their respective ligands may be locally present at low concentration and thus require affinities in the nanomolar range, high local concentration of interacting membrane bound molecules on opposing lipid bilayers may allow for intrinsic affinities several orders of magnitude lower, yet concomitantly exhibit very strong 2D affinities, as in the case of the TCR-pMHC interaction [38, 40].

1.3 TCR engineering

Recombinant TCR molecules have great potential as diagnostic and therapeutic agents as well as detection reagents in basic research. The TCR has evolved to recognize pMHC complexes specifically, and may therefore be a better candidate than the antibody in generation of specific ligands towards this class of antigen. However, there are two main issues that have hindered rapid development of such ligands, TCRs are hard to produce in soluble form and have low intrinsic affinity for its antigen [41].

1.3.1 Soluble TCRs

Expression of antibody fragments in *E. coli*, as well as in eukaryotic systems, has been highly successful [42]. Despite the resemblance in structure, soluble TCRs are generally much more unstable and hard to produce compared to antibodies. Antibody fragments are also readily expressed as fusion proteins to coat proteins of yeast and phage, while display of TCR has been more challenging [42]. The main reason seems to be the interface between the V and C domains, which is more extensive in TCRs than in antibodies [42]. When TCRs are expressed in soluble form, and in particular as scTCRs, hydrophobic patches are displayed which promote aggregation [43].

Many different strategies have been pursued in order to produce TCRs in a soluble form and as fusions to the coat proteins of yeast and phage. This includes the use of scTCR format [44], co-expression of chaperones i.e. Skp and FkpA [45-47] and introduction of an inter-chain disulfide bond to improve stability and folding [48]. Introductions of stabilizing mutations have also been important for increased expression yield and display level on yeast and phage [49, 50].

1.3.2 High affinity TCRs

The affinities of native TCRs for its cognate ligand falls within the range of 10^{-6} M to 10^{-4} M [39], with a binding half life of only a few seconds [38]. This range of affinities is too low to enable stable cell-surface adhesion of monovalent soluble TCRs [41]. One approach to increase the TCRs relative affinity is to produce multimeric proteins. MHC tetramer technology is a well-established method for making high avidity, multimeric pMHCs [51-54] and has also been successful for TCRs [55, 56]. These multimeric TCRs were used to stain APCs pulsed with peptide *in vitro*, which the monovalent form of the TCR could not [55, 56]. Multimeric TCRs may serve as a general approach to generate diagnostic and research tools, where immunogenicity is not a critical issue [55]. However, a potential problem with multivalent TCR complexes is that avidity binding might not occur at low cell-surface antigen densities observed physiologically [57].

The intrinsic affinity of TCRs can be increase by affinity maturation, which typically involved three steps: (i) introduction of diversity in the V genes of the TCR, thus creating a library of gene variants, (ii) selection of the higher affinity from the low affinity variants and (iii) screening to identify TCR variants featuring the desired affinity [58]. In this approach, the power of Darwinian selection is harnessed to evolve proteins with properties not found in nature [59]. Affinity maturation can be mediated by yeast and phage display technology and has been successful in obtaining high affinity TCRs capable of directly detecting pMHC complexes of APCs in soluble monomeric forms [24, 60, 61].

By using yeast surface display, Holler et al. [60] and Shusta et al. [62] produced variants of the 2C TCR by mutating the CDR3 α loop, and selected mutants with 100-fold increase in affinity, up to 9 nM. Wild type TCRs are not stable on yeast and thus prior to affinity selection, mutagenesis and stability selection is required to allow surface display on yeast [50]. Li et al. [24] was the first to report successful generation of high affinity TCRs using directed evolution and phage display. The selected variants contained mutations in the CDR3 loops primarily, which resulted in nanomolar and picomolar affinities and increased binding half life from 7 s to more than 15 h. The high affinity TCRs could bind to pMHC complexes on the surface of APCs, and showed no detectable cross-reactivity [24]. Furthermore, Dunn et al. [61] have been successful in selection of high-affinity TCR variants from libraries generated by mutations in the germ line encoded CDRs (CDR2s).

Thus, it does not appear to be an intrinsic affinity threshold preventing TCRs to be artificially engineered to obtain substantially higher monomeric affinities in solution than what is found in nature. As the peptide fine-specificity of such engineered TCRs is retained, naturally occurring TCRs therefore represent a very attractive starting point for affinity maturation to obtain highly sensitive pMHC-specific targeting molecules for a variety of application both in basic research and for therapy [63-66].

1.4 Celiac disease

CD is an inflammatory condition of the small intestine that affects approximately 1% of the population of most western countries [67, 68]. The disease is caused by an inappropriate immune response to dietary wheat gluten and similar proteins in barley and rye and leads to destruction of the small intestine epithelium. Selective tissue destruction is one of several key features that CD shares with autoimmune disorders. More strikingly is the presence of disease-specific, gluten-dependent autoantibodies specific for the enzyme tissue transglutaminase 2 (TG2). Furthermore, a strong association with certain HLA class II molecules is seen with more than 90% of the patients expressing HLA-DQ2.5 and the remainder expressing HLA-DQ8 [69, 70]. These particular HLA molecules have a unique ability to bind proline and glutamine rich gluten peptides that have been deaminated by TG2 [71, 72].

The importance of genes in CD is supported by familial clustering and by high concordance in twins [73]. Most people that have the disease-associated HLA class II molecules do not have CD, and HLA is thus considered a necessary but not a sufficient factor for disease development [74]. Genome-wide association studies have lead to the discovery of several non-HLA genes as susceptibility factors, many of which are related to the immune response [75].

The strong HLA class II association suggests that T cells are important for the pathogenesis. Upon ingestion of gluten one can see an abnormal influx of CD4⁺ T cells that recognize gluten-derived peptides in the context of HLA-DQ2.5 or HLA-DQ8 presented by APCs [76]. It is believed that these T cells orchestrate the immune response that leads to the destruction of the epithelium through the release of proinflammatory cytokines such as interferon- γ [77]. Most of the HLA-DQ2.5 restricted CD4⁺ T cells are reactive to either of two

immunodominant gluten peptides, gliadin- α 1a and gliadin- α 2 [78]. These are epitopes found within a 33mer peptide fragment that remains intact after gastrointestinal digestion of certain wheat proteins known as α -gliadins [79]. The high proline content of gluten proteins makes them resistant to digestion by gastrointestinal proteases. This results in long antigenic peptide fragments that pass through the epithelial barrier of the intestine where TG2 awaits [74, 79].

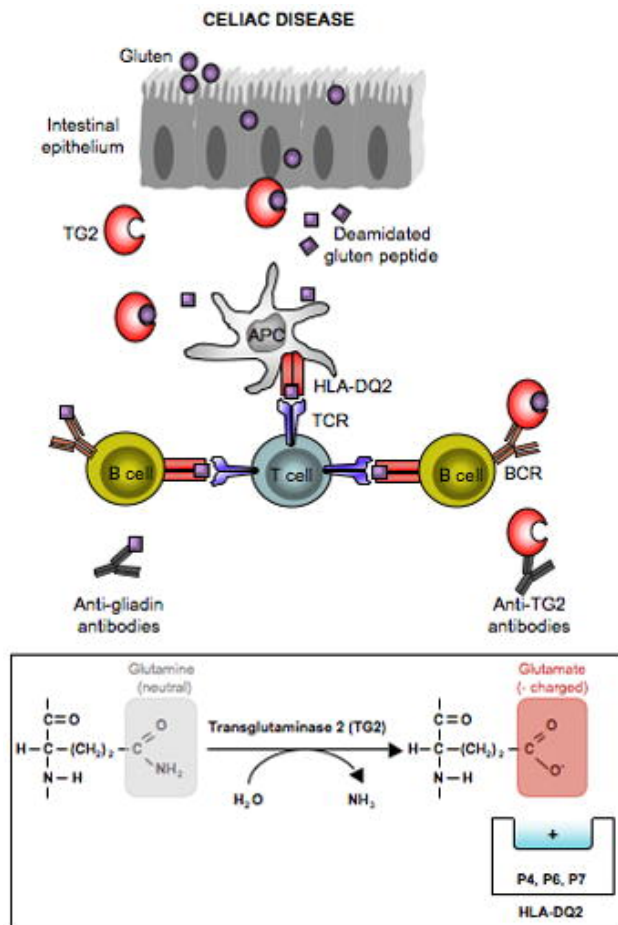


Figure 5: Schematic presentation of key features in the pathogenesis of CD. Gluten peptides cross the intestinal barrier into the lamina propria where they serve as substrates for TG2. Deamination of glutamine residues into glutamic acid creates antigens that are better suited to bind the disease-associated HLA molecules. Glutamate serves as an anchor residue at positions P4, P6 and P7 for peptides binding to HLA-DQ2.5. Antibodies recognizing gluten and TG2 are generated. The figure is adapted from [1].

1.4.1 APCs in the small intestine

Dendritic cells (DCs), macrophages and B cells are professional APCs that initiates adaptive immune responses, by presenting antigenic peptides bound to cell surface MHC class II molecules. Distinct subsets of APCs in the intestine have different origin and functions. Hence, some migrate to the lymph nodes to activate naïve T cells into becoming effector or regulatory T cells, while others stimulate activated T cells locally in the mucosa.

In the duodenum, the first part of the human small intestine, macrophages and DCs constitute the main population of APCs. These are mainly derived from monocytes and classical myeloid DCs precursors cells [80]. The HLA-DQ expressing APCs have been studied by Raki and Beitnes et al, and in the intestinal mucosa about 80% are CD163+ macrophages and 20% are CD11c+ DCs [81]. CD163 and CD11c are classical markers for macrophages and DC, respectively. The CD11c+ DC population can further be subclassified into cells expressing CD163, CD103 or CD1c. The CD11c+CD163+ DCs thus have an intermediate phenotype, expressing both the macrophage and DC markers, and represent roughly 7% of all HLA-DQ+ APCs [82].

The densities of the different subpopulations are characteristically altered in the active celiac lesion. The density of intermediary CD163+CD11c+ DCs increase, while the density of CD103+CD11c+ DCs, CD1c+CD11c+ DCs and CD163+CD11c- macrophages decreases [82]. The decreased intestinal density of CD103+CD11c+ DCs in CD has been hypothesized to at least partly be due to migration of these cells to lymph nodes. It has been demonstrated that equivalent CD103+CD11c+ DCs in mice are able to migrate to the draining lymph node and human CD103+CD11c+ DCs are readily detected in mesenteric lymph nodes [83, 84]. While the DC subgroup may migrate to the draining lymph node, present gluten peptides, and activate gluten-specific naïve T cells, the intermediate DC subgroup, which is increased in density in the active celiac lesion, may be responsible for driving intestinal pathology by activation gluten-specific memory T cells [85]. CD11c+ DCs, isolated from gluten-challenged mucosal biopsies, were found to be effective presenters of gluten peptides to gluten-reactive T cell clones [81]. Furthermore, the increased density of intermediate DCs precedes the typical changes in mucosal architecture, indicating that this DC subset may be involved in disease initiation [85].

The exact contribution of the different subgroups identified cannot be fully elucidated without including a DQ2.5-gluten peptide-specific detection reagent to complement previous studies. Moreover, the DQ2.2 vs. DQ2.5 discrepancy in CD predisposition has been suggested to rely on a spatial and temporal gradient in the tissue resulting in different peptide presentation ability to relevant T cells at activation sites [86]. Thus, analyzing gluten peptide presentation by the distinct APC subgroups, both where presentation occurs and how much is presented will be important for understanding CD.

1.4.2 TCRs in CD

A public T cell response, in which multiple individuals share identical or close to identical TCR repertoires towards the same antigenic epitope, has been observed in a variety of immune responses [87], including towards DQ2.5-glia- α 2 among CD patients. Sequencing of the TCR genes from dozens of gluten specific T cell clones, isolated from CD patients, have provided insight into the repertoire [88, 89].

The DQ2.5-glia- α 2 reactive T cells show a biased usage of the V β 6.7 chain, encoded by TCR β variable (TRBV) 7-2 gene segment. Furthermore, most of these TCRs express a non-germline encoded arginine residue in the CDR3 β loop, which has been shown to be critical for T cell recognition [88]. Moreover, the TRBV7-2 encoded β -chain show preferred pairing to a TCR α chain, encoded by the human TCR α variable (TRAV) 26-1 gene segment [89].

Very recently, the crystal structures of three distinct TCRs in complex with DQ2.5-glia- α 2 and one TCR in complex with DQ2.5-glia- α 1a were reported. Among other findings, the CDR3 β loop of the DQ2.5-glia- α 2 specific TCRs was found to sit above the peptide, and the main contributor to the CDR3 β -HLA-DQ2-mediated interaction was the conserved arginine, which arched down into the antigen binding cleft and interacted with the peptide [90]. The V β 6.7 chain footprint on HLA-DQ2 was maintained in the three crystals, suggesting an important role in mediating docking.

Compared to DQ2.5-glia- α 2 specific TCRs, a smaller degree of biased TCR usage against DQ2.5-glia- α 1a was observed [90]. However, TCRs of DQ2.5-glia- α 1a reactive T cells have also been found to carry the TRBV7-2 gene segment (Qiao et al. unpublished). For the DQ8-glia- α 1 specific T cells, a biased usage of the TRBV9 gene segment, coupled with a preferred pairing of the TRAV26-2 gene segment has been reported [91].

2. Aim of the thesis

The strong disease association of HLA-DQ2.5 indicates an important role of antigen presentation and T cell activation in CD. The TCR has evolved to recognize pMHC complexes specifically and thus represents the ideal ligand for their targeting. However, the low affinity of the TCR for pMHC prevents its direct use as trackers of peptide presentation.

The main objective was to develop an artificial high affinity HLA-DQ2.5-glia- α 1a specific reagent, by use of phage display engineering, based on a naturally occurring TCR derived from a CD lesion.

The V β 6.7+ TCR 380 specific for DQ2.5-glia- α 1a was chosen as the starting point. As describe by Molberg et al. [92] the T cell expressing TCR 380 was isolated from a CD patient using DQ2.5-glia- α 1a tetramers. The TCR V genes were cloned and employed to construct the scTCR s380 prior to this thesis (Løset et al., unpublished). Moreover, the wt V α -V β interface was engineered to reconstruct an optimal domain pairing resulting in both improved soluble expression and display propensity on pIX (Neuman and Høydahl et al., unpublished). The stabilized variant was named s380 and is described herein. In order to affinity mature scTCR s380, it was displayed on filamentous phage as a fusion to the phage coat protein pIX. Phage libraries were generated by individual randomization of the CDR3 α and CDR3 β loops prior to this thesis (Høydahl et al. unpublished, supplementary 1).

The aim of this thesis was to perform the scTCR s380 phage library selection on DQ2.5-glia- α 1a and to identify scTCR s380 clones with improved affinity.

3. Materials and methods

3.1 General material

3.1.1 Bacteria

The E. coli XL1-blue strain (recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' proAB lacIqZΔM15 Tn10 (Tet^r)] (Stratagene, CA, USA) was used for titration. The E. coli SS320 strain ([F' proAB+lacIqlacZΔM15 Tn10 (tet^r)] hsdR mcrB araD139 Δ(araABC-leu)7679ΔlacX74 galUgalK rpsL thi (Lucigen, WI, USA) was used for amplification of phage libraries in selection. The E. coli TOP10 F' strain (F'[lacI^q Tn10(tet^R)] mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 deoR nupG recA1 araD139 Δ(ara-leu)7697 galU galK rpsL(Str^R) endA1 λ⁻ (Invitrogen™, CA, USA) was used for amplification of multivalent pIX phage.

3.1.2 Plasmids

For phage display on pIX the previously described pGALD9ΔLFN phagemid was used [6]. In addition to all the other components of pGALD9ΔL, it contains a FkpA cassette that is controlled by its native promoter [45]. The pGALD9ΔLFN phagemid encoding scTCR s380 is illustrated in Figure 6. The scTCR is in a Vα-linker-Vβ format and is genetically fused N-terminal to the pIX gene.

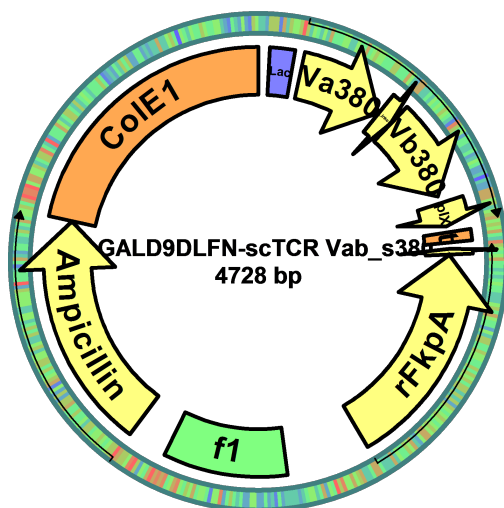


Figure 6: Schematic drawing of the pGALD9ΔLFN-scTCR s380 phagemid. The phagemid contain the following elements: a phage f1 packaging signal (f1), an ampicillin resistance-encoding gene, an origin of replication (ColE1), a lac promoter, a scTCR s380-encoding cassette fused to the pIX gene and a terminal signal, and a gene encoding the chaperone FkpA.

3.1.3 Helper phage

Two different helper phages have been used; M13K07 purchased from Amersham Bioscience (Uppsala, Sweden) and DeltaPhage, which have been constructed in Sandlie lab. Both have been described previously in [93] and [21], respectively.

3.1.4 Antibodies and additional reagents

Mouse anti-M13 HRP monoclonal antibody was purchased from GE Healthcare Life Sciences (Uppsala, Sweden). Rabbit anti-mouse-IgG HRP antibody was purchased from Sigma-Aldrich (St. Louis, MO, USA). The mouse anti-DQ2 monoclonal antibody 2.12.E11 was a kind gift from Professor Ludvig M. Sollid. Recombinant protein L (pL) immunoglobulin binding protein and NeutrAvidin (NA) biotin binding protein were both purchased from Thermo scientific (Rockford, IL, USA). Synthetic oligonucleotide primers were purchased from Eurofins Genomics (Ebersberg, Germany). Recombinant DQ2.5 with glia- α 1a or CLIP2 peptide were kind gifts from Professor Ludvig M. Sollid, and have been produced and purified as described [94]. The peptide is coupled to the N-terminal of DQ2 β -chain through a 15-residue linker [95].

3.2 Preparations for phage selection

3.2.1 Production of helper phage

E. coli XL1-Blue harboring either the M13K07 or the DeltaPhage genome was inoculated in 2x YT supplemented with 50 μ g/ml kanamycin (kan). The cultures were incubated with shaking at 220 rounds per minute (rpm) at 37°C overnight (ON) using INFORS HT thermal shaking incubator (VWR, Radnor, PA, USA). The following day supernatants were harvested by centrifugation at 3220xg/30min/4°C using Sorvall RC3R plus centrifuge (VWR) and sterile filtered using 0.2 μ m filters (Millipore, Billerica, MA, USA). Virion particles were precipitated by mixing the supernatant with 1/5 volume of PEG/NaCl (20% polyethylene glycol (PEG) 8000 (Sigma-Aldrich, Oslo, Norway) and 2.5 M NaCl), followed by incubation in ice-slurry for 3 hours. The precipitated phage was pelleted by centrifugation at 3220xg/30min/4°C using the Sorvall centrifuge and the pellets were re-suspended in 1x PBS pH 7.4. To remove any remaining debris, the phage solutions were centrifuged at 13000xg/5min/RT and the supernatants were transferred to new tubes and stored at 4°C.

3.2.2 Spot titration of phages

Phage stocks rescued by the helper phages M13K07 or DeltaPhage were titrated by standard spot titration [96]. *E. coli* XL1-Blue was inoculated in 2x YT supplemented with 30 µg/ml tetracyclin (tet) and incubated with shaking at 200 rpm/37°C/ON using SI-600R incubator (Lab companion, Seoul, Korea). For each phage stock a dilution series was prepared in 1xPBS pH 7.4 according to Table 1. 10 µl of each phage dilution was transferred to a 96-well microtiter plate (Corning Incorporated, NY, USA). The XL1-blue ON culture was reinoculated to A_{600nm} 0.025 in 2x YT supplemented with 30 µg/ml tet and incubated with shaking at 220rpm/37°C until OD_{600nm} 0.5 was reached. 190 µl of the XL1-blue culture was transferred to each phage dilution in the microtiter plate and was further incubated at 200rpm/37°C/30min using Titramax 1000 (Heidolph, Schwabach, Germany). Volumes of 3 µl of each preparation were spotted onto BA85/20 nitrocellulose membranes (Whatman, Dassel, Germany) that were laid on top of LB-agar dishes supplemented with either 100 µg/ml ampicillin (amp) or 50 µg/ml kan. The dishes were incubated at 37°C/ON. The number of colony forming units (cfu)/ml was calculated using the formula $(cfu \times dilution^{-1} \times 20 \times 1000)/3$. Cfu/ml calculated from LA-amp dishes equals to the number of phagemid-carrying phage in the stock solution and cfu/ml calculated from the LA-kan dishes corresponds to the number of helper phage genome-carrying phage.

Table 1: Overview of dilutions made in spot titration of phage

Dilution	Gradient	1x PBS	Transfer
A	10^{-2}	495 µl	5 µl from stock
B	10^{-4}	495 µl	5 µl from A
C	10^{-5}	450 µl	50 µl from B
D	10^{-6}	450 µl	50 µl from C
E	10^{-7}	450 µl	50 µl from D
F	10^{-8}	450 µl	50 µl from E
G	10^{-9}	450 µl	50 µl from F

3.3 Selection of phage libraries

3.3.1 Pre-selection on pL

Maxisorp™ immunotubes (Nunc, Roskilde, Denmark) were coated with 30 µg pL in 3 ml 1xPBS and incubated ON/4°C. The immunotubes were blocked with 3 ml 1xPBS containing 0.05% Tween 20 (Sigma, MO, USA) and 4% skim milk (AppliChem, Gatersleben, Germany) (PBSTM) for 30min/RT on a SB3 rotator (Stuart). Volumes of 100 µl phage library were

blocked under the same conditions in 3 ml PBSTM. Following blocking, the phage was transferred to the immunotubes and incubated for 1h/RT on a rotating wheel. 1 ml of the phage solution was channeled into the selection procedure described in 3.3.2.

3.3.2 Selection

Four rounds of selection were preformed. Prior to each round all components were incubated with blocking agent dissolved in 1xPBS supplemented with 0.05% Tween 20 (PBST) for minimum 30 minutes/RT. The blocking agent used was alternated each round between 4% skim milk and 2% bovine serum albumin (BSA) (Sigma).

The Dynabeads MyOne Streptavidin T1 (InvitrogenTM) were washed and blocked prior to use. The beads were resuspended in the stock solution by vortexing for 30 seconds before the required volume was transferred to a 1.5ml eppendorf tube. The beads were washed twice by addition of 1 ml PBS, pH 7.4 and brief vortexing. By placing the tubes in a DynaMagTM magnet rack (Life technologiesTM, CA, USA) the beads accumulated on the tube wall and the solution could be removed. After blocking, the beads were observed in a light microscope (Nikon TMS, Japan) as single entities floating in the fluid.

Selection was executed by incubating the phage with biotinylated pMHC target, starting with 60 nM pMHC in R1 followed by 10x decrease per round, in 1 ml blocking solution for 1h/RT on a SB3 rotator (Stuart). The phage libraries were first incubated with biotinylated HLA-DQ2.5-CLIP2 (referred to as negative selection) and the resulting phage-antigen complexes were captured onto streptavidin-magnetic beads by incubation for 30min/RT on a SB3 rotator (Stuart). The beads containing bound complexes were concentrated on the tube wall using the DynaMagTM magnet rack (Life technologies) and the supernatant was transferred to new 1.5 ml tubes. Biotinylated HLA-DQ2.5-glia- α 1a was added to the remaining phage in the supernatant and incubated (referred to as positive selection), before the resulting phage-antigen complexes were captured onto beads.

In the first round of selection, positive selection was directly followed by washing bead-captured phage-antigen complexes; ten times with 1xPBST and then ten times with 1x PBS, with a brief vortex each time.

Starting from the second round of selection, positive selection was followed by addition of free soluble DQ2.5-glia- α 1a in excess in two rounds. The beads were incubated with free antigen in 1ml blocking solution on a rotating wheel for 30 min, before the solution was removed like before and stored (later referred to as eluate 1, or E1). New free target was added in 1ml blocking solution and incubated on a rotating wheel for 60 min, followed by removal and storage of the solution (later referred to as eluate 2, or E2). Prior to the first and the second addition of soluble DQ2.5-glia- α 1a, the beads were washed by the same procedure as described above, two times with 1xPBST and two times with 1xPBS. Sequential washing of the beads was performed after off-rate selection in the same way as in the first round of selection.

Phage bound to the beads through interactions with biotinylated DQ2.5-glia- α 1a were eluted by incubating the samples with 0.5 ml 100 mM TEA pH 11 (Sigma) at RT/20min. using a SB3 rotator (Stuart), followed by placement of the sample tubes in the magnet rack and transfer of the solution to 0.5 ml 1 M tris-HCl, pH 7.5 for neutralization (later referred to as eluate 3, or E3).

3.3.3 Infection of *E. coli* SS320 with selection output and phagemid rescue

Phage-containing solutions obtained during the selection procedure were used to infect log-phase *E. coli* SS320 cultures that were estimated to comprise approx. 2.5×10^{10} cells. The cultures were incubated at 80rpm/37°C in SI-600R incubator (Lab companion) for 25 minutes after addition of the phage. The infected cells were pelleted by centrifugation at 3220xg/15min/RT using Centrifuge 5810R (Eppendorf, Hamburg, Germany), carefully resuspended in 2 ml 2xYT-medium and plated onto large 24.5x 24.5 cm Bio-Assay Dishes (Nunc) containing LB-agar supplemented with 30 μ g/ml tet, 100 μ g/ml amp and 0.1 M glucose (TAG). The bacteria were grown at 37°C/ON.

To determine the selection output, volumes of 10 μ l infected culture were removed prior to centrifugation and transferred to a microtiter plate. Dilution series were prepared for each culture in 2x YT according to Table 2. 3 μ l of each dilution was spotted onto a BA85/20 nitrocellulose membrane (Whatman) that was laid on top of LA-amp dishes. The dishes were incubated at 37°C/ON. The number of cfu/ml present in the original culture was calculated using the formula (cfu x dilution⁻¹ x 1000)/3.

Table 2: Overview of dilutions made in titration of panning output

Dilution	Gradient	2 x YT	Transfer
A	10^{-1}	90 μ l	10 μ l from culture
B	10^{-2}	90 μ l	10 μ l from A
C	10^{-3}	90 μ l	10 μ l from B
D	10^{-4}	90 μ l	10 μ l from C
E	10^{-5}	90 μ l	10 μ l from D
F	10^{-6}	90 μ l	10 μ l from E
G	10^{-7}	90 μ l	10 μ l from F

The phagemid-containing bacteria were scraped from the large bio-assay dishes using 10 ml 2xYT per dish and a glass spreader. Scraped bacteria were diluted to OD_{600nm} 0.05 in 50 ml 2xYT supplemented with TAG and incubated with shaking at 220rpm/37°C in INFORS HT (VWR) until OD_{600nm} reached 0.1. Helper phage M13K07 or DeltaPhage was then added to the cultures at multiplicity of infection (MOI) 20. The cultures were further incubated at 80rpm/37°C/30min and subsequently at 200rpm/37°C/30min. The bacteria cells were pelleted by centrifugation at 3220xg/15min/RT using Centrifuge 5810R (Eppendorf). The supernatants were discarded and the pellets were gently re-suspended in 50 ml 2xYT supplemented with 100 μ g/ml amp and 50 μ g/ml kan. The cultures were incubated at 220rpm/30°C/6h, before virion-containing supernatants were harvested by centrifugation at 3220xg/4°C/30min and further sterile filtrated using 0.22 μ m filters (Sarstedt, Nümbrecht, Germany). The supernatants were mixed with 1/5 volume of PEG/NaCl, and incubated in ice slurry at 4°C/ON to precipitate the virions. The precipitated virions were concentrated and treated as in 3.2.1.

3.3.4 Calculation of phage enrichment

Enrichment factor (EF) is defined as ratio from current selection round divided by the ratio from previous round. The ratio was obtained by dividing the phage output (cfu^{amp}/ml) on the phage input (cfu^{amp}/ml) in a selection round.

The total EF was calculated by multiplying the EFs found for each selection round.

Total enrichment factor: I x II x III, where I = round 2/round 1, II= round 3/round 2, III=round 4/round 3.

3.4 Analysis of phage libraries

3.4.1 Analysis of library content by PCR

The primers were ordered from Eurofins Genomics, Ebersberg.

ColE1_fwd2:	5'-TGGATAACCGTATTACCGCCTTTG-3'	Tm 58.81°C
scTCR380_Va_rv:	5'-GTGGCTTTCAGGAGGAGCTG-3'	Tm 59.11°C
scFv_hVκ_rv:	5'-GTGAGAGTGAATTCTGTCCCAGATC -3'	Tm 59.04°C

Annealing temperatures were calculated using Oligonucleotide properties calculator and nearest neighbor (<http://www.basic.northwestern.edu/biotools/oligocalc.html>). Amplification was done with Phusion HotStart DNA polymerase (Thermo Scientific). PEG precipitated phage libraries were used as template and each library sample was tested with both primer pairs. The PCR reactions were set up according to Table 3.

Table 3: PCR reaction setup

Reagent	1 μl Phage library		NC	NC	PC/NC		Final Conc.
Template	1 μl Phage library			1 μl L1R1	1 μl Control Plasmid		Virions Or DNA
dH ₂ O	35.5 μl	35.5 μl	36 μl	36.5 μl	35.5 μl	35.5 μl	-
dNTP	2 μl	2 μl	2 μl	2 μl	2 μl	2 μl	0.2mM
ColE1_fwd2	0.5 μl	0.5 μl	0.5 μl		0.5 μl	0.5 μl	0.5 μM
scTCR_380_rv	0.5 μl		0.5 μl		0.5 μl		0.5 μM
scFv_hVκ_rv		0.5 μl	0.5 μl			0.5 μl	0.5 μM
5x Phusion HF buffer	10 μl	10 μl	10 μl	10 μl	10 μl	10 μl	1x
Phusion DNA Pol'	0.5 μl	0.5 μl	0.5 μl	0.5 μl	0.5 μl	0.5 μl	0.02U/μl
Total	50 μl	50 μl	50 μl	50 μl	50 μl	50 μl	

The PCR program used is given in Table 4 and was carried out using Doppio PCR cycler (VWR). To visualize the PCR products 1 μl of each reaction sample was prepared for gel electrophoresis (described in detail in 3.5.2).

Table 4: PCR program used for amplification of phagemid or DNA plasmid

Step	Temperature	Time
Initial denaturation	98°C	3min
Touchdown		
Denaturation	98°C	10sec
Annealing	Start: 72°C	30sec
	End: 58°C	
	Steps: 7	
Extension	72°C	15sec
Rep: 20		
Denaturation	98°C	10sec
Annealing	58°C	30sec
Extension	72°C	15sec
Final extension	72°C	5min
Hold at	4°C	24h

3.4.2 Polyclonal ELISA

Maxisorp™ microtiter plates (Corning Inc.) were coated with 1 µg/well NA (Thermo scientific) in 100 µl PBS pH 7.4 at 4°C/ON. The wells were blocked with 300 µl/well PBST containing 4% w/v nonfat dried milk powder (AppliChem) for 1 hour at RT. 125 ng biotinylated pMHC was added per well in 100 µl PBST (corresponding to 20 nM/well) and incubated for 1h/RT. 25 µl phage library sample diluted in 75 µl block solution was added to the appropriate wells and incubated for 1h/RT. Bound phage were detected with anti-M13-HRP (1:5000) in 100 µl PBST for 1h/RT. Following incubation of each layer the wells were washed automatically 3x with 250µl PBST using a Skan washer (Molecular devices). The wells were developed by adding 100 µl TMB soluble substrate (Calbiochem, Darmstadt, Germany) to each well and the absorbance was measured at 620nm after 25 min using a sunrise ELISA reader device (TECAN). The reaction was stopped after 30 min. by adding 100 µl 1M HCl to the wells and the absorbance was measured at 450nm.

3.4.3 Single colony screening of selection output

Single clone phagemid rescue

Single colonies originating from selection output clones were randomly picked from LA-amp plates and grown overnight in 400 µl 2xYT-TAG in MegaBlock 96 well 2.2ml plates (Sarstedt, Nümbrecht, Germany) at 1100rpm/37°C in Titramax 1000 (Heidolph). The plates were sealed with AirPore tape sheet (Qiagen, Hilden, Germany). The cultures were re-inoculated by transferring 10 µl to fresh deep well plates containing the same volume per well

of 2xYT-TAG and incubated for three hours at 1100rpm/37°C. The cultures were superinfected with 10^9 cfu^{kan} of DeltaPhage and incubated for 30 min with no agitation at 37°C, followed by 30 min at 1100rpm/37°C. The cultures were pelleted by centrifugation at 3220xg/15min/RT using Centrifuge 5810R (Eppendorf) and the supernatants were discarded. The pellets were gently resuspended in 400 µl pre-warmed 2xYT supplemented with 100 µg/ml amp and 30 µg/ml kan, and the cultures were incubated with shaking at 900rpm/30°C/ON, before cells were pelleted by centrifugation and the supernatant used in ELISA.

Single clone ELISA

MaxisorpTM microtiter plates (Corning Inc.) were coated with 1 µg/well NA (Thermo scientific) in 100 µl PBS pH 7.4 at 4°C/ON. The plates were incubated with 4% PBSTM for 1h/RT to block the wells. 63 ng/well biotinylated DQ2.5-glia-α1a and DQ2.5-CLIP2 (corresponding to 10 nM/well in a 100 µl volume) was added to separate plates and incubated for 1h/RT. Supernatants were transferred from the deep well plates to corresponding wells in two microtiter plates and thus incubated with both pMHC targets for 2h/RT. Following incubation of each layer the wells were washed automatically 3x with 250µl PBST using a Skan washer (Molecular devices). Phage detection and development was done as described in 3.4.2, and absorbance was measured at 620nm after 25 min using a sunrise ELISA reader device (TECAN). The reaction was stopped after 30 min. by adding 100 µl 1M HCl to the wells and the absorbance was measured at 450nm.

3.5 General methods

3.5.1 Miniprep of single clones

Single clones were inoculated from glycerol stock in 5 ml 2xYT-TAG and incubated with shaking at 220rpm/37°C/ON. 2 ml of the overnight cultures were centrifuged at 3220xg/15min/RT using Centrifuge 5810R (Eppendorf). The plasmids were isolated from the bacteria using QIAprepTM Spin Miniprep Kit (Qiagen) and the DNA concentrations were measured using DS-11+ Spectrophotometer (DeNovix, Wilmington, Delaware). The clones were sequenced by the ABI lab core facility (Dept. of Biosciences, University of Oslo, Norway). To visualize the isolated plasmids 1 µl of each sample was prepared for gel electrophoresis.

3.5.2 Gel electrophoresis

A 1% gel was prepared using 0.5 g agarose (Sigma-Aldrich), which was dissolved in 50 ml 1xTAE buffer (40 nM Tris-acetate, 1 mM ethylenediaminetetraacetic acid (EDTA)) by heating the solution to boiling point. 5 µl SYBR safe DNA gel stain (Life technologies) was added to the agarose solution before casting. DNA samples were mixed with 2 µl of 6x sample buffer and dH₂O to a total volume of 12 µl and added to the wells in the gel. A DNA ladder was added in parallel (New England Biolabs, MA, USA). The gel electrophoresis was carried out in 1xTAE buffer at 90V for 45 minutes using Power pac 300 (Biorad) and the DNA was visualized using Gel DocTM EZ Imager (Biorad).

3.5.3 Sequencing analysis

DNA sequencing of the POI-encoding cassette was done using the two forward primers pQE-FP (5'-CGGATAACAATTTTCACACAG-3') and Yol_frwd (5'-GTGAATTTTCAGAAGCACGC-3'). Samples were prepared for sequencing by mixing app. 20 ng/µl miniprep with milli-q H₂O in a total volume of 9 µl and then adding 1 µl of a 10 µM primer solution. The ABI-lab core facility at Dept. of Biosciences, University of Oslo, Norway was used and the data obtained was analyzed using the tools listed in Table 5.

Table 5: Tools used for sequence analysis

Analysis	Program	Company/Software/ Developer	Link
Raw data analysis	Sequence scanner	Applied Biosystems	http://www3.appliedbiosystems.com
Multiple sequence alignment	Clustal Omega	The European Bioinformatics Institute	https://www.ebi.ac.uk/Tools/msa/clustalo/
Multiple sequence alignment editor	BioEdit V 7.1.3	Tom Hall	http://www.mbio.ncsu.edu/BioEdit/bioedit.html
TCR/IG V gene analysis	IMG/QUEST	IMG	http://www.imgt.org/IMGT_vquest/share/textes/

4. Results

4.1 Phage selection on DQ2.5-glia- α 1a

4.1.1 Background

In order to employ TCRs as trackers of antigen presentation the affinity has to be improved. This may be achieved by making phage libraries of TCRs with randomized amino acids in CDR loops, and by performing selections on specific target. For the development of such a reagent, scTCR s380 reactive to the immunodominant DQ2.5-glia- α 1a epitope, a naturally occurring TCR derived from a CD lesion, was chosen to be the starting point for phage library construction.

The library construction is described in supplementary 1. Briefly, the CDR3 α and CDR3 β amino acid loops of scTCR s380 were separately randomized. Different parts of the loops were targeted for mutagenesis to generate several sub-libraries, which were packaged into virion particles by rescued with M13K07 and DeltaPhage giving mono- and multivalent display, respectively. The scTCR s380 variants were expressed as fusion proteins to the pIX capsid.

The first attempt to affinity mature scTCR s380 is described in supplementary 2. At that time, a naïve scFv phage library had recently been panned against the same target, DQ2.5-glia- α 1a, using the very same laboratory environment (Høydahl et al., unpublished). Notably, this particular scFv library had been pre-enriched for scFvs, that performed particularly well in *E.coli*, by functional pL purification [97]. Thus, these intersecting panning campaigns led to contamination of the scTCR s380 phage libraries with various DQ2.5-glia- α 1a specific scFv clones, as revealed in the target screening following panning of the scTCR libraries. Backtracking of the contamination using PCR revealed that the original scTCR s380 phage library stocks were clean, and thus could be used in a second attempt to affinity mature scTCR s380 (supplementary 3).

Phages from sub-libraries that were randomized in the same CDR3 loop were pooled before selection, resulting in the libraries listed in Table 6.

Table 6: Libraries used for panning

Library no	Library name ^a
L1	scTCR s380 CDR3 α – M13K07
L2	scTCR s380 CDR3 β – M13K07
L3	scTCR s380 CDR3 α – DeltaPhage
L4	scTCR s380 CDR3 β – DeltaPhage

^aLibraries were prepared by Høydahl (unpublished).

Initial experiments were performed to prepare an optimized selection protocol (supplementary 4-6).

4.1.2 Selection round one and two

To limit the number of phage with unspecific binding properties, the libraries were first negatively selected on biotinylated HLA-DQ2.5 harboring the irrelevant peptide CLIP2. Phages that remained unbound were subsequently subjected to positive selection on the relevant peptide antigen *glia- α 1a* bound in the context of biotinylated HLA-DQ2.5.

In order to capture the majority of phages displaying scTCR variants with the relevant specificity, HLA-DQ2.5-*glia- α 1a* was given in great excess compared to the number of phages in the first round. However, to increase the chance of isolating high affinity scTCRs, the concentration of antigen in the positive selection was reduced by a tenfold for each round of selection.

To select for increased off-rate, free soluble DQ2.5-*glia- α 1a* (referred to as competitor) was added in excess in two rounds to the bead-captured phage-antigen complexes to capture the dissociating scTCR phage, and thus preventing them from re-binding to the antigen on the bead. This was done starting from the second round of selection.

Phages that were eluted in the second addition of competitor (E2) and phages that were eluted from the beads by TEA (E3) were used to infect *E.coli*. To limit growth bias caused by clonal differences, the infected bacterial cells were further grown on large LB-TAG Bio-assay dishes. Thus, starting from the second round of selection, every library that was subjected to selection, became two sub-libraries. Both were subsequently channeled into the following selection round (Figure 7 A). An overview of the selection strategy is shown in Figure 7 B.

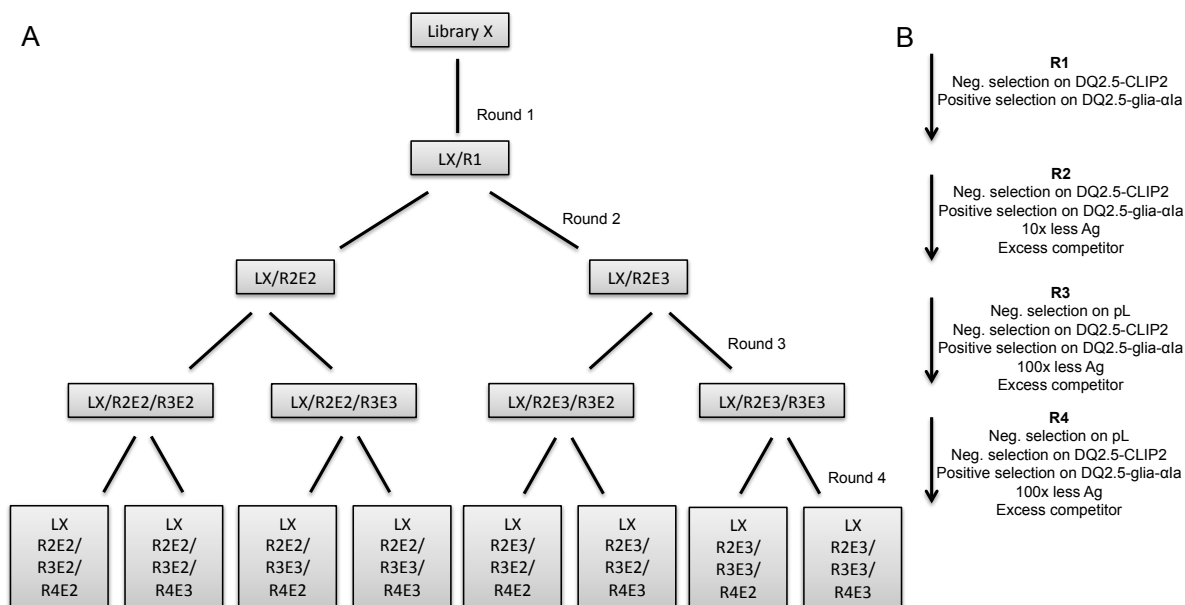


Figure 7: (A) Diagram showing how one library evolves through each round of selection to give several sub-libraries. (B) Overview of the selection strategy.

4.1.3 Detection of scTCR- and scFv-encoding phagemid by PCR

Due to the scFv phage library contamination (supplementary 2), the scTCR phage libraries were monitored throughout the new selection. The phage libraries resulting from each round of selection were screened for presence of phagemid harboring scTCR s380 and phagemid harboring human scFv with kappa light chain. This was conducted by PCR (described in 3.4.1). A common forward primer that anneal to the same vector element in both phagemids was also used. Furthermore, in order to discriminate between the two phagemids, the reverse primers were design to anneal to different regions in the scTCR and scFv cassettes to yield products of different sizes. The reaction samples were analyzed by gel electrophoresis (described in 3.5.2).

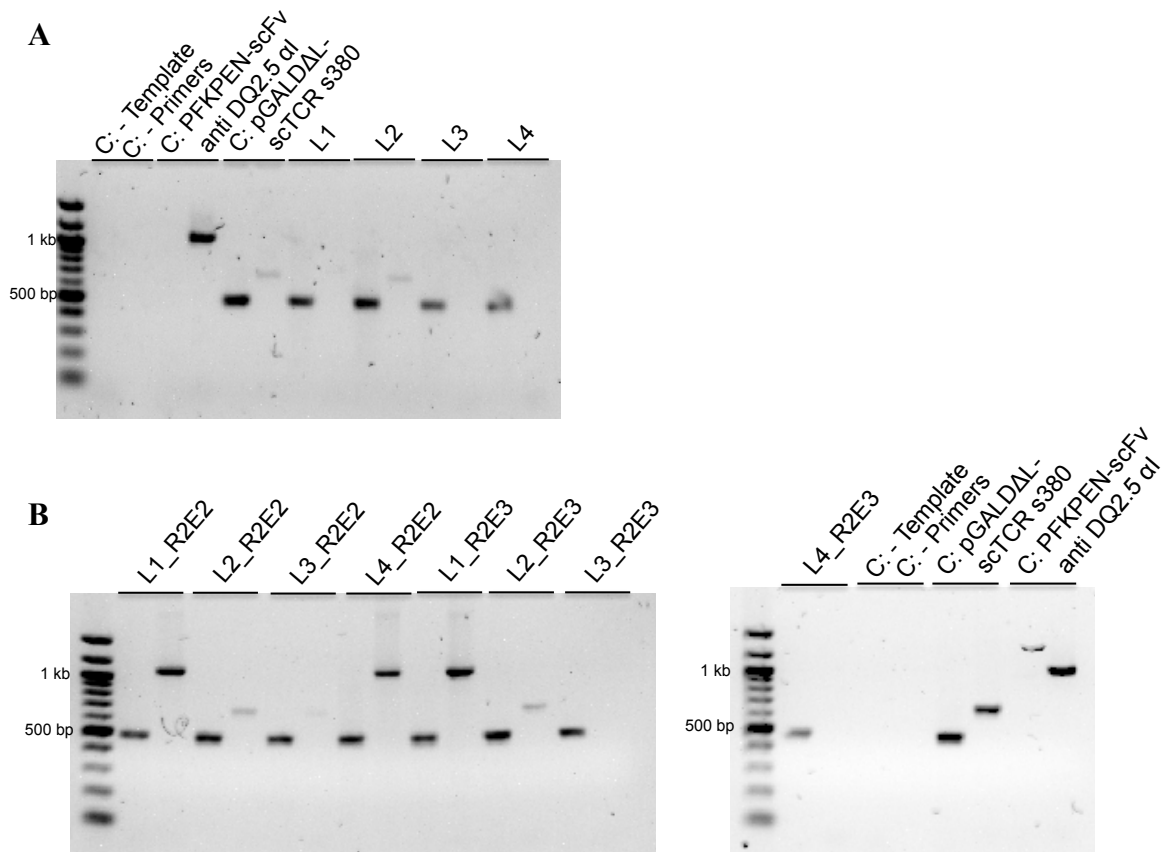


Figure 8: Gel electrophoresis showing the result of PCR analysis of the phage libraries resulting from selection R1 (A) and selection R2 (B). Two and two wells are marked according to the library template that was used. The first and the second well corresponds to the PCRs performed with primer set specific for scTCR s380- and scFv-encoding phagemid, respectively. Six controls were included in the PCRs and are shown. 100 bp DNA ladder was included in all gels and the relevant fragments are marked.

As seen, PCR products were not generated in absence of template or primers (Figure 8 A-B). When purified plasmids encoding scTCR s380 and human scFv also were used as template, both primer sets resulted in amplification of fragments of expected size (Figure 8 A-B). An additional band corresponding to a fragment of app. 700 bp was obtained with the scFv primer set when pGALD9ΔL scTCR s380 was used as template. No band was observed when the scTCR primer set was used with the scFv-encoding plasmid.

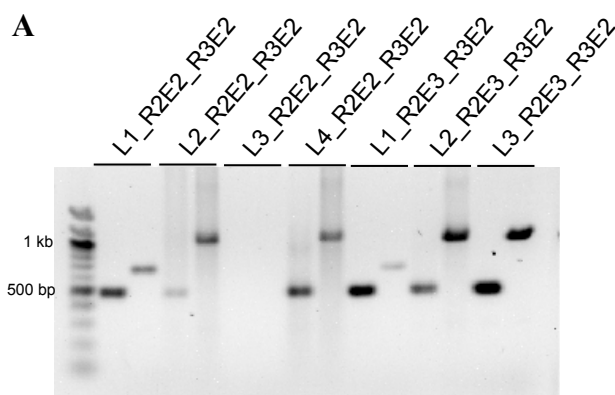
For the library samples resulting from selection R1, bands of expected size of about 500bp were obtained using the scTCR primer set (Figure 8 A), indicating presence of scTCR s380 phagemid throughout. With the scFv primer-set no bands of expected size were obtained for any of the library samples, indicating that scFv phagemid was not present. However, again, the weak band at about 700 bp was observed for one of the library samples.

PCR analysis of the phage libraries from selection R2 revealed that three of eight libraries contained scFv-encoding phagemid, as bands corresponding to fragments of 1000bp were obtained using the scFv primer set (Figure 8 B). All eight libraries were also positive for scTCR-encoding phagemid, with clear bands of expected sizes obtained with the scTCR primer set. The eight libraries originated from the original four libraries. The contamination was found in two sister libraries, L1E2 and L1E3, and in L4E2. In L4E3, the sister library of L4E2, no contamination was found however.

4.1.4 Negative selection of scFv-displaying phages

In order to prevent or limit the propagation of scFv phagemids within and between the scTCR s380 CDR3 α and CDR3 β libraries, an additional step was included in the selection protocol as of round three. Prior to negative selection on DQ2.5-CLIP2, the libraries were negatively selected on pL (described in 3.3.1). pL interacts with high affinity with variables domains of the kappa light chain family (except V domains of the V κ II family) [98, 99] and have previously been used for functional purification of phage libraries, including the scFv library in question here [97]. Only phages that were not bound to pL were channeled into R3 and R4 selections as before.

To find out whether or not pre-selection on pL had been successful in preventing scFv propagation, the libraries resulting from the third and fourth round of selection was analyzed by PCR as in 4.1.3 and as described in methods (3.4.1). The reaction samples were visualized by gel electrophoresis (described in 3.5.2).



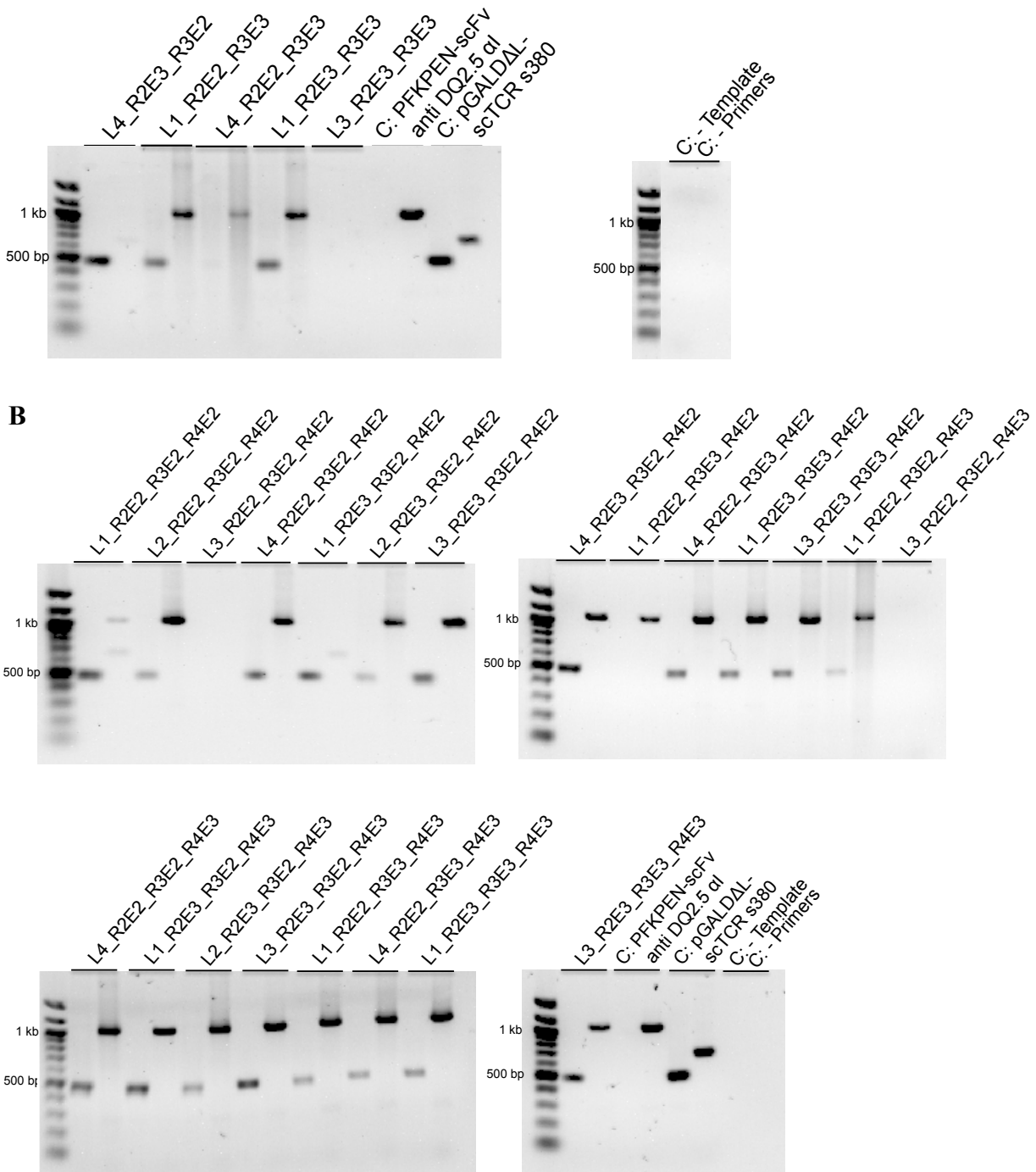


Figure 9: Gel electrophoresis showing the result of PCR analysis of the phage libraries resulting from selection R3 (A) and from selection R4 (B). Two and two wells are marked according to the library template that was used. The first and the second well correspond to the PCRs performed with primer set specific for scTCR s380- and scFv-encoding phagemid, respectively. Six controls were included in the PCRs and are shown. 100 bp DNA ladder was included in all gels and the relevant fragments are marked.

Seven of the twelve libraries were positive for scFv-encoding phagemid, as the PCR resulted in amplification of a 1000 bp fragment with the scFv primer set. Furthermore, nine of the twelve libraries derived from selection R3 were positive for scTCR-encoding phagemid, as the PCR resulted in amplification of a 500 bp fragment with the scTCR primer set. For two of the libraries no bands were obtained with either of the primer sets. Thus, the contamination was indeed present after negative selection on pL, but as the scTCRs were also present, the scFv displaying phages had not overgrown the culture.

Of the 22 libraries derived from selection R4, one L1 sub-library was positive for scTCR-encoding phagemid only. 18 libraries were positive for both scTCR-encoding and the scFv-encoding phagemids. Bands of expected size and of varying strengths were obtained with both primer-sets. For two L3 sub-libraries no bands were obtained with either of the primer sets.

Analysis after selection R3 and R4 revealed that scTCR is present, even if the occurrence of scFv-encoding phagemid increased with every round.

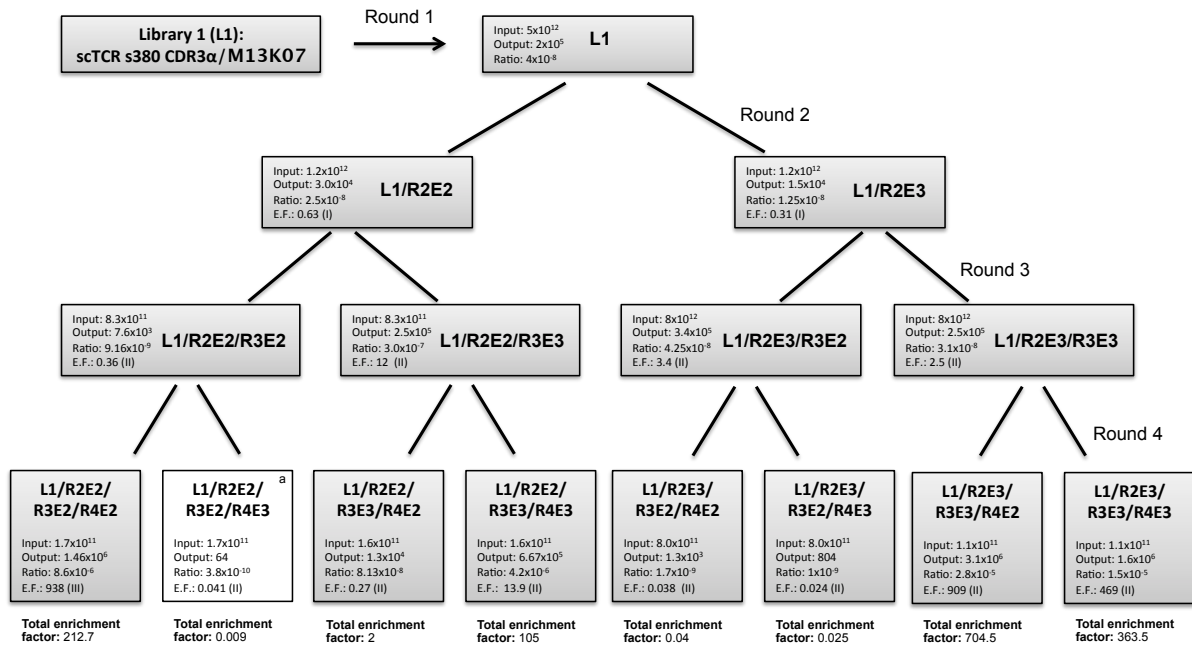
4.1.5 Phage enrichment

The selection process is designed to enrich for target specific clones, and whether selection has lead to enrichment, can be predicted by calculating the EF. In order to calculate the EF, the number of phages that are added to each selection round (input) and the number of phages that are recovered in each round (output) has to be determined.

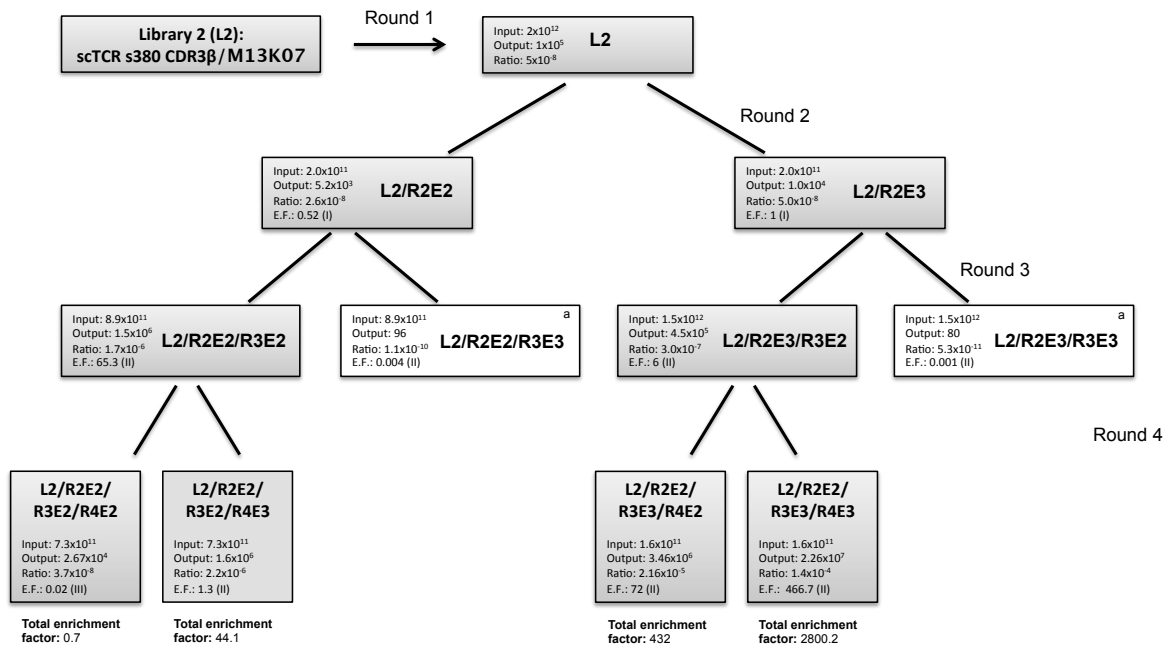
The initial phage library titers (R0) were determined prior to selection in order to perform selection round one with a complexity of 1000 based on library diversity. The calculated inputs corresponding to this complexity were based on titers obtained by spot titration (described in 3.2.2) of the generated libraries. Starting from round two, 100 µl amplified and precipitated phage output from the previous round was used as input in the following round. The concentration in this input volume was determined by spot titration after all four selection rounds were completed. The phage output was determined directly after each selection round, as described in 3.3.3. The enrichment factors were calculated for the various libraries between the selection rounds and are shown in Table 7.

Table 7: Phage enrichment

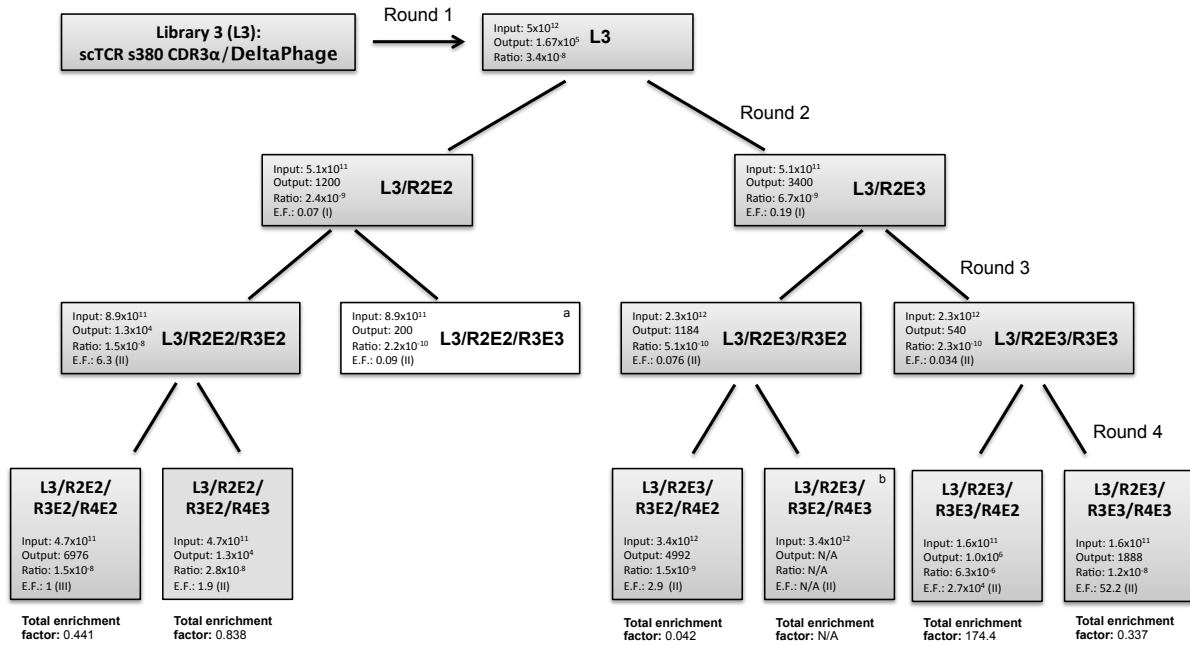
A



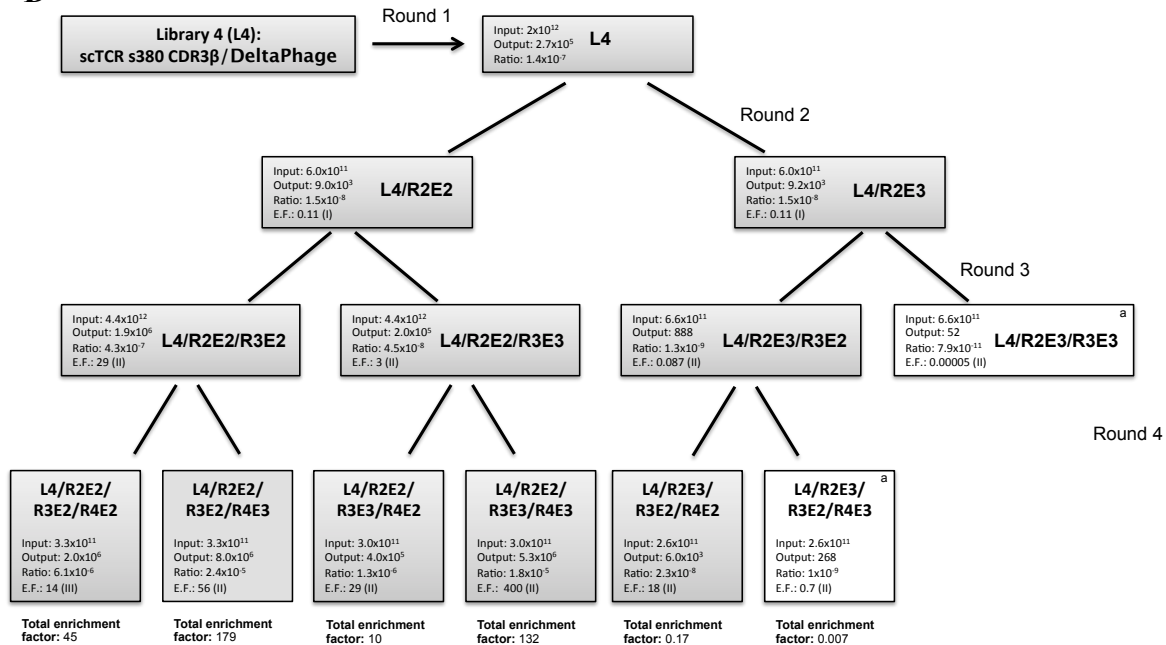
B



C



D



^a The libraries in the white boxes were not rescued due to low phage numbers and were therefore not propagated into the next round of selection

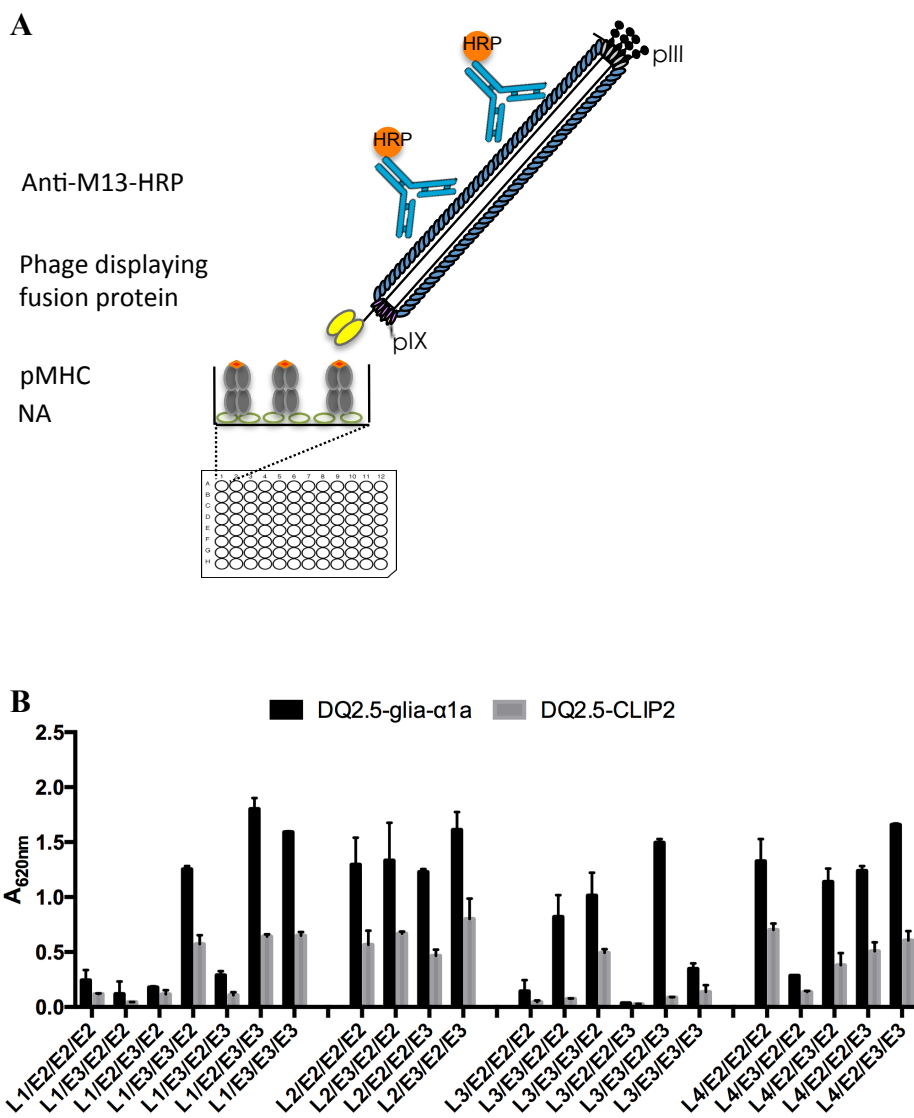
^b The enrichment factor for one of the L3 sub-libraries could not be calculated because the output from R4 was not available

Enrichment was found in four of eight L1 and in three of four L2 subgroups. The same was found in one of five L3 sub-groups and in four of six L4 sub-groups. Thus, the results do indeed indicate enrichment of target specific clones.

4.2 Screening of scTCR phage libraries

4.2.1 Polyclonal ELISA of M13K07-rescued R4 phage output

To investigate whether or not any of the phage libraries contained binders to DQ2.5-glia- α 1a, a polyclonal ELISA (described in 3.4.2) was performed as shown in Figure 10 A. In short, the output from round four was rescued with M13K07 and precipitated phages were tested for binding to DQ2.5-glia- α 1a and DQ2.5-CLIP2. Binding of scTCR s380 wt displayed on phage to DQ2.5-glia- α 1a is not detected in ELISA, and is probably due to the low affinity. The same ELISA can therefore be used to check for specific binders with improved affinity.



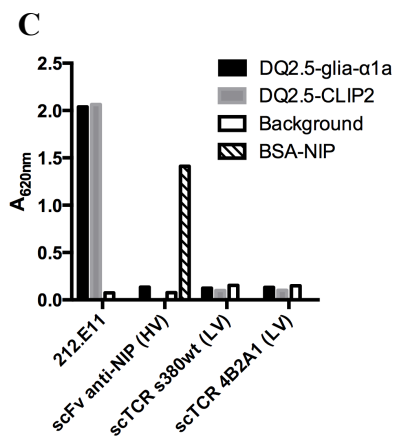


Figure 10: Polyclonal ELISA of phage libraries from selection R4. (A) Schematic setup of ELISA used for testing of phage library binding to DQ2.5-glia- α 1a and DQ2.5-CLIP2. (B) ELISA binding of the various sub-libraries. The library name was shortened by removal of RX. (C) ELISA binding of control samples. The experiment was performed in duplicate and the mean \pm sd is shown. The phage libraries were also added to empty wells to monitor binding to the blocking agent (data not shown).

Many of the M13K07-rescued phage libraries showed preferential binding to DQ2.5-glia- α 1a (Figure 10 B). Three of seven L1 subgroups and three of six L3 subgroups were positive for binding, and so were all four L2 subgroups and four of five L4 subgroups. Two of the reactive L3 libraries had particularly low background binding to DQ2.5-CLIP2.

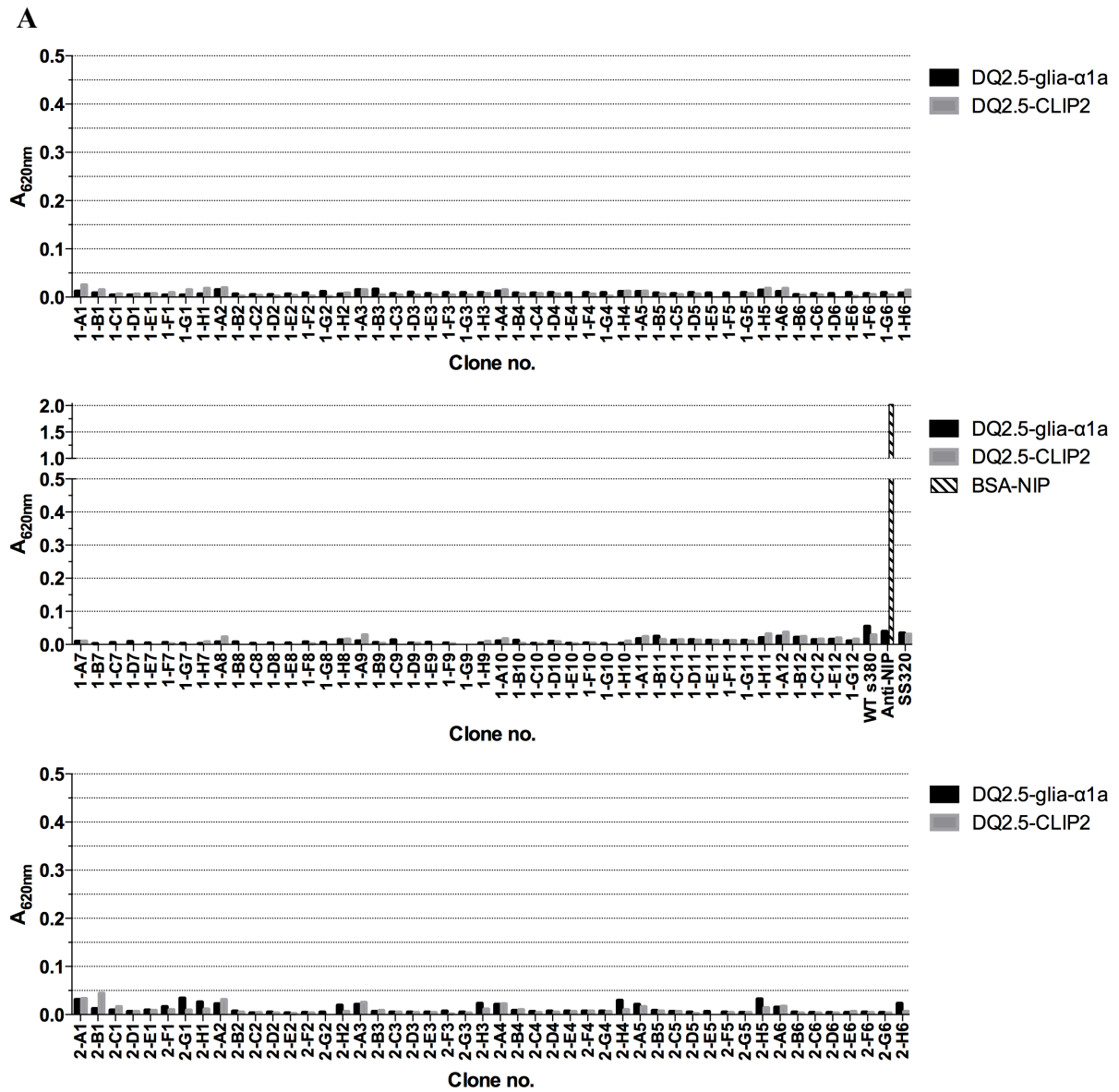
Both DQ2.5-glia- α 1a and DQ2.5-CLIP2 complexes were detected with anti-DQ2 2.12.E11 antibody (Figure 10 C) at high and similar signal intensity, indicating that equal and sufficient amounts of target molecules were available in the wells for phage library binding. Phages displaying the I-E^d restricted scTCR 4B2A1 at low valence were negative for binding to DQ2.5-glia- α 1a [100]. Phages displaying scFv anti-NIP at high valence showed strong antigen-specific binding to BSA-NIP.

Phages displaying wt scTCR s380 on pIX at low valence showed no reactivity in line with unpublished results from the research group. Thus, the positive binding signals obtained for several of the phage libraries might be caused by the presence of specific clones with higher affinity than the wt clone.

4.2.2 Phage packaging of single clones and ELISA

Since L2/E3/E2/E2 showed preferential binding to HLA-DQ2.5 glia- α 1a in the polyclonal ELISA, this output was selected for further investigation at single clone level. To screen for high affinity binders, a total of 186 single clones were separately grown and rescued with

DeltaPhage to achieve multivalent display of the fusion protein, and the packaged virions were subsequently tested in ELISA for binding to HLA-DQ2.5-glia- α 1a and -DQ2.5-CLIP2 (described in 3.4.3). The clones were grown in a total of two deep well plates and the following three controls were included in separate wells in each plate; pGALD9 Δ L-scFv anti-NIP, wt pGALD9 Δ LFN- scTCR s380 and *E. coli* SS320 cells without phagemid.



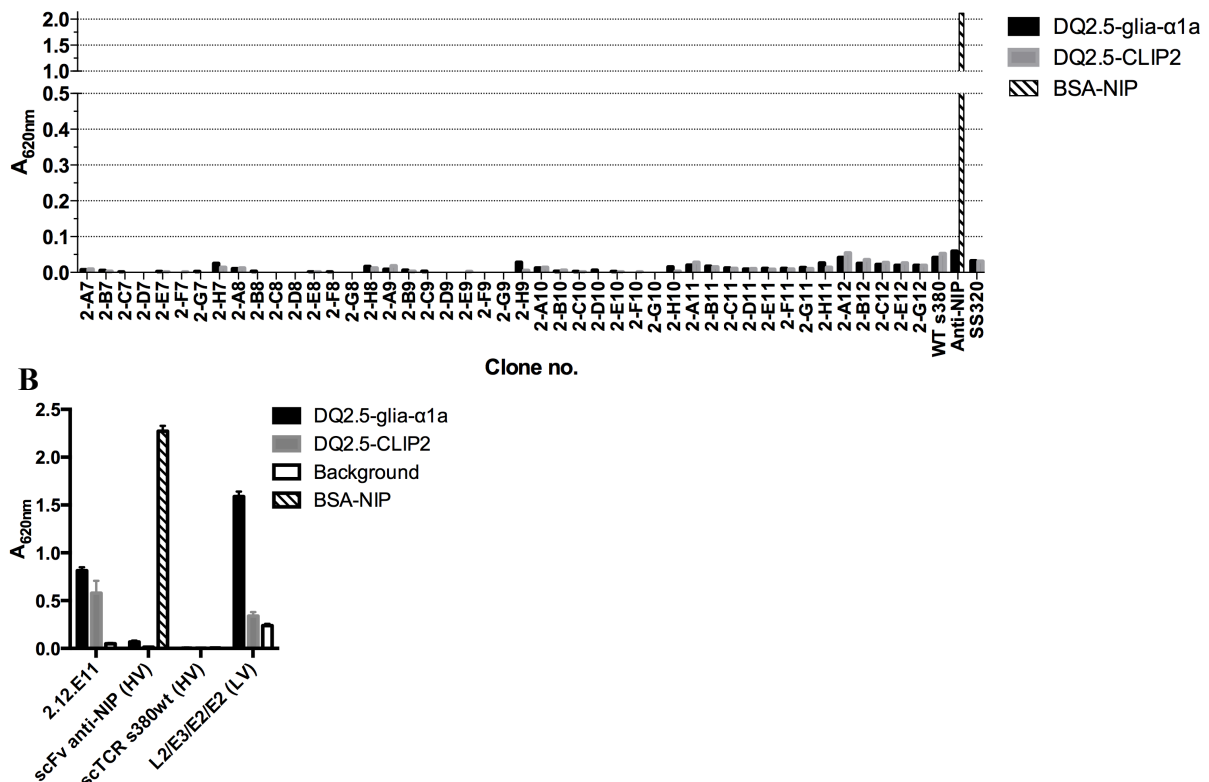


Figure 11: Single colony screening of L2/E3/E2/E2 from selection R4. The same ELISA setup as depicted in Figure 10 A was used. (A) ELISA measurements of single clones that were grown in deep well plates and named according to plate number and position in the plate, e.g. 1-A1; plate number 1, well A1. The controls are shown as the three last bars in the second and fourth graph. (B) ELISA measurements for control samples that were included for the ELISA setup.

Background binding of the negative controls to HLA-DQ2.5 glia- α 1a was measured up to A_{620nm} 0.06. The single clones that were screened were all negative for binding to HLA-DQ2.5 glia- α 1a, showing A_{620nm} values below the background signal. The pGALD9 Δ L-scFv anti-NIP controls that were included in the phage packaging showed strong antigen-specific binding (Figure 11 A), indicating that the conditions were satisfactory for phage production.

Both DQ2.5-glia- α 1a and DQ2.5-CLIP2 complexes were detected with anti-DQ2 2.12.E11 antibody (Figure 11 B), with signal intensities in the same range indicating that the target molecules were present in roughly equal amounts per well. As seen before (4.2.1), preferential binding to DQ2.5-glia- α 1a was observed for the phage library, that the single clones were derived from, L2/E3/E2/E2, which also confirms that sufficient quantities of pMHC were available for phage binding. In line with unpublished data from the research group, phages displaying wt scTCR s380 on pIX at high valence were negative for binding to

DQ2.5-glia- α 1a. Again, phages displaying scFv anti-NIP at high valence showed strong antigen-specific binding towards BSA-NIP.

Even though this method is known to give false negatives and positives (Løset G.Å., personal communication), it was surprising that there were no positive hits among the 186 clones that were tested, especially because the phage library that the clones came from showed strong preferential binding for the second time. Further investigation was needed.

4.2.3 Sequencing of single clones

To determine the identity of a portion of the screened clones, 22 single phagemids were isolated from ON cultures (described in 3.5.1), and the POI-encoding cassette was sequenced (described in 3.5.3).

The POI-encoding cassettes of all 22 phagemids were identical in sequence. This sequence could not be aligned with the scTCR s380 wt sequence, but alignment with three relevant scFv anti DQ2.5-glia- α 1a clones gave a match, namely, a scFv anti DQ2.5-glia- α 1a clone that was dominating the R3 output of the recent scFv phage library selection. Specific binding of the scFv anti DQ2.5-glia- α 1a in soluble form has been detected in ELISA repeatedly. This was not observed in the library screening (4.2.2) however.

4.3 Trouble shooting

4.3.1 Spot titration of scFv phage cultures

To investigate how efficiently the scFv anti DQ2.5-glia- α 1a clone was produced upon DeltaPhage rescue in the screening, spot titration (described in 3.2.2) was performed on the supernatants of the same 22 samples that were sequenced (4.2.3). The two pGALD9 Δ L-scFv anti-NIP controls were included in the titration. The results are illustrated in Figure 12 and revealed that the end titers obtained for the clone were low compared to the two controls.

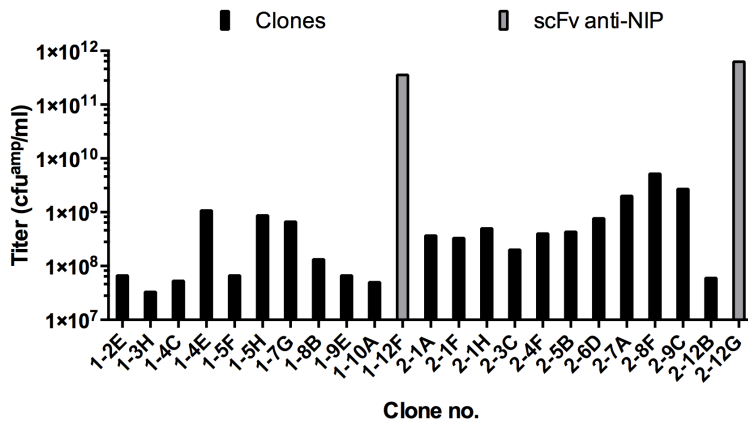


Figure 12: End titers of the scFv anti DQ2.5-glia- α 1a clone after DeltaPhage-rescue in screening. The results are given as amp resistant colony forming units (cfu^{amp}) per ml.

Even though a source for pIX capsid was missing when the scFv clone, encoded in pSEX for display on pIII, was rescued with DeltaPhage in the amber non-suppressor host *E. coli* SS320, infectious phage particles were produced. However, the appearance and structure of these particles is unknown. The lack of signal in the single clone ELISA (4.2.2) could thus most likely be explained by the low titers, if the rescued phages display the fusion protein at all.

4.3.2 Phagemid rescue of pSEX scFv anti-DQ2.5-glia- α 1a and ELISA

To study the specific binding properties of scFv anti-DQ2.5-glia- α 1a, an ELISA was performed. In short, the phagemid was rescued with either M13K07 or DeltaPhage in multiple parallels using the same conditions as for library rescue, and PEG precipitated phages were tested using the same setup and phage input as in the polyclonal ELISA (4.2.1).

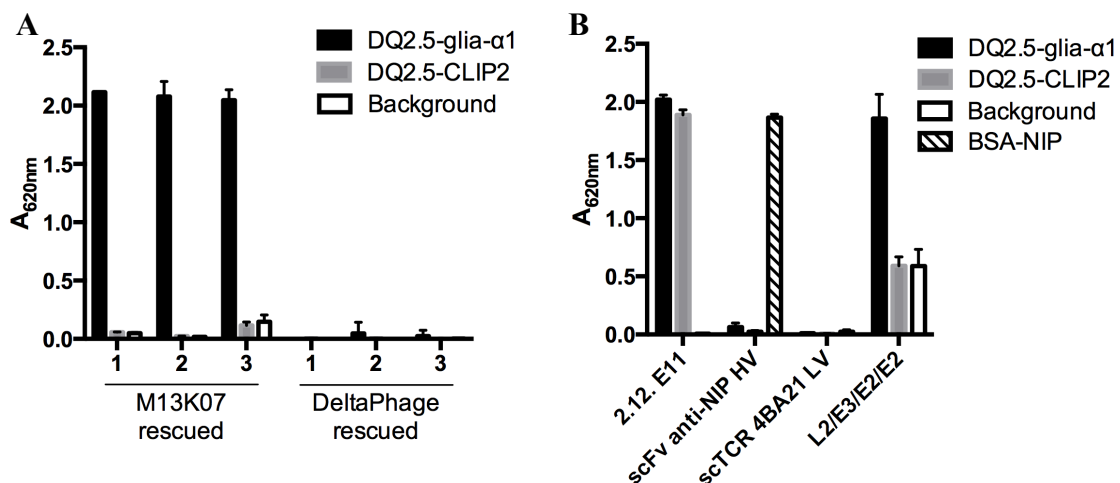


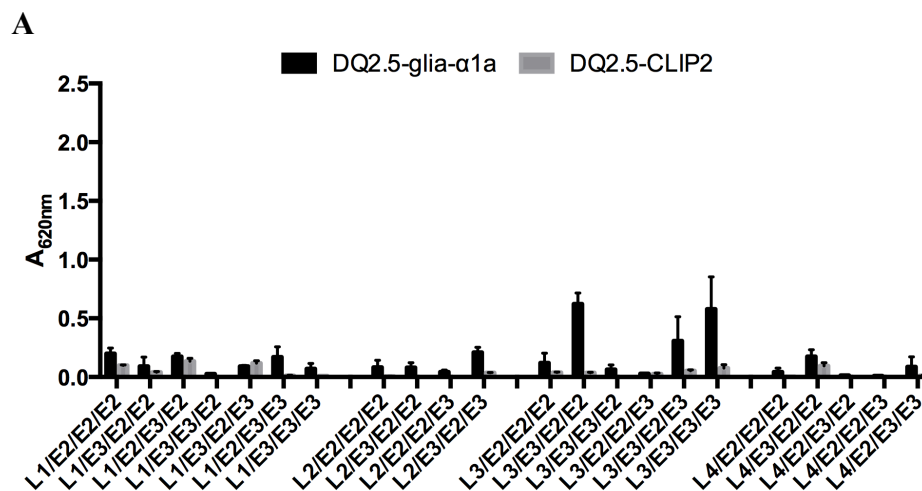
Figure 13: Phage ELISA on scFv anti-DQ2.5-glia- α 1a. (A) ELISA binding of scFv anti-DQ2.5-glia- α 1a phages to DQ2.5-glia- α 1a, DQ2.5-CLIP2 and to the blocked coat (termed background), including three M13K07-rescued and three DeltaPhage-rescued samples of the clone. (B) ELISA binding of control samples. The ELISA experiment was performed in duplicate and the mean \pm sd is shown.

The results confirmed that when the scFv-encoding pSEX phagemid is rescued with M13K07, strong preferential binding to HLA-DQ2.5-glia- α 1a is detected (Figure 13 A). However, antigen binding was not detected when rescue was performed with DeltaPhage. This could possibly be exploited to limit the rescue of scFv phagemids present in the scTCR phage libraries, and to knock down the signals caused by scFv clones in the polyclonal ELISA.

4.4 Screening of scTCR phage libraries with new discovery

4.4.1 Polyclonal ELISA of DeltaPhage-rescued R4 phage output

To further investigate if any of the phage libraries contained antigen specific scTCR s380 clones, the output from selection round four was rescued with DeltaPhage and precipitated phages were tested in ELISA for binding to DQ2.5-glia- α 1a and DQ2.5-CLIP2.



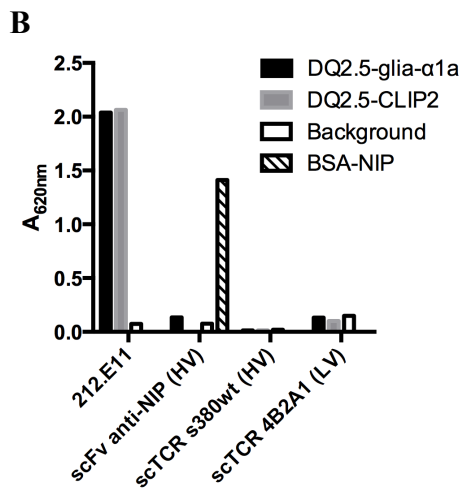
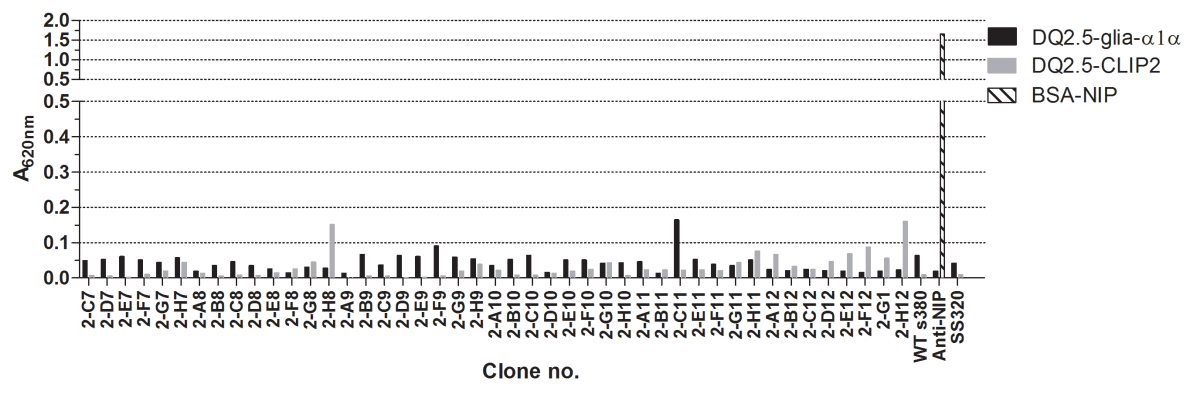
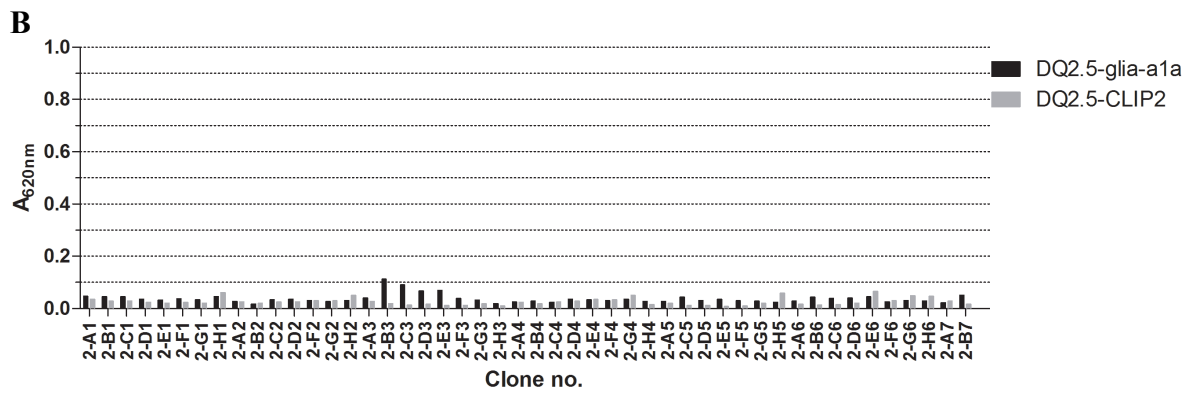
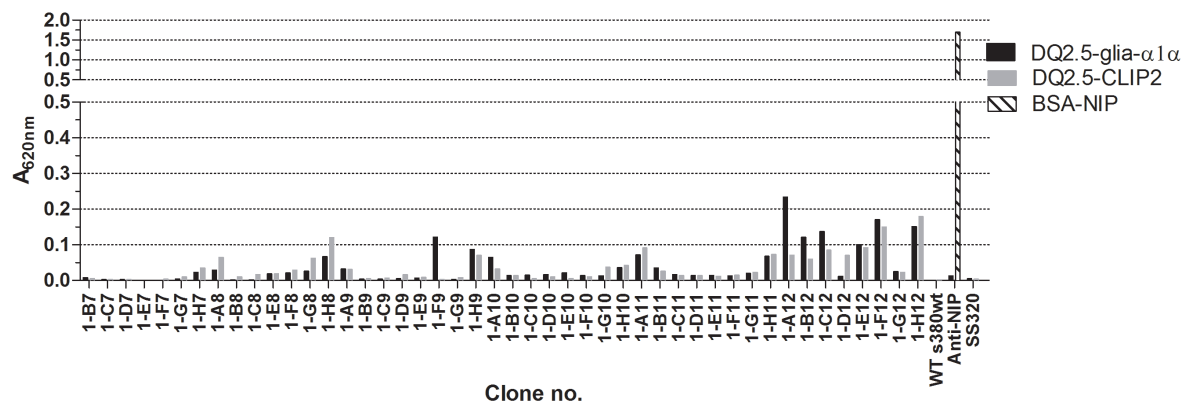
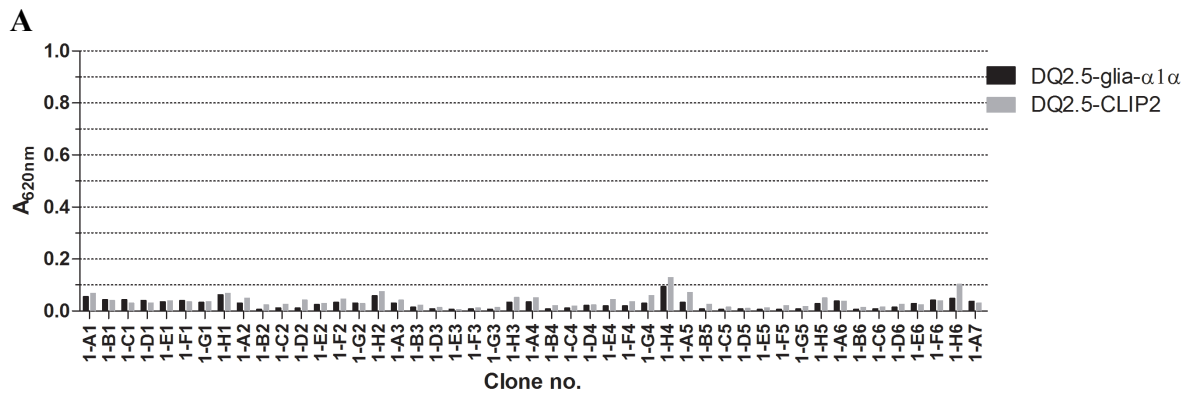


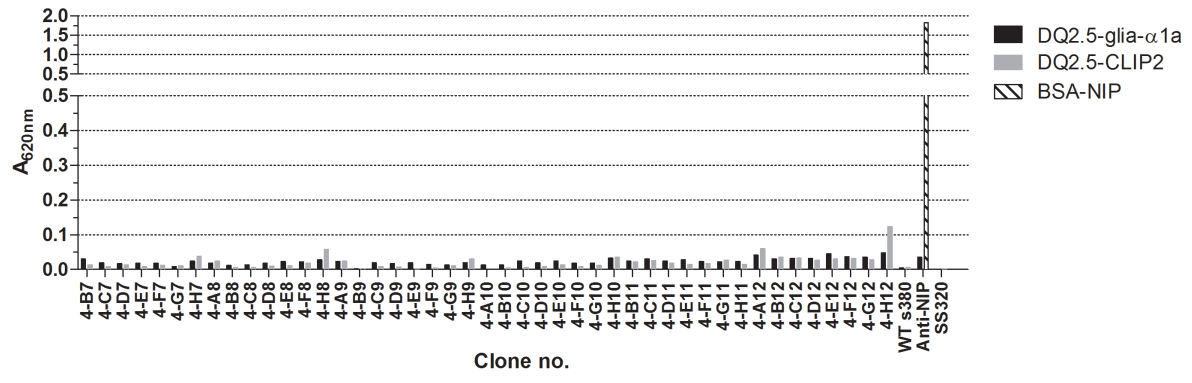
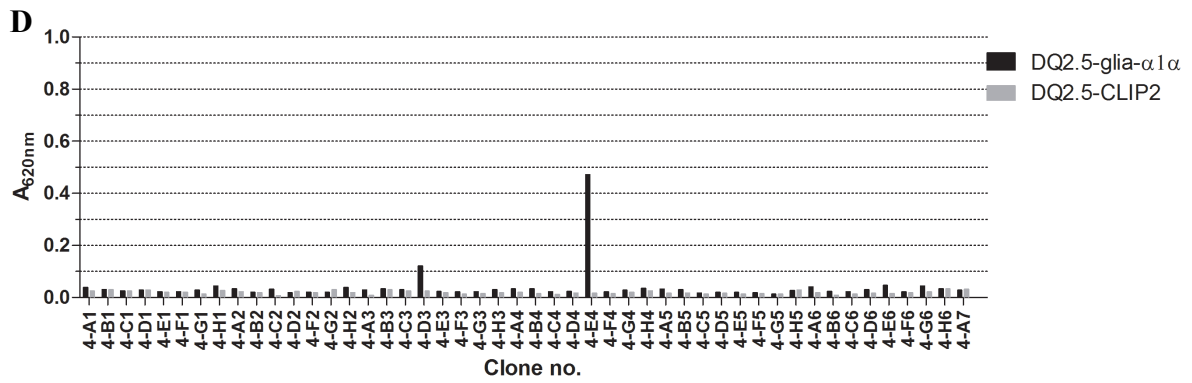
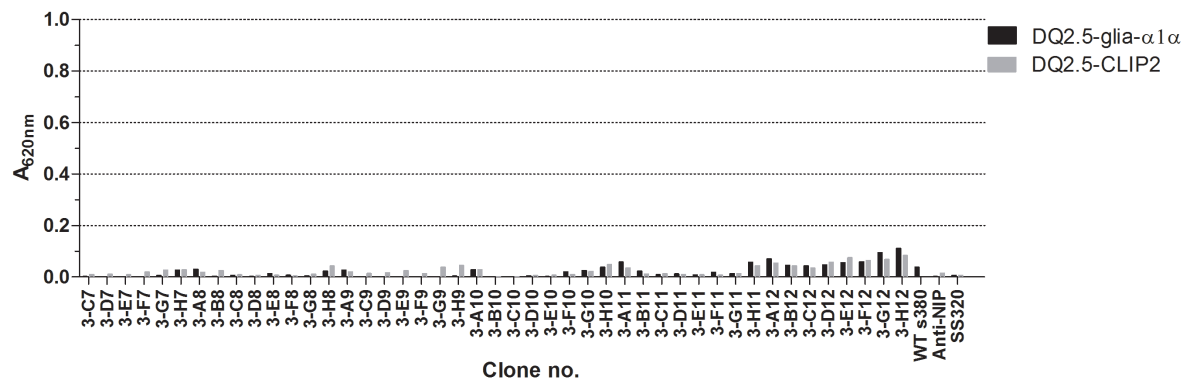
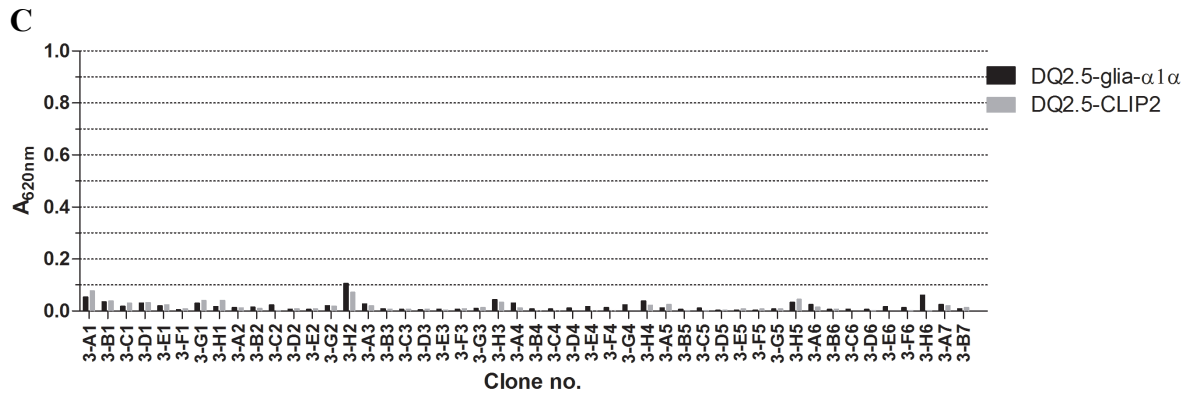
Figure 14: Polyclonal ELISA of R4 phage libraries rescue by DeltaPhage. The same ELISA setup as depicted in Figure 10 was used. (A) ELISA binding of the various sub-libraries to DQ2.5-glia- α 1a and DQ2.5-CLIP2. (B) ELISA binding of control samples. The experiment was performed in duplicate and the mean \pm sd is shown. The phage libraries were also added to empty wells to monitor binding to the blocking agent (data not shown).

Compared to ELISA results obtained with M13K07-rescued R4 phage output, most of equivalent DeltaPhage-rescued libraries showed reduced or no reactivity towards DQ2.5-glia- α 1a. However, three subgroups of L3 were positive and one of them even showed increased binding to DQ2.5-glia- α 1a. Interestingly, L3 is a CDR3 α library that was rescued with DeltaPhage initially, and has been through two rounds of selection with high valence display.

4.4.2 Phage packaging of single clones and ELISA

The two libraries with strongest signal in the polyclonal ELISA (4.4.1), L3/E3/E2/E2 and L3/E3/E3/E3, were selected for further investigation at single clone level. The screening for high affinity binders was conducted in the same manner as in 4.2.2. Thus, 186 clones were analyzed from each of the two libraries and grown in a total of four deep well plates. The following three controls were scattered to different wells in each deep well plate; pGALD9 Δ L-scFv anti-NIP, wt pGALD9 Δ LFN- scTCR s380 and *E. coli* SS320 cells without phagemid.





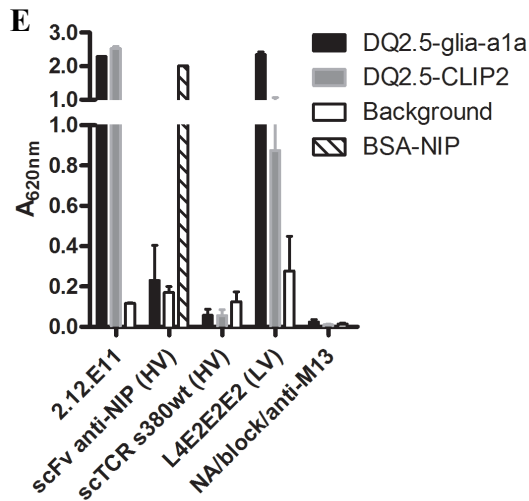


Figure 15: Single colony screening of L3/E3/E2/E2 (A-B) and L3/E3/E3/E3 (B-C) from selection R4. ELISA measurements for the individual clones are shown. The clones are named according to plate number and position in the plate, e.g. 1-A1; plate number 1, well A1. The three controls are indicated by name. (E) ELISA measurements for control samples that were included for the ELISA setup. The same ELISA setup as depicted in Figure 10 A was used.

Two distinct clones, namely 1-A12 and 4-E4, showed A_{620nm} values above the highest background signal, making them interesting candidates. To determine their identity, their phagemids were isolated from ON cultures and the POI-encoding cassette was sequenced. To get an overview of the clone population, an additional 22 clones were sequenced. The quality of the obtained sequences was low. However, the identity of the two candidate clones were revealed and proved to be clones of scFv origin.

5. Discussion

The low affinity of native TCRs for pMHC prevents the direct use of a soluble TCR as a detection reagent [41]. The DQ2.5-glia- α 1a restricted TCR 380, which has all the characteristics of a disease driven T cell response, was chosen for affinity maturation. As the glia- α 1a T cell epitope is one of two immunodominant gluten peptides [78], a specific DQ2.5-glia- α 1a tracker should be highly useful in the characterization of antigen presentation in CD. In addition to the intrinsically low affinity of the TCR for pMHC, the lack of reliable expression systems for soluble TCRs have slowed down their development as recombinant tools.

We have previously shown that FkpA overexpression has a major effect on soluble periplasmic expression of scTCRs as well as on the display levels on the surface of phage [45, 46]. FkpA encoding phagemids were therefore used, enabling chaperone co-expression during selection. Several reports have been published, highlighting the effect of introducing stabilizing mutations in the scTCR format affecting both yield and protein stability. In line with this, the wt TCR 380 was stabilized by introduction of three point mutations [42, 49], which resulted in increased display levels on pIX capsid and increased yield of soluble scTCR (Neuman, Høydahl et al, unpublished).

The scTCR s380 was cloned into the phagemid vector for pIX display. We have previously reported a side-by-side comparison of display on pIII with display on pVII and pIX, and shown that affinity selections of folded domains are particularly efficient when displayed on pVII or pIX [6].

The CDR3 α and CDR3 β loops of s380 were selected for initial randomization to generate phage libraries based on the assumption that standard diagonal docking mode applied. Crystal structures of numerous of TCR-pMHC complexes have shown that most peptide contacts are made by the CDR3 loops of the TCR and thus are likely to be the key determinants of TCR specificity. Targeting of the CDR1 and CDR2 loops, which contacts the MHC molecule primarily, could lead to loss of MHC restriction and thus specificity [2]. Furthermore, recently reported crystal structures of TRAV7-2+ TCRs in complex with DQ2.5-glia- α 1a and DQ2.5-glia- α 2 were indeed shown to use standard TCR-pMHC docking [90].

Phage library selection was performed with addition of competitor, which serves two purposes. In addition to being a way of selecting for increased off-rate, by removing fast-dissociating phages and thus leaving behind binders of higher dissociation rate [101], it is also a way to functional elute phages. Different parameters, like incubation time and concentrations of antigen and phage, may be adjusted to elute clones with desired affinities [102]. However, very long incubation times may be required to elute high affinity binders, and thus other strategies may be more convenient. Hence, 100 mM TEA solution at pH 11 was used to elute phages that remained bound after incubation with competitor. This step was modified from standard protocols by extending the incubation time to increase the change of retrieving potential high affinity clones [103].

In the selection strategy used, both eluates resulting from competitive elution and from elution with high pH were used to infect *E.coli* for amplification and amplification. The competitor eluate (E2) is expected to include a high portion of phages displaying functional scTCRs, however with moderate off-rates. Clones of higher affinities in the picomolar range are expected to be in the TEA eluate (E3), but together with more background clones that are released simultaneously.

The scTCR phage libraries were monitored for presence of scTCR s380 clones and human scFv clones with kappa light chain using PCR. Despite continued precaution, scFv-encoding phagemid was detected in three of eight libraries after the second round of selection. Effort was made to limit the propagation of the scFv-encoding phagemid by pre-selecting the phage libraries on pL. Nevertheless, the occurrence of scFv-encoding phagemid increased with every round. Fast-growing sequences, called parasite clones, can have 1000x enrichment during amplification in *E. coli* [104]. Escape of a few scFv clones in negative selection may thus be sufficient for subsequent retrieval in positive selection and propagation. The scTCR-encoding phagemids were also detected in the libraries however, and thus the scFv displaying phages had not overgrown the cultures.

Enrichment of target specific clone or clones was found in 12 of the 22 sublibraries. These 12 libraries were positive for both the scTCR-encoding phagemid and the scFv-encoding phagemid in the PCR. Nine of the sub-libraries that showed positive enrichment were also positive for binding to the relevant target in ELISA after M13K07 rescue, while the last three were negative. The assumption that target specific scFv binders caused the positive signals

obtained in the ELISA performed with the M13K07-rescued phage libraries is supported by the great reduction or total loss of signal when the same libraries were tested for binding after DeltaPhage rescue. Rescue of pIX-scTCR-encoding phagemid with DeltaPhage is expected to result in production of phage with high valence display of the scTCR, which in turn is expected to amplify the signal in ELISA due to avidity effects [21]. This was not observed however.

When rescue of pIII-scFv-encoding phagemid was performed with DeltaPhage, relatively low end titers were achieved. This aspect could possibly be exploited to limit proliferation of scFv-displaying phages during the amplification step. However, this would result in high valence display of the scTCR, which is not favorable in affinity selection.

Furthermore, when rescued with M13K07, a higher portion of the CDR3 β libraries showed preferential binding to DQ2.5-glia- α 1a in ELISA than the CDR3 α libraries. In the context of the newly reported crystal structures [90], this is an interesting finding. For the CDR3 β loop of DQ2.5-glia- α 2 restricted TCRs that express the TRVB7-2 gene segment, a semi-conserved sequence, including a highly conserve arginine, is observed. This conservation suggests an important role of the CDR3 β loop in mediating binding to the HLA molecule, and thus mutations in this loop could potentially lead to the loss of binding ability. Indeed, the CDR3 β loop may be more sensitive to sequence alterations than the CDR3 α loop, and thus library generation by randomization of the CDR3 β loop could lead to a greater portion of non-functional clones, and furthermore easier access of the scFv clone, compared to CDR α phage libraries.

Single clone screening was conducted in small cultures in 96 deep well plates. Based on results obtained in the research group, high valence display of the fusion protein is considered necessary to uncover binders in this type of screening assay. Even a clone with relatively high monomeric affinity has proved hard to detect when displayed on pIX at low valence (unpublished, Nilssen, N. R. et al.). Screening of two L3 sublibraries identified two potential candidate clones, one from each sublibrary, which revealed by sequencing was the scFv clone. However, sequencing also revealed that scTCR clones were present in the clone population, as indicated by the PCR analysis. The assay is known to give false positives and negatives and, as observed repeated times by other group members (unpublished), the signal

strengths of one unique clone may vary greatly, even in the same experiment. Thus, repeating the screening could lead to other candidate clones.

The specificity of the scFv clone partly explains how the clone was selected in parallel with the scTCR clones during selection, but characterization of the scFv clone revealed a relatively low affinity for DQ2.5-glia- α 1a (unpublished, Høydahl, L.S.). However, unlike the scTCR that was displayed on pIX, the scFv clone was displayed on pIII and fusion proteins are displayed at higher frequency on pIII than on pIX when rescue is performed with M13K07 [21, 105]. Thus avidity effects, caused by heterogeneous oligovalent display on pIII, provide further explanation for the efficient retrieval of scFv-displaying phage during selection.

Growth bias arises during the amplification step between selection rounds, because different sequences amplify in bacteria at different rates [104]. Small differences in sequence can have large impact on the bacterial host viability and hence on the amplification. There are evident differences between TCRs and antibodies when it comes to expression of these proteins as recombinant molecules in *E.coli*. Compared to scTCR, scFv is typically much more amenable to production, both as soluble molecules and as fusion proteins on phage [42], and thus appears to be less toxic to the host. On top of this, the scFv clone was derived from a refined scFv phage library, which featured particularly high scFv expression and display levels [97]. Together, these factors result in considerable advantages for the scFv over the scTCR, and hence provide an explanation for the outcome of scTCR phage library selection.

6. Future perspectives

The outcome of an affinity selection is dependent on numerous factors, including the quality of the start-up libraries and the selection conditions used. Libraries comprising high diversity are more likely to result in retrieval of high affinity ligands compared to libraries with lower diversity [15].

A PCR protocol was established to detect scFv-encoding and scTCR-encoding phagemids in phage library samples. This enabled us to monitor their presence throughout the selection and observe that scTCR-encoding phagemid remained despite the contamination by scFv-encoding phagemid. Furthermore, a pre-selection step on pL was design to counteract scFv propagation.

Both the scTCR and the scFv encoding phagemids harbored the same antibiotic resistance gene. By cloning the scTCR-encoding cassette into a phagemid that contain a chloramphenicol resistance-encoding gene, which is available in our lab, one could to distinguish between the two phagemids and hence alleviate the scFv.

The scTCR s380 phage libraries used in the selection have some weaknesses that could be improved to increase the chance of succeeding in isolating high affinity scTCRs. Hence, the phage libraries comprised 40% TAA template background due to limited mutagenesis efficiency. In new libraries, a highly specialized template, which upon randomization is forced to make a palindrome cryptic RE site due to hairpin formation, may be exploited to remove the template after library mutagenesis [106]. Moreover, the mutagenic efficiency could be increased to approx. 80% with optimal conditions and a modified Kunkel protocol, which has resulted in 100% mutagenized libraries, could be used [107]. Members of the research group are presently working to optimize library construction (unpublished).

In addition, the phage phenotype-genotype linkage was disrupted when the DNA library transformation was conducted with high phagemid to cell ratio, which caused 20-30% of the *E.coli* population to contain more than one phagemid. This could be avoided by electroporation of smaller amounts of library DNA.

The selection conditions used in the affinity selection were modified throughout the process as new information was obtained, and thus several parameters have been optimized for our system, including the washing step and elution conditions. The same selection strategy would be applied in a new attempt to affinity mature the scTCR, preferably in parallel with selection conducted without off-rate selection, as different strategies tend to select different clones.

Identification of TCR candidate clones would need further validation, including investigation of expression yield in soluble form, and testing of binding specificity and affinity by surface plasmon resonance. Furthermore, independent mutations in the CDR3 α and CDR3 β could be combined to yield much higher affinities.

7. Supplementary results

7.1 Supplementary 1

Construction, selection and screening of CDR3 α and CDR3 β scTCR libraries performed by Lene Støkken Høydahl

Overview:

- CDR3 α and CDR3 β mutagenesis and library packaging
- Selection and polyclonal ELISA
- Illumina sequencing
- Library analysis by sequencing – library quality

CDR3 α and CDR3 β mutagenesis and library packaging

Assuming conventional TCR docking onto pMHC, the CDR3 loops of the TCR should primarily contact the peptide. These loops were therefore chosen for affinity maturation by random mutagenesis using a modified Kunkel mutagenesis protocol adapted from Siddhu and Tonikian et al [14, 108]. Briefly, by using uracil-containing ssDNA as template for library generation by complementary strand synthesis using degenerate primers, followed by *E. coli* SS320 electroporation, the uracil-containing template should be degraded. However, as the mutagenesis frequency is not 100% and not all template DNA will be degraded in *E. coli*, a TAA stop codon was inserted into CDR3 α and CDR3 β of the template by QuikChange mutagenesis (oligos in Table 9) to ensure that remaining template background would be rendered non-functional. Mutagenesis was done using templates both with and without FkpA-encoding gene to see if chaperone co-expression influence in the selection process. Various oligos were used to target different parts of the CDR3 loops (Table 8, Table 10).

CDR3 α loop	CDR3 β loop
CALSDHY <u>SSGS</u> SARQLTF	CASSTAV <u>LAGG</u> PQYF

Figure 16: CDR3 α and CDR3 β loops of TCR 380. CDR3 α and CDR3 β loop amino acid sequences are shown according to IMGT loop definition. Bold; residues actually forming the loop. Underlined; residues replaced by TAA stop codon.

Table 8: Mutagenic oligos for randomization of the CDR3 α and CDR3 β loops

CDR3α oligos			
<i>Oligo name</i>	<i>Sequence^a</i>	<i>Theoretical diversity</i>	<i>Mutagenesis strategy^b</i>
<i>Oligo 1</i>	<i>N-D<u>HYSSGSARQ</u>-C</i>	<i>6-mer (6.40x 10⁷)</i>	<i>NNK</i>
<i>Oligo 2</i>	<i>N-D<u>HYSSGSARQ</u>-C</i>	<i>7-mer (1.28x 10⁹)</i>	<i>NNK</i>
<i>Oligo 3</i>	<i>N-D<u>HYSSGSARQ</u>-C</i>	<i>6-mer (6.40x 10⁷)</i>	<i>NNK</i>
<i>Oligo 4</i>	<i>N-D<u>HYSSGSARQ</u>-C</i>	<i>7-mer (1.28x 10⁹)</i>	<i>NNK</i>
<i>Oligo 5</i>	<i>N-D<u>HYSSGSARQ</u>-C</i>	<i>6-mer (1.77x 10⁶)</i>	<i>NWW</i>
<i>Oligo 6</i>	<i>N-D<u>HYSSGSARQ</u>-C</i>	<i>7-mer (1.28x 10⁹)</i>	<i>NNK+fixed</i>
<i>Oligo 7^b</i>	<i>N-D<u>HYSSARQ</u>-C</i>	<i>6-mer (6.40x 10⁷)</i>	<i>NNK</i>
CDR3β oligos			
<i>Oligo name</i>	<i>Sequence^a</i>	<i>Theoretical diversity</i>	<i>Mutagenesis strategy^b</i>
<i>Oligo 8</i>	<i>N-T<u>AVLAGGP</u>-C</i>	<i>6-mer (6.40x 10⁷)</i>	<i>NNK</i>
<i>Oligo 9</i>	<i>N-T<u>AVLAGGP</u>-C</i>	<i>6-mer (6.40x 10⁷)</i>	<i>NNK</i>
<i>Oligo 10</i>	<i>N-T<u>AVLAGGP</u>-C</i>	<i>6-mer (6.40x 10⁷)</i>	<i>NNK</i>
<i>Oligo 11</i>	<i>N-T<u>AVLAGGP</u>-C</i>	<i>6-mer (1.77x 10⁶)</i>	<i>NWW</i>
<i>Oligo 12</i>	<i>N-T<u>AVLAGGP</u>-C</i>	<i>7-mer (1.28x 10⁹)</i>	<i>NNK</i>
<i>Oligo 13</i>	<i>N-T<u>AVLAGGP</u>-C</i>	<i>6-mer (6.40x 10⁷)</i>	<i>NNK+fixed</i>

^a Underlined; residues targeted by oligo, ^b Degenerate codons, N \rightarrow any nucleotide, K \rightarrow T or G, NNK \rightarrow any amino acid, NWW \rightarrow hydrophobic or charged amino acids

^b Shortens loops with two amino acids

Sublibraries representing each oligo were made separately on DNA level, but to reduce the number of *E. coli* SS320 aliquots needed for transformation, DNA libraries made from CDR3 α 6-mers or CDR3 β 6-mers were mixed before electroporation, while 7-mers were kept separate. Electroporated *E. coli* SS320 was grown in culture 30min, before each culture was split in two. Phagemid rescue was performed by addition of helper phage (as described in 3.3.2), either M13K07 or DeltaPhage, to package all libraries with low or high valence display of the scTCR fusion.

Before the initial culture was split, each electroporated library was titrated as described in 3.2.3 to determine library size. A CDR3 α library size of 1.80×10^{10} individual transformants was obtained, 4.47 times higher than the theoretical diversity of 4.03×10^9 individual clones. Obtained CDR3 β library size was 1.13×10^{10} individual transformants, 7.34 times higher than

the theoretical diversity of 1.54×10^9 individual clones. For all sublibraries electroporated, the obtained number of transformants covered the theoretical sizes of each library (results not shown).

Table 9: Oligos to introduce stop codon in the CDR3 α and CDR3 β loops

Oligo name	Oligo sequence
Q_CDR3alphaSTOP_fw	5'-CTGTGCTCTGAGTGATCACTATTAATCTGGTTCTGCAAGGCAACCTGACC-3'
Q_CDR3alphaSTOP_rv	5'-GGTCAGGTTGCCTTGCAGAACCAGATTAATAGTGATCACTCAGAGCACAG-3'
Q_CDR3betaSTOP_fw	5'-CCAGCAGCACAGCGTAACTAGCGGGAGGGCCG-3'
Q_CDR3betaSTOP_rv	5'-CGGCCCTCCCCTAGTTACGCTGTGCTGCTGG-3'

Table 10: Mutagenic oligos for randomization of the CDR3 α and CDR3 β loops at DNA level

Oligo name	Oligo sequence ^a
CDR3α oligos	
Oligo 1	5'-TTC TGT GCT CTG AGT NNK NNK NNK NNK NNK TCT GCA AGG CAA CTG-3'
Oligo 2	5'-CTG AGT GAT CAC TAT NNK NNK NNK NNK NNK NNK CTG ACC TTT GGA TCT-3'
Oligo 3	5'-GCT CTG AGT GAT CAC NNK NNK NNK NNK NNK NNK AGG CAA CTG ACC TTT-3'
Oligo 4	5'-TGT GCT CTG AGT GAT NNK NNK NNK NNK NNK NNK AGG CAA CTG ACC TTT-3'
Oligo 5	5'- GCT CTG AGT GAT CAC NWW NWW NWW NWW NWW NWW AGG CAA CTG ACC TTT-3'
Oligo 6	5'- TGT GCT CTG AGT GAT NNK TAT NNK NNK NNK NNK NNK AAA CTG ACC TTT GGA TCT-3'
Oligo 7	5'-TGT GCT CTG AGT GAT NNK NNK NNK NNK NNK NNK CAA CTG ACC TTT GGA-3'
CDR3β oligos:	
Oligo 8	5'-CTC TGT GCC AGC AGC NNK NNK NNK NNK NNK NNK GGG CCG CAG TAC TTC-3'
Oligo 9	5'-GCC AGC AGC ACA GCG NNK NNK NNK NNK NNK NNK CAG TAC TTC GGG CCG-3'
Oligo 10	5'- TGT GCC AGC AGC ACA NNK NNK NNK NNK NNK NNK CCG CAG TAC TTC GGG-3'
Oligo 11	5'- TGT GCC AGC AGC ACA NWW NWW NWW NWW NWW NWW CCG CAG TAC TTC GGG-3'
Oligo 12	5'- TGT GCC AGC AGC ACA NNK NNK NNK NNK NNK NNK CAG TAC TTC GGG CCG -3'
Oligo 13	5'- CTC TGT GCC AGC AGC CTG NNK NNK NNK NNK NNK NNK GAA CAG TAC TTC GGG CCG-3'

^aDegenerate codons, N \rightarrow any nucleotide, K \rightarrow T or G, NNK \rightarrow any amino acid, NWW \rightarrow hydrophobic or charged amino acids

Selection and polyclonal ELISA

Phages from CDR3 α and CDR3 β phage sublibraries were pooled before selection, resulting in the following libraries selected in R1.

- L1. scTCR s380 CDR3 α /scTCR s380 CDR3 β – M13K07 rescue
- L2. scTCR s380 CDR3 α /scTCR s380 CDR3 β – Deltaphage rescue
- L3. scTCR s380 CDR3 α FkpA/scTCR s380 CDR3 β FkpA – M13K07 rescue
- L4. scTCR s380 CDR3 α FkpA/scTCR s380 CDR3 β FkpA – Deltaphage rescue

In R1 the 4 libraries, L1-L4, were selected as described in 3.3.2. In R2 and R3, each of the 4 libraries was selected in parallel using two different selection strategies, with (alternative 1, A1) or without (alternative 2, A2) competitor in solution. After R1-R3, polyclonal ELISA was performed as described in 3.4.2 (Figure 17).

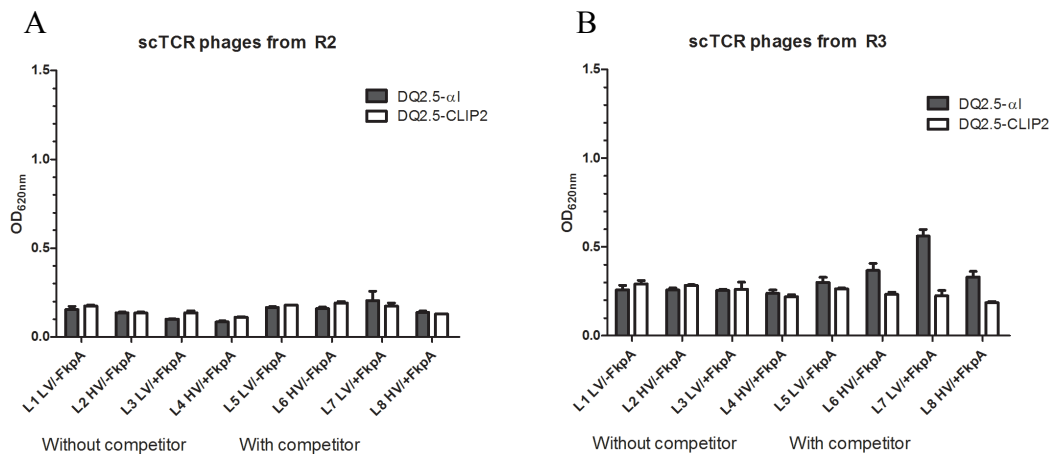


Figure 17: Polyclonal ELISA of phage libraries from selection R2 (A) and R3 (B). Binding of the phage libraries to DQ2.5-glia- α 1a and DQ2.5-CLIP2 is shown. The experiment was performed in duplicate and the mean \pm sd is shown.

Illumina sequencing

Encouraged by the results from polyclonal ELISA, CDR3 loops regions were amplified by PCR and sequenced by Illumina (Norwegian Sequencing Centre). Single clones expression and binding analysis is labor intensive and well known for false positives and negatives. In addition, it is usually necessary to screen a high number of clones. Using a high-throughput sequencing platform, such as Illumina sequencing, up to 1.6×10^8 reads with read lengths of 100-150 nucleotides are obtained. Several papers have employed high-throughput sequencing technologies combined with selection. In line with this, Ravn et al. compared Illumina sequencing with classical screening. Based on sequence frequency after Illumina sequencing, they retrieved the most potent clone and valuable candidates that were not identified with classical screening (Ravn, U., et al., 2010).

The results showed that the diversity of the library was still high, and no clear enrichment of any sequences could be found. Surprisingly, a high percentage of the library was the template used to generate the libraries, representing between 10-30% of the sequences. In order to decrease the diversity and to enrich for specific binders, it was decided that two additional round of selection would be performed on the libraries.

7.2 Supplementary 2

Selection R4 and R5 and screening performed by Jeannette Nilsen

Overview:

- Selection and polyclonal ELISA
- Batch-cloning from phagemid to pFKPEN-vector
- Screening of soluble scTCR from small scale expression

Selection and polyclonal ELISA

Eight phage libraries, L1-L8, were selected a fourth (R4) and a fifth round (R5). These selection rounds were performed essentially as in R3 (supplementary 1), using the two strategies, with and without competitor in solution for L1-L4 and L5-L8, respectively. After R4-R5, polyclonal ELISA was performed as described in 3.4.2, with the exception that the phage library input was normalized and 1×10^{11} virions/well was used.

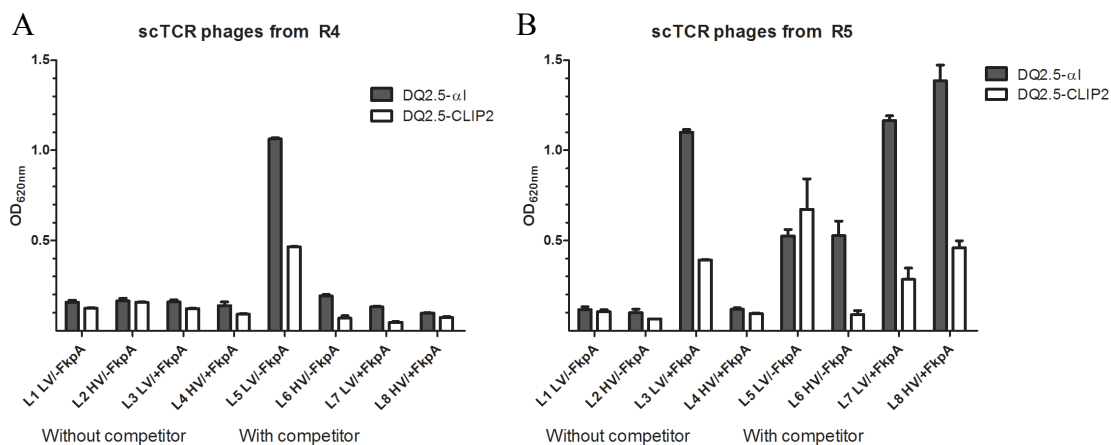


Figure 18: Polyclonal ELISA of phage libraries from selection R4 (A) and R5 (B). Binding of the phage libraries to DQ2.5-glia- α 1 and DQ2.5-CLIP2 is shown. The experiment was performed in duplicate and the mean \pm sd is shown.

Increased signals from R2-R5 indicated enrichment of target specific binders.

Batch-cloning from phagemid to pFKPEN-vector for soluble expression of scTCR

Phagemids in library L7 after the fifth round of panning were isolated, from *E.coli* that had been infected with the library output and plated onto solid media on Q-trays (described in 3.3.3), using miniprep as described in 3.5.1. The scTCR-encoding cassettes were isolated

using Nco I and Not I (NEB) restriction sites and batch-cloned into the expression vector pFKPEN. The pFKPEN-vector is a derivative of the pHOG-dummy described previously [97], which contains the FkpA-encoding gene that is controlled by its native promoter [45].

The isolated phagemids were digested using the restriction enzymes Nco I and Not I (NEB), followed by separation of the products by gel electrophoresis (described in 3.5.2). The bands corresponding to the scTCR cassettes were isolated from the gel and purified using QIAquick Gel extraction kit (Qiagen). pFKPEN-phox vector was digested with the same restriction enzymes and treated with calf intestinal alkaline phosphatase (CIP).

The scTCR cassettes were ligated into the pFKPEN expression vectors with an insert:vector ratio of 3:1. In addition, the ligation mixture was also prepared without insert, to monitor the vector background. The ligation mixtures were transformed into approx. 40 μ l electrocompetent XL1-blue cells (stratagene). Electroporation was performed at 1.3V and the cells were plated onto LA-amp dishes for ON incubation at 37°C. The transformation resulted in high number of transformants and low vector background of approx. 2.85%.

Small-scale expression of soluble scTCR and screening

186 single clones from L7 were expanded in 400 μ l expression cultures, separated into the medium and periplasmic fractions as described in [46], and tested for binding to in ELISA as described in 3.4.3. No binders to DQ2.5-glia- α 1a were found. 18 single clones were sequenced as described in 3.5.3, and sequencing revealed that the clones were not scTCRs, however they were scFv clones of varying sequences.

In parallel with scTCR phage library selection R1-R3, a scFv-pIII phage library was selected against the same target, DQ2.5-glia- α 1a. This selection identified three DQ2.5-glia- α 1a specific clones (Høydahl, unpublished).

Thus, these intersecting panning campaigns had led to contamination of the scTCR s380 phage libraries with various DQ2.5-glia- α 1a specific scFv clones.

7.3 Supplementary 3

Analysis of initial phage libraries (R0) by PCR

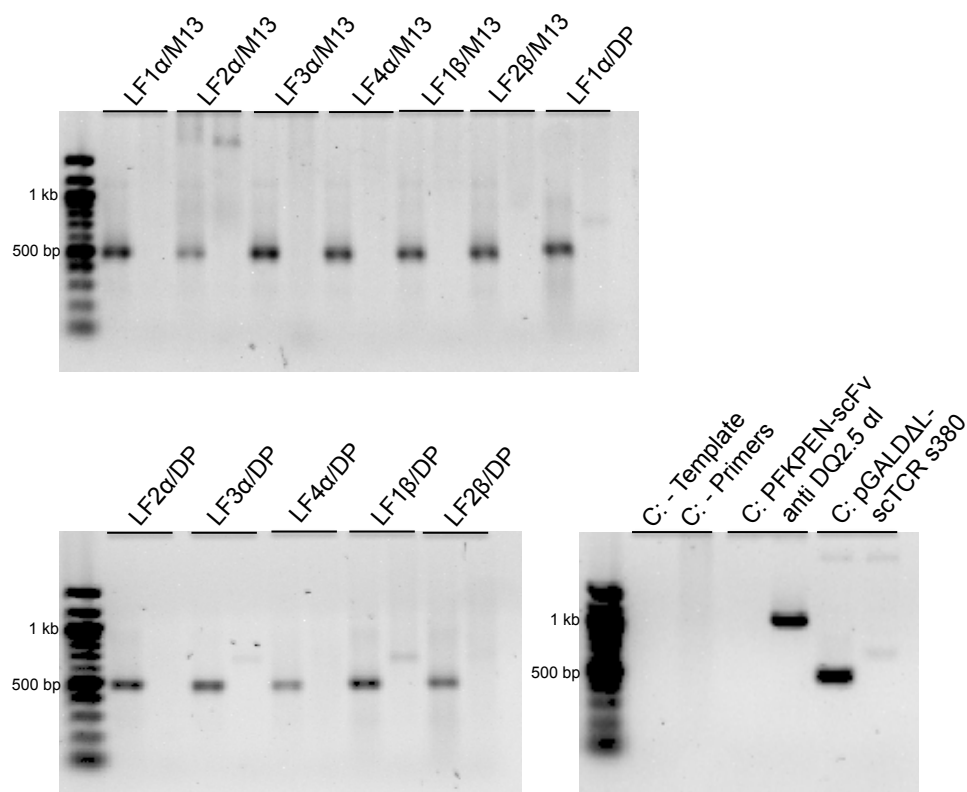


Figure 19: Gel electrophoresis showing the result of PCR analysis of the initial phage libraries. Two and two wells are marked according to the library template that was used. The first and the second well correspond to the PCRs performed with primer set specific for scTCR s380- and scFv-encoding phagemid, respectively. Six controls were included in the PCRs and are shown. 100 bp DNA ladder was included in all gels and the relevant fragments are marked.

The initial phage libraries were screened for presence of phagemid harboring scTCR S380 and phagemid harboring human scFv with kappa light chain by PCR (described in 3.4.1). Clear bands of expected size of about 500bp were obtained using the scTCR primer set (Figure 19), indicating presence of scTCR s380 phagemid. With the scFv primer-set no bands of expected size were obtained for any of the library samples, indicating that scFv phagemid was not present.

7.4 Supplementary 4

Titration

Phagemids are introduced to *E. coli* for amplification and subsequent super infection with helper phage is required for phage production. Two kinds of helper phages were prepared (described in 3.2.1), and in order to use the appropriate amount in phagemid rescue, the virion concentration was estimated by spot titration (described in 3.2.2) and by optical density at $A_{268\text{nm}}$ [14]. The titer based on $A_{268\text{nm}}$ was obtained using the following calculation: $A_{268\text{nm}} \times 2.5 \times 10^{12} = X$ virions/ml, which is modified from [14] based on the size difference to the phGHam-g8 phagemid.

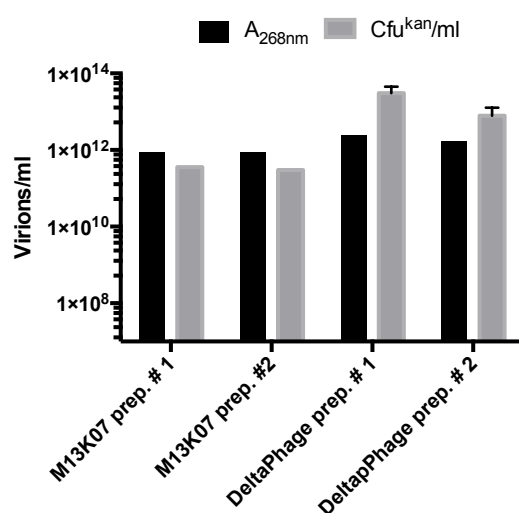


Figure 20: End titers of M13K07 and DeltaPhage after amplification and PEG precipitation.

The helper phage preparations were not made simultaneously. A slightly higher OD titer than infection titer normally observed [93]. This is also seen for the M13K07 preparations, in which case approx. 3×10^{11} $\text{cfu}^{\text{kan/ml}}$ and 9×10^{11} v/ml were determined with spot and OD titration, respectively (Figure 20). For the DeltaPhage preparations, however, the opposite is observed. By spot titration, the titer was estimated to be more than three and ten times higher than by OD titration, for preparation numbered 1 and 2, respectively. Thus, indicating overestimated infection titers. The OD titers were used in down stream experiments.

Agarose gel electrophoresis analysis

Polyphage can be produced during phage packaging [109] and may interfere with phagemid rescue if present in large quantities. Gel electrophoresis was performed on 25 μl helper phage preparations. The phage samples were mixed with 5 μl 6x sample buffer and added to the

wells of a stain-free 1%w/v agarose gel. The sample content was separated at 60V/3h in 1xTAE buffer using power pac 300 (Biorad, CA, USA). The virion capsids were denatured by incubating the gel in 200 ml 0.2 M NaOH for 1h, which was further rinsed in 100 ml dH₂O for 5 min and incubated in 100 ml 1 M Tris-HCl, pH 7 for 15 min. The ssDNA genomes in the gel were stained by further incubation for 1h in 100 ml dH₂O with SYBR safe DNA gel stain (1:10000) (Life technologies) and then visualized using Gel Doc™ EZ Imager (Biorad) (Figure 21).

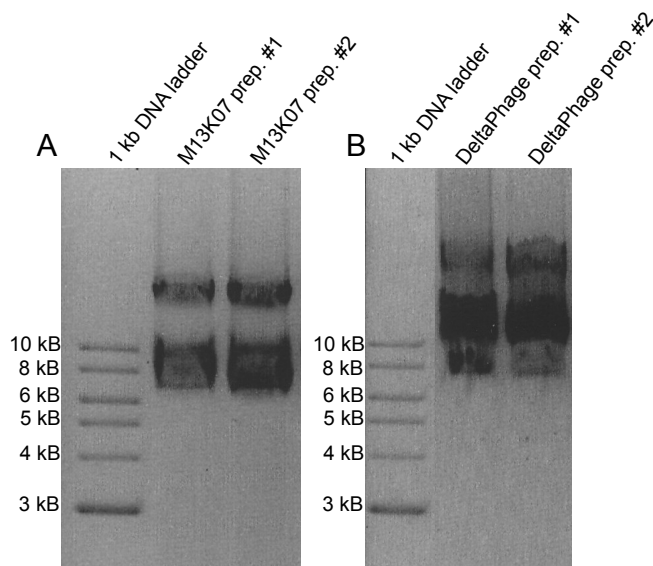


Figure 21: Agarose gel electrophoresis analysis of M13K07 (A) and DeltaPhage (B) preparations. The theoretical genome sizes for M13K07 and DeltaPhage are 8669 bp and 8678 bp, respectively. 1kb ladder was included in both gels.

For all preparations only minor fractions appeared to be of multi-length phage genomes (polyphage) as normally seen in any helper phage system. The genome sizes of the two helper phages differ only by nine bp, and thus would normally migrate to the same point on the gel. However, the preparations were not run on the same gel, which may explain the disparity.

7.5 Supplementary 5

Determining the growth curve of *E. coli* SS320

After a selection round, *E. coli* are transduced with the eluted phages to enable library amplification. The ratio between *E. coli* and phage is of importance to get full recovery of library members and to maintain genotype-phenotype link. Furthermore, superinfection with helper phage is done at MOI 20 to ensure rescue of all library clones (described in 3.3.3). In order to determine the relationship between absorbance and the number of cells/ml, the growth curve of *E. coli* SS320 was monitored.

E. coli SS320 cells were inoculated in 2x YT supplemented with 30 µg/ml tet and incubated with shaking at 220rpm/37°C/ON. SS320 cells were re-inoculated to OD_{600nm} 0.025 in 100 ml 2x YT supplemented with 30 µg/ml tet and incubated with shaking at 220rpm/37°C using SI-600R incubator (Lab companion). 1 ml of the culture was removed every 20 min for 5 h and the absorbance was measured at 600nm using spectrophotometer WPA SL05 (Biochrom, Cambridge, UK). Each time the absorbance was measured, a dilution series was prepared for the culture in 2x YT according to Table 2. 3µl of each dilution was spotted onto a BA85/20 nitrocellulose membrane (Whatman) that was laid on top of LB-agar dishes. The dishes were incubated at 37°C/ON. The number of cfu/ml present in the original culture was calculated using the formula $(\text{cfu} \times \text{dilution}^{-1} \times 1000)/3$. The growth curve was made by plotting LOG (cfu/ml) as function of OD_{600nm} and by performing linear regression in Prism 6 (GraphPad, CA, USA).

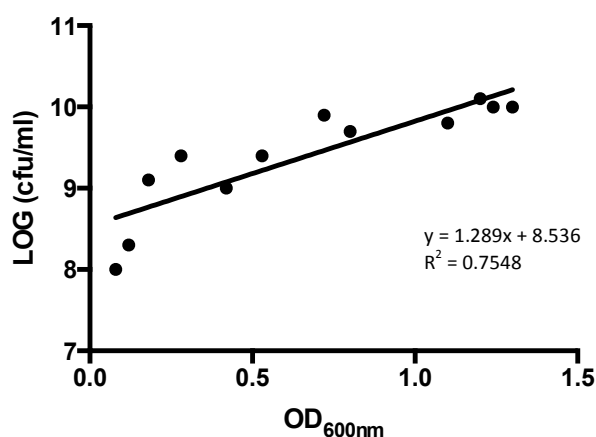


Figure 22: *E. coli* SS320 growth curve presented in a semi-log graph

From the regression equation, the number of cells per ml in a culture when OD_{600nm} is 0.1 was calculated to be approx. 4×10^8 cells/ml. This knowledge was used to determine the culture size needed to exceed the number of eluted phage after a selection round. A 10 ml SS320 culture at OD_{600nm} 0.5 was estimated to comprise a large surplus of *E. coli* compared to the output size that are expected from selection. The number of helper phage needed for rescue at MOI20 was also calculated.

7.6 Supplementary 6

Evaluation of phage elution with TEA

Exposure to a high pH solution, such as 100 mM TEA at pH 11, is a commonly used strategy to elute binders after selection. Short incubation time of 5-10 min is adequate to release the majority of the phages, but high-affinity binders may still be bound to the antigen and thus longer incubation time might be required [103]. However, high pH can influence protein structure by altering electrostatic interactions between amino acids. To investigate if prolong incubation at high pH could have a negative effect on phage infectivity, phages were incubated with 100 mM TEA pH 11 for various time periods before neutralization and then titrated using spot titration (described in 3.2.2).

Equal aliquots of M13K07-rescued phages displaying wild type (wt) scTCR s380 on pIX were transferred to seven 1.5 ml tubes. 500 μ l 0.1 M triethylamine (TEA) pH 11 (Sigma) was added to six of the phage-containing tubes and these were incubated on a SB3 rotator (Stuart, Staffordshire, UK) in RT for 5, 10, 15, 20, 25 and 30 minutes, before neutralization with 500 μ l 1 M Tris-HCl pH 7.5. Each TEA treated phage sample was spot titrated as described in 3.2.2. The graph was made by plotting the number of infectious particles (given as cfu^{amp})/ml as a function of time using Prism 6 (GraphPad).

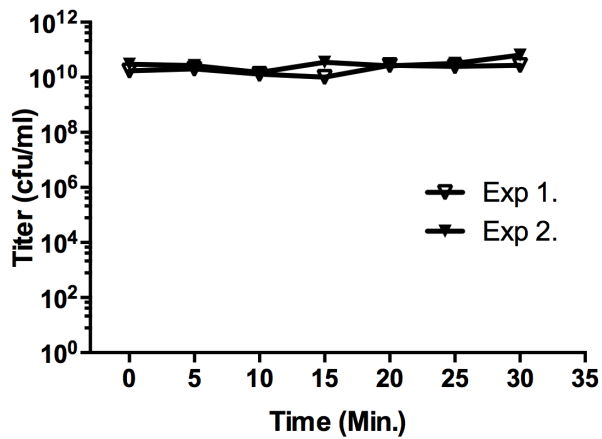


Figure 23: Evaluation of the elution conditions when using TEA at pH 11. The number of infectious particles is shown as a function of incubation time. The titers are given as amp resistant colony forming units (cfu^{amp}) per ml. Two independent experiments are shown.

The results showed that the infectivity is not negatively influenced by prolonged exposure to high pH (Figure 23). Thus to increase the chance of retrieving potential high affinity clones, the selection protocol was modified and selected phages were eluted by treatment with TEA pH 11 for 20 minutes.

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