UNIVERSITY OF LJUBLJANA
BIOTECHNICAL FACULTY
ACADEMIC STUDY IN BIOTECHNOLOGY

Katja ŠUSTER

In vitro AFFINITY MATURATION OF RECOMBINANT V_HH ANTIBODIES SPECIFIC FOR A 16kDa PROTEIN PRESENT IN *Mycobacterium tuberculosis*

GRADUATION THESIS

University studies

Ljubljana, 2012

UNIVERSITY OF LJUBLJANA
BIOTECHNICAL FACULTY
ACADEMIC STUDY IN BIOTECHNOLOGY

Katja ŠUSTER

In vitro AFFINITY MATURATION OF RECOMBINANT V_HH ANTIBODIES SPECIFIC FOR A 16kDa PROTEIN PRESENT IN *Mycobacterium tuberculosis*

GRADUATION THESIS
University studies

In vitro ZORENJE AFINITETE REKOMBINANTNIH PROTITELES V_HH, SPECIFIČNIH ZA 16kDa PROTEIN BAKTERIJE *Mycobacterium tuberculosis*

DIPLOMSKO DELO
Univerzitetni študij

Ljubljana, 2012

This thesis work is a completion of university studies in biotechnology. The work was carried out in the laboratory of Plant Research International (PRI) in the Department of Bioscience, Wageningen University, Wageningen (The Netherlands).

The Council of the Interdepartmental Programme in Biotechnology appointed Professor Mojca Narat, PhD, as supervisor, Jules Beekwilder, PhD, as co-advisor, and Professor Peter Dovč, PhD, as reviewer.

Committee for evaluation and defense of graduation thesis:

Chairman:	Prof. Dr. Branka JAVORNIK University of Ljubljana, Biotechnical Faculty, Department of Agronomy
Member:	Prof. Dr. Mojca NARAT University of Ljubljana, Biotechnical Faculty, Zootechnical Department
Member:	Dr. Jules BEEKWILDER Wageningen University and Research centre, PRI, Bioscience
Member:	Prof. Dr. Peter DOVČ University of Ljubljana, Biotechnical Faculty, Zootechnical Department

Date of defense: 10. 9. 2012

The results of this thesis are a result of the candidate's own work. Signed agree to the publication of his thesis on the website of the Digital Library of the Biotechnical Faculty. I declare that the work that I submitted in electronic form is identical to the printed version.

Katja Šuster

Diplomsko delo je zaključek Univerzitetnega študija biotehnologije. Opravljeno je bilo v laboratoriju inštituta Plant Research International na Oddelku za bioznanost Univerze v Wageningenu, Wageningen (Nizozemska).

Študijska komisija dodiplomskega študija biotehnologije je za mentorja diplomskega dela imenovala prof. dr. Mojco Narat, za somentorja dr. Jules Beekwilderja in za recenzenta prof. dr. Petra Dovča.

Komisija za oceno in zagovor:

Predsednica:	prof. dr. Branka JAVORNIK Univerza v Ljubljani, Biotehniška fakulteta, Oddelek za agronomijo
Članica:	prof. dr. Mojca NARAT Univerza v Ljubljani, Biotehniška fakulteta, Oddelek za zootehniko
Član:	dr. Jules BEEKWILDER Univerza v Wageningenu, PRI, Oddelek za bioznanost
Član:	prof. dr. Peter DOVČ Univerza v Ljubljani, Biotehniška fakulteta, Oddelek za zootehniko

Datum zagovora: 10. 9. 2012

Naloga je rezultat lastnega raziskovalnega dela.Podpisana se strinjam z objavo svojega diplomskega dela na spletni strani Digitalne knjižnice Biotehniške fakultete. Izjavljam, da je delo, ki sem ga oddala v elektronski obliki, identično tiskani verziji.

Katja Šuster

KEY WORDS DOCUMENTATION

DN	Dn
DC	UDC 579:577.27(043.2)=111
CX	immunology/antibody affinity maturation/V_HH fragments/error-prone PCR/phage display/microbiology/*Mycobacterium tuberculosis*/16 kDa heat shock protein
CC	AGRIS /
AU	ŠUSTER, Katja
AA	NARAT, Mojca (supervisor)/BEEKWILDER, Jules (co-supervisor)
PP	SI-1000 Ljubljana, Jamnikarjeva 101
PB	University of Ljubljana, Biotechnical faculty, Academic Study in Biotechnology
PY	2012
TI	*In vitro* AFFINITY MATURATION OF RECOMBINANT V_HH ANTIBODIES SPECIFIC FOR A 16kDa PROTEIN PRESENT IN *Mycobacterium tuberculosis*
DT	Graduation thesis (University studies)
NO	XIII, 52 p., 26 tab., 20 fig., 2 ann., 46 ref.
LA	en
AL	en/sl
AB	Affinity maturation is the process by which B cells produce antibodies with increased affinity for antigen during the course of an immune response. Like the natural prototype, the *in vitro* affinity maturation is based on the principles of mutation and selection. It has successfully been used to optimize antibodies, antibody fragments or other peptide molecules. The object in the present graduation thesis was to produce recombinant V_HH fragments with a higher affinity for the immunodominant 16kDa heat shock protein of *Mycobacterium tuberculosis* using the method error-prone PCR. For the error-prone PCR methods used the rates of producing mutations were 3.8×10^{-3} and 5.2×10^{-3} errors/bp. The selection was carried out by panning of the V_HH-displayed phage library, containing approximately 10^7 individual clones, against *M. tuberculosis* lysate. After three rounds of panning, plasmids from randomly selected phage pools were extracted and bulk-ligated into a PRI-VSV expression vector. Constructs were introduced into *E.coli* BL-21-Al which expressed recombinant V_HH fragments in the presence of L-arabinose. V_HH fragments were purified using Ni-NTA metal-affinity chromatography. In the ELISA nine of the tested recombinant V_HH fragments resulted in a higher affinity for the antigen than original V_HH fragments.

KLJUČNA DOKUMENTACIJSKA INFORMACIJA

ŠD	Dn
DK	UDK 579:577.27(043.2)=111
KG	imunologija/protitelesa/zorenje afinitete protiteles/V_HH fragmenti/error-prone PCR/fagna predstavitev/mikrobiologija/*Mycobacterium tuberculosis*/16 kDa stresni protein
KK	AGRIS /
AV	ŠUSTER (KORDEŽ KOROŠEC), Katja
SA	NARAT, Mojca (mentor)/BEEKWILDER, Jules (somentor)
KZ	SI-1000 Ljubljana, Jamnikarjeva 101
ZA	Univerza v Ljubljani, Biotehniška fakulteta, Študij biotehnologije
LI	2012
IN	*In vitro* ZORENJE AFINITETE REKOMBINANTNIH PROTITELES V_HH, SPECIFIČNIH ZA 16kDa PROTEIN BAKTERIJE *Mycobacterium tuberculosis*
TD	Diplomsko delo (univerzitetni študij)
OP	XIII, 52 str., 26 pregl., 20 sl., 2 pril., 46 vir.
IJ	en
JI	en/sl

AI Zorenje afinitete protiteles je proces v katerem B celice tekom imunskega odziva proizvajajo protitelesa z višjo afiniteto do antigena. Tudi zorenje afinitete *in vitro* temelji na principu mutacij in selekcije. Slednje je uspešno pri optimizaciji protiteles, njihovih fragmentov ali drugih peptidnih molekul. Cilj te diplomske naloge je bil proizvesti rekombinantne fragmente V_HH z višjo afiniteto do imunodominantnega stresnega proteina, z molsko maso 16kDa iz bakterije *Mycobacterium tuberculosis* z uporabo metode »error-prone« verižne reakcije s polimerazo. Izkazalo se je, da sta bili stopnji izzvanih mutacij 3,8 x 10^{-3} in 5,2 x 10^{-3} mutacij na bazni par. Selekcija je potekala v treh stopnjah. Lizat *M. tuberculosis* je bil izpostavljen fragmentom V_HH, predstavljenih na površini fagov iz primarne knjižnice, ki je vsebovala okoli 10^7 individualnih klonov. Kloni, ki se niso vezali na antigen, so bili po vsaki stopnji izločeni iz knjižnice, kloni, ki so se vezali na antigen pa so predstavljali sekundarno in terciarno knjižnico, s katerima sta bili izvedeni druga in tretja selekcija. Plazmidi iz naključno izbranih fagov iz zadnje selekcije so bili nato ekstrahirani in vstavljeni v PRI-VSV ekspresijski vektor. Konstrukti so bili vstavljeni v *E.coli* BL-21-Al, ki je ob prisotnosti L-arabinoze proizvedla rekombinantne fragmente V_HH. Čiščenje fragmentov je potekalo na kovinski afinitetni kromatografiji Ni-NTA. Z ELISA smo dokazali, da ima 9 testiranih V_HH fragmentov po vstavljanju naključni mutacij višjo afiniteto do antigena kakor nemutirani V_HH fragmenti.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

p.

LIST OF ANNEXES

ABBREVIATIONS AND SYMBOLS

BSA	Bovine serum albumine
CDR	Complementary-determining region
CIP	Alcaline calf intestinal phosphatase
DNA	Deoxyribose nucleic acid
DNAse I	Deoxyribonuclease I
dNTP	Deoxyribonucleotide triphosphate
ELISA	Enzyme-linked immunosorbent assay
Fab	Fragment antigen-binding
HA	Human influenza hemagglutinin
His	Histidine
LB	Luria-Bertani medium
LN23	Llama nanobody number 23
LN23+	Llama nanobody number 23 gained from error-prone PCR where added also $MnCl_2$
LN50	Llama nanobody number 50
LN50+	Llama nanobody number 50 gained from error-prone PCR where added also $MnCl_2$
$MgCl_2$	Magnesium chloride
$MnCl_2$	Manganese (II) chloride
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Pfu	Plaque-forming unit
scFv	Single-chain variable fragment
StEP	Staggered extension process
T_a	Annealing temperature
Taq-polymerase	DNA polymerase of thermophilic bacterium Thermus aquaticus
TB	Tuberculosis
TBE	Tris/Borate/EDTA buffer
TBS	Tris buffered saline
V_HH	Variable domain of heavy chain of llama heavy chain antibodies

1 INTRODUCTION

For centuries tuberculosis (TB) presented a serious health problem all over the world. In 2010 there were 8.8 millions new cases of TB and 1.4 million of deaths caused by the infection with the bacteria *Mycobacterium tuberculosis*. Lack of diagnostic capacity has been a crucial barrier preventing an effective response to the challenges of HIV-associated and drug resistant TB, with only 7 % of the estimated global burden of multi-drug resistant TB being detected, the consequence of critical gaps in laboratory capacity for culture and drug susceptibility testing. Therefore, the expanded capacity to diagnose TB and multi-drug resistant TB is a global priority for TB control (Global tuberculosis …, 2011).

The current diagnostic methods for TB include DNA-based, biochemical and serological approaches (Ferrara, et al. 2009) but none of these methods is yet appropriate for the high-throughput, rapid, and low-cost detection of TB in an affordable point of care test (Trilling *et al*., 2011). Research on new TB diagnostic tools has been therefore accelerated over the last few years and the diagnostic pipeline has been growing rapidly as a result (Global tuberculosis …, 2011)

A device with high sensibility – a biosensor with integrated antibodies specific for *M. tuberculosis* could rapresent a new diagnostic tool for the classification of TB. Biosensors are small in size and could be used as lab on a chip for rapid, cheap and accurate diagnostic test for TB (Trilling *et al*., 2011).

Recombinant llama antibody fragments (V_HH) specific for *M. tuberculosis* could be used in such biosensors as they are compact in size (15 kDa) (Harmsen and De Haard 2007). Trilling *et al*. (2011) shown that this kind of antibodies are able to distinguish *M. tuberculosis* from other mycobacterium species. They produced recombinant llama antibodie V_HH fragments and all selected recognized the species-specific 16 kDa protein of *M. tuberculosis*.

Even thow the specificity of produced antigen specific V_HH fragments was already proved (Trilling *et al*., 2011), higher affinity of V_HHs could mean a higher sensibility of the test. That could be reached by affinity maturation of the present antibodie fragments.

Affinity maturation is the process by which B cells produce antibodies with increased affinity for antigen during the course of an immune response. The first exposure to a given antigen entices clones of B cells displaying antigen-specific antibodies to undergo a rapid phase of multiplication and mutation resulting in an expanded series of B cell clones displaying antibodies more specific and with higher affinity for the antigen. With repeated exposures to the same antigen, a host will produce antibodies of successively higher affinities. A secondary response can elicit antibodies with higher affinity than in a primary response. The main principles of the *in vivo* affinity maturation, namely somatic hyper mutation and antigen selection of high-affinity clones, are utilized for the biotechnological approach of the *in vitro* affinity maturation.

Like the natural prototype, the *in vitro* affinity maturation is based on the principles of

mutation and selection. It has successfully been used to optimize antibodies, antibody fragments or other peptide molecules like antibody mimetics. The antibody affinity for their antigen is mainly dependent on the conformation of the amino acids present in the complementary determining regions (CDRs) of both the light and heavy chains of the antibody. Therefore, to change (improve) affinity, random or site-directed mutations inside the CDRs are introduced using several methods like UV iradiation, chemical mutagens, error-prone polymerase chain reaction (PCR), bacterial mutator strains, mutational hot spots, parsimonious mutagenesis, chain shuffling, deoxyribose nucleic acid (DNA) shuffling by random fragmentation and reassembly or staggered extension process. Two or three rounds of mutation and selection using display methods like phage display, yeast display or ribosome display usually result in antibody fragments with affinities in the low nanomolar range.

Random mutagenesis consists of randomly mutating the antibody gene, whereas site-directed mutagenesis generally "directs" or assigns mutations to chosen positions along the antibody gene sequence (Sheedy *et al.*, 2007) .

1.1 HYPOTHESES AND OBJECTIVES

The objective of the present graduation thesis was production of recombinant V_HH fragments with a higher affinity for the immunodominant 16kDa heat shock protein of *M. tuberculosis* using the method of error-prone PCR.

In a previous study of Trilling *et al.* (2011) a 3- year old female llama *Vicugna alpacos* (GDL, Utrecht University, The Netherlands) was immunized with *M. tuberculosis* lysate. Primary phage library was generated from lymphocyte RNA and phages, displaying V_HH fragments, specific for chosen antigen were selected and characterized. All characterized V_HHs bound to the same target – the 16 kDa *M. tuberculosis* antigen.

We predicted that introducing some point mutations in the sequences of V_HH fragments isolated by Trilling *et al.* (2011) with the method error-prone PCR, will resut in newly generated V_HHs that will have different sequence and consequently a higher affinity, the same affinity, lower affinity or will completely lose the affinity for the chosen antigen.

As the starting material were V_HH fragments that already went through the affinity maturation process *in vivo*, we thought about the chance that we won't be able to generate a V_HH with a higher affinity than the original one. But because the *in vivo* affinity maturation and the evolution itself are based on the principles of mutation and selection, hypothetically we should at some point be able to provoke such a mutation that would result in a V_HH variant with a higher affinity.

2 STATE OF THE ART

2.1 CAMELID'S HEAVY CHAIN ANTIBODIES

In 1989 a group of biologists led by Raymond Hamers at the Free University of Brussels investigated the immune system of dromedaries. In addition to the expected four-chain antibodies, they identified simpler antibodies consisting only of two heavy chains – heavy chain antibodies. This discovery was published in *Nature* in 1993 (Hamers-Casterman *et al.*, 1993).

The discovery that camelids produce functional antibodies devoid of light chain formed a further breakthrough because their single N-terminal domain (V_HH, also referred to as Nanobody® by Ablynx – the developper) binds antigen without requiring domain pairing (Harmsen and De Haard, 2007).

A heavy chain antibody is an antibody which consists only of two heavy chains and lacks the two light chains usually found in antibodies. In common antibodies, the antigen binding region consists of the variable domains of the heavy and light chains (V_H and V_L). V_HH fragments are antibody-derived proteins that contain the unique structural and functional properties of naturally-occurring heavy-chain antibodies. These heavy-chain antibodies contain a single variable domain (V_HH) and two constant domains (CH2 and CH3). Importantly, the cloned and isolated V_HH domain is a perfectly stable polypeptide harbouring the full antigen-binding capacity of the original heavy-chain antibody (Harmsen and De Haard, 2007; Deffar *et al*., 2009).

A single-domain antibody (V_HH or also refered to as sdAb) is an antibody fragment consisting of a single monomeric variable antibody domain. Although single-domain antibodies were later also identified in particular cartilaginous fish (Greenberg *et al.*, 1995, cited by Harmsen and De Haard, 2007), most research on the biotechnological application of single domain antibodies was done using camelids because they are easier to handle and camelids are easier to immunize than fish (Harmsen and De Haard, 2007). The heavy chain antibodies from cartilaginous fish are called IgNAR (immunoglobulin new antigen receptor), from which single domain antibodies called V_{NAR} fragments can be obtained (Stanfield *et al*., 2007).

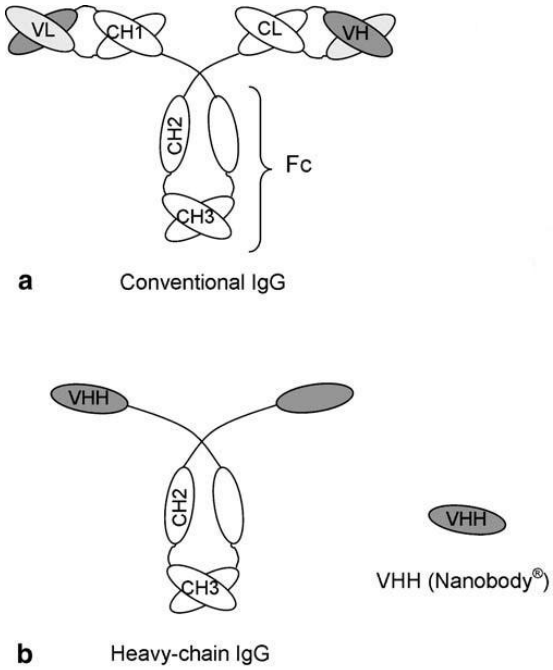

Figure 1: The comparison of a conventional antibody (a), a heavy chain antibody (b) and the V_HH fragment (Harmsen and De Haard, 2007: 14)

V_HH fragments have many advantages for biotechnological applications and several result from their single domain nature. For example V_HH libraries generated from immunized camelids retain full functional diversity whereas the conventional antibody libraries result in a diminished diversity (because of reshuffling of V_L and V_H domains during library construction). Important advantages are also a high microbial production level during the process of recombinant protein production and resistance to high temperatures (they remain functional at 90 °C (Van der Linden *et al.*, 1999)) (Harmsen and De Haard, 2007).

Table 1: Advantages of camelid single-domain antibody fragments as compared to conventional antibody fragments (Harmsen and De Haard, 2007: 15)

Advantage	Molecular basis
Facile genetic manipulation	Single-domain nature
Increased functional size of immune libraries	No decrease in library size because of reshuffling of VL and VH domains
Facile production of multivalent formats	More flexible linker design and no mispairing of VL and VH domains
Facile production of oligoclonal preparations from single cells	No mispairing of VL and VH domains
High physicochemical stability	Efficient refolding due to increased hydrophilicity and single-domain nature
High solubility	Increased hydrophilicity
Recognition of hidden antigenic sites	Small size and extended flexible CDR3
Rapid tissue penetration, fast clearance	Small size
Well expressed	Efficient folding due to increased hydrophilicity and single-domain nature

2.2 POSSIBILITIES FOR *IN VITRO* AFFINITY MATURATION

With *in vitro* mutagenesis we try to mimic the natural affinity maturation process that takes place during the secondary immune response.

The domain structure of antibody molecules permits their division into functional subunits, which can be mixed and matched to create novel molecules with a specific subset of functional properties (Hayden *et al.*, 1997; Sheedy *et al.*, 2007).

The affinity of llama heavy chain antibody variable domain (V_HH) for their antigen is mainly dependent on the conformation of the amino acids present in the CDRs of heavy chains of the antibody.

Techniques such as random mutagenesis, bacterial mutator strains passaging, site-directed mutagenesis, mutational hotspots targeting, parsimonious mutagenesis, antibody shuffling (chain, DNA and staggered extension process) have been used with various degrees of success to affinity mature or modify different kinds of antibodies (Sheedy *et al.*, 2007).

2.2.1 Random mutagenesis

Random mutagenesis consists in the introduction of mutations randomly throughout the gene. It can be sub grouped into error-prone PCR and bacterial mutator strains, but we can also introduce random mutations by using UV iradiation or chemical methods like deamination, alkylation or base-analog mutagenesis.

Error-prone PCR uses low-fidelity polymerization conditions to introduce a low level of point mutations randomly over a gene sequence (Stemmer, 1994; Sheedy *et al.*, 2007).

Bacterial mutator strains passaging has been used mainly to mutate antibodies binding to proteins. It consists of the selection of antibodies from a library followed by mutagenesis through amplification in a bacterial mutator strain (for example: *Escherichia coli*). Such mutator strains can produce a large number of mutant antibodies which can be selected afterwards by phage-display or other methods (Irving *et al.*, 1996; Sheedy *et al.*, 2007). To obtain high-affinity mutants, four to ten passages through mutator cells are required (Coia *et al.*, 2001; Sheedy *et al.*, 2007).

2.2.2 Site-directed mutagenesis

Mutations are directed to specific CDRs or framework regions or in other words: selected residues are mutated.

Mutational hot spots targeting is based on the theory that DNA encoding the variable domains of antibodies contains "mutational hot spots", or nucleotid sequences naturally prone to hypermutations during the *in vivo* affinity maturation process (Chowdhury and

Pastan, 1999; Sheedy *et al.*, 2007).

In parsimonious mutagenesis all three CDRs of a variable gene region can be simultaneously and thoroughly searched for improved variants in libraries of manageable size (Balint and Larrick, 1993; Sheedy *et al.*, 2007). Synthetic codons are used to mutate about 50% of all targeted amino acids while keeping the other 50% of targeted residues intact (wild type) (Chames *et al.*, 1998; Sheedy *et al.*, 2007).

2.2.3 Antibody shuffling

Antibody shuffling can be accomplished in several ways, using chain shuffling, DNA shuffling by random fragmentation and reassembly, staggered extension process (StEP) or variations of these techniques.

The chain shuffling method consists in shuffling heavy and light chain variable regions of antibodies. The heavy and light chains isolated from an immune library can be recombined, thereby generating a vast number of functional antibodies from an initially limited subset of antibodies (Kang *et al.*, 1991; Sheedy *et al.*, 2007). Since shuffling approaches mimic somatic hyper mutation, they are believed to be more efficient than random or site-directed mutagenesis in producing functional antibodies (Ness *et al.*, 2002; Sheedy *et al.*, 2007). Prior to shuffling, the pre-isolation of antigen-binding antibodies from an immune library is required (Park *et al.*, 2000; Sheedy *et al.*, 2007).

DNA shuffling by random fragmentation and reassembly involves the digestion of a large antibody gene with deoxyribonuclease I (DNAse I) to create a pool of random DNA fragments. This fragments can then be randomly reassembled into full -length genes by repeated cycles of annealing in the presence of DNA polymerase. The fragments prime mutually based on homology, and recombination occurs when fragments from one copy of gene prime another copy , causing a template switch (Stemmer, 1994; Sheedy *et al.*, 2007).

Staggered extension process (StEP) is a type of antibody shuffling that consists of priming of template sequences followed by several cycles of denaturation and shortened annealing extension. During each cycle, the DNA fragments can anneal to different templates based on sequence complementarity, and extend further to create recombinant genes, in that way this approach allows the combination of CDRs that are originally from different antibodies into one antibody while sampling and entire pool of available CDRs (Zahao *et al.*, 1998; Jirholt *et al.*, 1998; Sheedy *et al.*, 2007). The whole process can be performed in a single PCR reaction and results in a pool of mutants, the majority of which are functional (Zahao *et al.*, 1998; Sheedy *et al.*, 2007).

2.3 CHOSEN METHODS

Most frequently used strategies for *in vitro* maturation of antibodies are those of site-directed mutagenesis and error-prone PCR (Sheedy *et al.*, 2007). As error-prone PCR is the most common and simple to use, we decided that this will be the method we will use to affinity maturate V_HH fragments. The second method we thought about using was one of

the methods of site-directed mutagenesis. As the V_HH antibody consists of only heavy chains, chain shuffling was not an option. But we sereously thought about using the strategy of DNA shuffling by random fragmentation and reassembly. For this method we do not need primers, so it is also more convenient than the StEP method.

DNA shuffling also offers several advantages over other traditional mutagenesis strategies. Compared to methods such as site-directed mutagenesis and even error-prone PCR, DNA shuffling can be used with longer DNA sequences, and also allows for the selection of clones with mutations outside the binding or active site of the antibody, whereas site-directed mutagenesis is limited to a given region of the antibody due to the limitation in library size that can be efficiently transformed to construct the mutant library (Stemmer, 1994; Sheedy *et al.*, 2007).

At the end we decided that error-prone PCR is the best option for our propose as it is easier to handle and not so time consuming.

2.3.1 Random mutagenesis by error-prone PCR

Error-prone PCR is a normal PCR that is typically performed using conditions that reduce the fidelity of Taq DNA polymerase during DNA synthesis to introduce a low level of point mutations randomly over a gene sequence. That can be done in several ways, for example increasing the concentration of magnesium chloride ($MgCl_2$) in the reaction mixture, adding manganese chloride ($MnCl_2$), using unequal concentrations of each nucleotide and in that way varying the ratios of nucleotides in the reaction, including a nucleotide analog such as 8-oxo-dGTP or dITP, or also by combining two or more of this options for one reaction. One of the mechanisms for inducing randomized nucleotide sequences via PCR is also the use of mutagenic (overhanging) primers. Even thou the Taq polymerase itself has a naturally high error rate, with errors biased toward AT to GC changes (Pitchard *et al.*, 2005; Cirino *et al.*, 2003).

Table 2: Different errror rates for Taq polimerase reported in the literature and the corresponding reference citations (Taq DNA polymerase (native and recombinant), 2012)

1.1 x 10^{-4} base substitutions/bp	Tindall, *et. al*. (1988) Biochemistry 27, 6008. Assay = Reversion of Opal Suppression in LacZ.
2.4 x 10^{-5} frameshift mutations/bp	Tindall, *et. al*. (1988) Biochemistry 27, 6008.
2.1 x 10^{-4} errors/bp	Keohavong, *et. al*. (1989) PNAS 86, 9253. Assay = Denaturing Gradient Gel Electrophoresis.
7.2 x 10^{-5} errors/bp	Ling, *et. al*. (1991) PCR Methods Appl 1(1), 63.
8.9 x 10^{-5} errors/bp	Cariello, *et. al*. (1991) Nucleic Acids Research 19(15), 4193. Assay = DGGE
2.0 x 10^{-5} errors/bp	Lundberg, *et. al*. (1991) Gene 108, 1. Assay = Loss of LacI function.
1.1 x 10^{-4} errors/bp	Barnes, *et. al*. (1992) Gene 112, 29. Assay = Loss of LacZ function.

For our purpose we decided to use the error-prone PCR protocol developed by McCullum *et al.* (2010). They modified the standard PCR protocol to include: increased concentration of *Taq* DNA polymerase, increased polymerase extension time, increased concentration of $MgCl_2$ ions, increased concentration of dNTP substrates and the reaction was supplemented with $MnCl_2$ ions. To minimize mutational bias in the amplified sequences,

they used unbalanced ratio of nucleotides. The mentioned protocol uses a several serial dilution (and amplification) steps in which a portion of the amplified material (approximately 10 %) is successively transferred in a new tube after every fourth amplification cycle for a fresh PCR reaction. In that way it is possible to generate a mutagenic library that contains a range of single-nucleotide point mutations. Of course a larger amount of starting template is required. The process consists of a total of 64 cycles of PCR or 16 serial transfer steps. This serial of dilution steps enable the experimenter to control the level of mutagenesis incorporated into the pool. Consequently, it is very easy to generate pools of variants with increasing degrees of mutations while simultaneously avoiding the PCR saturation problem. When all 16 serial dilution steps are used, this technique produces an average error rate of approximately 3.5 % per nucleotide per PCR reaction. This number can vary between different templates (McCullum *et al.*, 2010).

2.3.2 Mutagenesis by DNA shuffling for random fragmentation and reassembly

Using DNA shuffling the libraries can be created by random fragmentation of a pool of related genes, followed by reassembly of the fragments by self-priming PCR. This process causes crossovers between homologous sequences, due to template switching. The whole process consists of 5 steps. The first step is the preparation of parent genes. In DNA shuffling starting from a single gene as the parent template, diversity originates from random point mutation, due to the limited fidelity of the polymerases used in PCR.These point mutations may provide useful diversity, but the high mutation rate decreases the frequency of active clones. There is also an other version of DNA shuffling, called family shuffling, that allows more than 2 genes (also genes from different species) to be used as the parental sequences. In contrast with single gene DNA shuffling that differs by only a few point mutations, the block-exchange nature of family shuffling creates chimeras that differ in many positions. That is why family DNA shuffling can provide a greater functional diversity, but homologies of at least 80 % in DNA family shuffling using 2 genes and 60 % in DNA family shuffling using 3 or more genes are necessary (Stemmer, 1994).

The second step involves digesting parent genes with DNAse I to a pool of random DNA fragments. Following that is the third step that consists of running the DNA fragments on a low-melting-point agarose gel to then excise DNA fragments of specific molecular size ranges. After that fragments should be purified, for example by electrophoresis onto DE81 ion-exchange paper (Whatman). Continuing with the fourth step of the process, fragments are reassembled into a full-length gene by repeated cycles of annealing in the presence of DNA polymerase. The fragments prime each other based on homology, and recombination occurs when fragments from one copy of a gene prime on another copy, causing a template switch. This process is called self-priming PCR and no primers are used (Stemmer, 1994).

2.4 ANTIBODY DISPLAY TECHNOLOGY

The first used displaying technology was phage display, described in 1985 by Smith. Since then, this technique has become an essential toolkit in protein engineering where diverse libraries of peptides or proteins containing hundreds of millions of mutations can be

rapidly created and the best candidates selected. The major advantages of phage display technology are its robustness, simplicity and the stability of phage particles (which enables selection on cell surface), tissue sections and even *in vivo* (Dufner at al., 2006). But also phage display has its limitations. Because the coupling of genotype and phenotype (i.e. protein synthesis and assembly of phage particles) takes place in bacteria, the DNA needs to be imported artificially. Library size is therefore restricted by transformation efficiency (Dufner *et al.*, 2006). Realization of these limits of phage display has spawned a number of new methods that use the same principle but exploit the cellular machinery in a cell-free environment.

In 1994 Mattheakis *et al.* presented a library of synthetic peptides displayed in the surface of ribosomes and selected them for binding to a specific antibody. The ribosome display is a cell-free expression system. It is a most widely used alternative to phage display. The DNA library that encodes peptides or proteins is transcribed and translated *in vitro* using prokaryotic or eukaryotic cell-free expression systems. The combination of the absence of a stop codon, an elevated level of magnesium ions and low temperature stalls the ribosome at the end of the mRNA while the nascent polypeptide folds and is presented outside the ribosome tunnel (Dufner *et al.*, 2006). This technique has a particular advantage in comparison to phage display and also all other cell-surface display technologies: the DNA does not have to be imported into a host because phenotype-genotype coupling and amplification both take place *in vitro*.

The yeast display of antibodies was first published by Boder and Wittrup (1997). In yeast cell surface displays, functional proteins of interest are genetically fused to an anchor protein and expressed on the cell surface. The yeast *Saccharomyces cerevisiae* is the most commonly used organism in yeast display (Tanaka *et al.*, 2012). The main advantage of this type of display is that yeasts are eukaryotes and offer post-translational modification and processing machinery similar to that of mammals (Boder and Wittrup, 1997). But on the other hand this method includes disadvantages like smaller mutant library sizes compared to alternative methods and differential glycosylation in yeast compared to mammalian cells (Boder *et al.*, 2000).

There are quite some more display technologies in use (i.e. mRNA display, bacterial surface display) but in this graduation thesis we will focus on phage display.

2.4.1 Phage display

Bacteriophages were first described by Frederick Twort in 1915 and Felix d'Hérelle in 1917. D'Hérelle named them bacteriophages because they could lyse bacteria on the surface of agar plates (phage: from the Greek, "to eat").

Phage display is a method for the study of protein-protein, protein-peptide, and protein-DNA interactions that uses bacteriophages to connect proteins with the genetic information that encodes them. In other words: phage display describes the display of foreign (poly) peptides on the surface of phage particle. It was originally invented by George P. Smith in 1985 and he demonstrated the display of peptides on filamentous phage by fusing the

encoding gene for peptide of interest on to gene3 of filamentous phage (Bratkovič, 2010; Smith, 1985).

The idea is to display a protein of our interest on the surface of a phage. This can be done by splicing a gene encoding such a protein into a gene that encodes a capsid structural protein. As the phage has several proteins building its capsid, it is possible to display a protein of interest on any of them.

2.4.1.1 Filamentous bacteriophage

The most commonly used phage is M13 (a single-stranded filamentous DNA bacteriophage) or other filamentous phage. It infects only male bacterial cells, after attachment to the male-specific pilus (F pilus), that is present in suitable host cells with the genotype *E. coli* F'. When entering the cell, the phage is stripped of its protein coat and the single-stranded DNA is converted into a double stranded replicative form followed by DNA replication and assembly of new particles. This tipe of phage is released from the infected cell without causing the death of its host.

STRUCTURE:

The tube-like capsid is composed of several thousand copies of tightly packed major coat protein (gene VIII product (p8)), capped by five copies of p3 and p6 on one end and five copies of p7 and p9 on the opposite end.

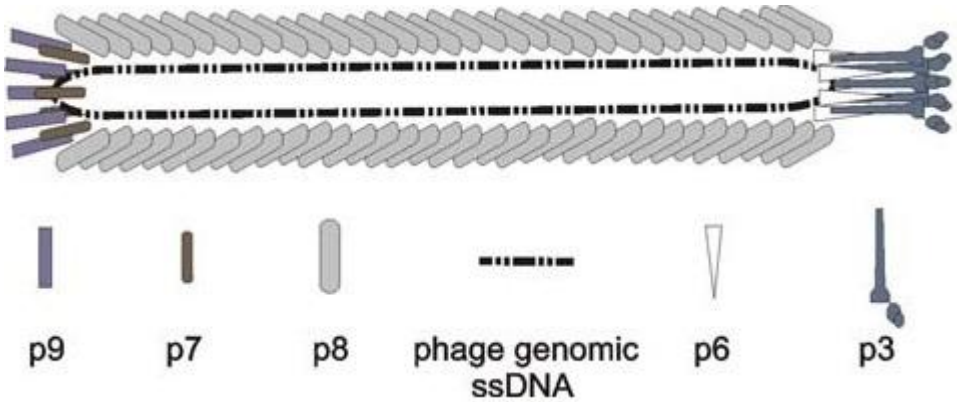

Figure 2: Filamentous phage structure (Bratkovič, 2010: 750)

In 1985 Smith demonstrated that fusions to the minor capsid protein p3 (product of gene III) of the non-lytic filamentous phage f1 were fairly well tolerated. He cloned a fragment of the *Eco*RI restrictase gene in the middle section of the gene III.
All five capsid proteins in the phage virion have so far been utilized for display purposes. The most common approach for peptide display is to fuse the foreign sequences to the amino terminus of pIII or pVIII, while proteins are usually displayed from pIII. Peptide and protein fusions to the amino termini of pVII and pIX have been reported, as well as fusions to the carboxy termini of pVI, an artificial pVIII, and pIII (Kehoe and Kay, 2005).

2.4.1.2 Phage display vectors and phagemids

A number of phage vectors are used in DNA and cDNA cloning.

An "expression vector," including a phage-display vector, has an additional feature compared to vectors in general: the foreign DNA is "expressed" as a protein. That is, it programs machinery of the *E. coli* host cell to synthesize a foreign peptide whose amino acid sequence is determined (*via* the genetic code) by the nucleotide sequence of the insert. Phage display differs from conventional expression systems, however, in that the foreign gene sequence is spliced into the gene for one of the phage coat proteins, so that the foreign amino acid sequence is genetically fused to the endogenous amino acids of the coat protein to make a hybrid "fusion" protein. The hybrid coat protein is incorporated into phage particles ("virions") as they are released from the cell, so that the foreign peptide or protein domain is displayed on the outer surface of the phage coat (Smith and Petrenko, 1997).

A phagemid (also called phasmid) is a type of cloning vector developed as a hybrid of the filamentous phage M13 and plasmids to produce a vector that have plasmid properties (i.e., carry antibiotic resistance and enable replication of dsDNA), and with features of phage vectors (i.e., allow for production and packing of ssDNA into virions). Phagemids contain an origin of replication (ORI) for double stranded replication, as well as an f1 ORI to enable single stranded replication and packaging into phage particles. Many commonly used plasmids contain an f1 ORI and are thus phagemids. Similarly to a plasmid, a phagemid can be used to clone DNA fragments and be introduced into a bacterial host by a range of techniques (transformation, electroporation). However, infection of a bacterial host containing a phagemid with a 'helper' phage, for example VCSM13 or M13K07, provides the necessary viral components (absent in the phagemid) as well as a defective origin of DNA replication. This origin of DNA replication is sufficiently active to permit propagation of the phage, but it is much weaker than the origin contained in phagemid vectors. As a result, infection of phagemid-containing bacterial cells with helper phage results in the packaging of only the phagemid. In other words, phagemids replicate as plasmids in *E. coli*, and they can also be packaged as recombinant M13 phage in the presence of helper phage (Bratkovič, 2010; Smith and Scott, 1993).

For purpose of this graduation thesis the phagemid pComb3XSS was used (see Figure 3 below for structure).

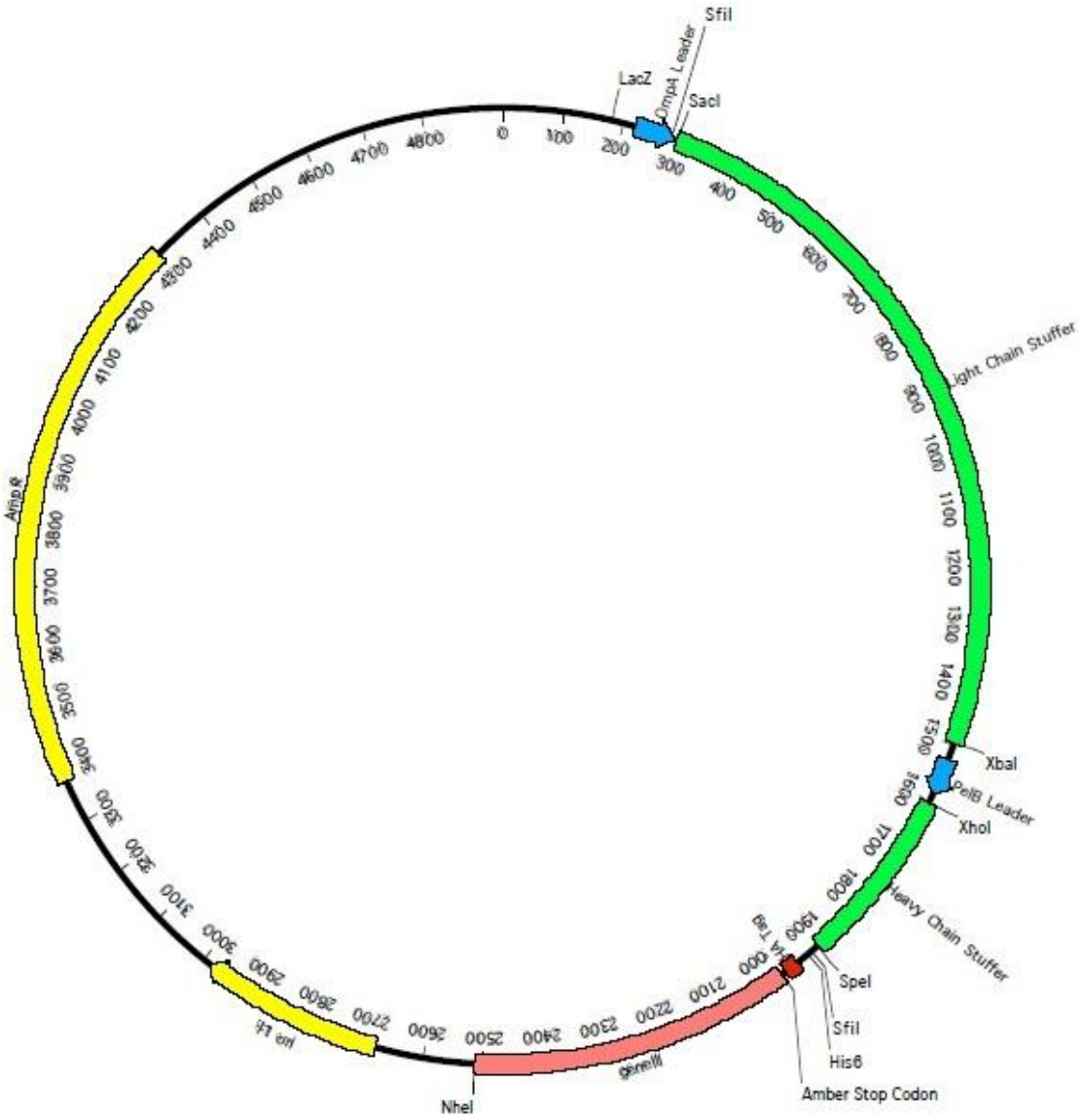

Figure 3: Structure of the phagemid pComb3XSS (pComb3X maps, 2012)

The pComb3XSS phagemid has an increased stability over the other pComb3 vectors and contains *Sfi*I cassette for cloning of full fragment antigen-binding (Fab), single-chain variable fragment (scFv), peptide and other protein for phage display. 6x histidine (His) and human influenza hemagglutinin (HA) tags allow for purification and detection of the later produced protein. An amber stop codon is used to turn-off expression of the pIII fusion protein by switching to a non-supressor strain of *E. coli* allowing production of soluble protein without subcloning. Alternatively, the gene for phage protein pIII can be removed by *Spe*I/*Nhe*I enzymatic digest. The "SS" refers to the double stuffer, a 1200 bp stuffer in the Fab light chain cloning region bounded by SacI and *Xba*I restriction sites and a 300 bp stuffer in Fab heavy chain cloning region bound by *Xho*I and *Spe*I restriction sites. Also, the 1600 bp double stuffer (both stuffer plus the leader sequence between the Fab light chain and heavy chain cloning regions) can be removed by *Sfi*I digest so that non-Fab genes of interest can also by cloned (pComb3X Family, 2012).

The pComb3XSS vector sequence is available on GeneBank, accession # AF268281.

2.5 AFFINITY SELECTION

The most common selection pressure imposed on phage-displayed peptide populations is affinity for a target receptor. Affinity selection is ordinarily accomplished by minor modifications of standard affinity purification techniques commonly in use in biochemistry. The receptor (for example an antigen in the case of antibody-display) is immobilized to a solid support, and the phage mixture (library phage) is passed over this surface. Those phages (usually a tiny minority) whose displayed peptides bind the receptor are captured on the surface or matrix, allowing unbound phages to be washed away. Finally, the bound phages are eluted in a solution that loosens receptor peptide bonds, yielding an population of phages (eluate) that is greatly enriched (often a million fold or more) for receptor-binding clones. The eluted phages are still infective and are propagated simply by infecting fresh bacterial host cells, yielding an "amplified" eluate that can serve as input to another round of affinity selection. Phage clones from the final eluate (typically after 2-3 rounds of selection) are propagated and characterized individually. The amino acid sequences of the peptides responsible for binding the target receptor are determined simply by ascertaining the corresponding coding sequence in the viral DNA (Smith and Petrenko, 1997; Bratkovič, 2010).

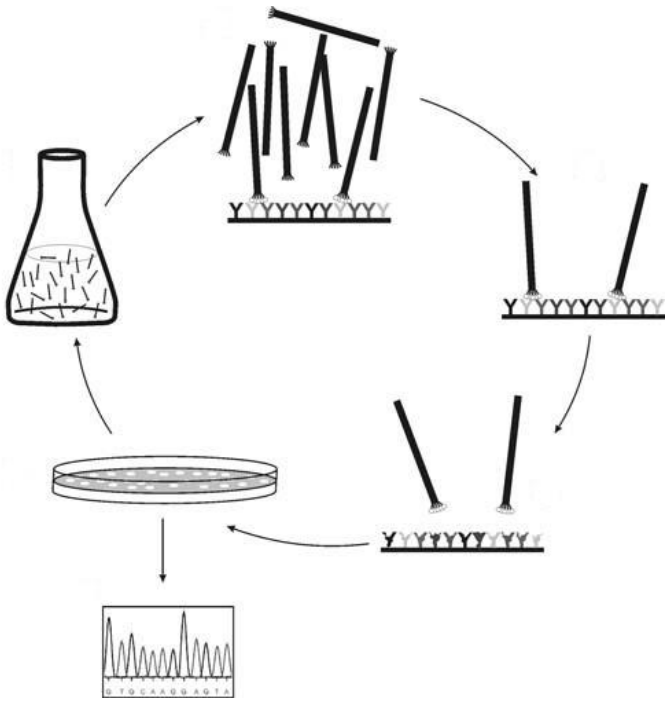

Figure 4: Library screening (Bratkovič, 2010: 750)

3 MATERIALS AND METHODS

3.1 BIOLOGICAL MATERIAL

3.1.1 Bacterial strains

Escherichia coli (XL1-blue Electroporation-competent cells) were provided by Stratagen (cat. # 200228).

Escherichia coli (BL21-Al™ One Shot® Chemically competent cells) were provided by Invitrogen (cat. # C6070-03).

Mycobacterium tuberculosis lysate was provided by the Royal Tropical Institute, Amsterdam, The Netherlands. The bacteria were grown in Middlebrook 7H9 medium (Difco, BD, Sparks, MD, USA) supplemented with 10% OADC (BBL, BD) and heat-killed at 80 °C. After 2 washing steps with PBS to remove all media the bacteria pellet was resuspended in PBS. 500 µL of bacterial suspension was lysed with 0.6 g zirkonia/silica 0.1 mm (BioSpec Products Inc, Bartlesville, OK, USA) in a Retch MM 301 (Retch GmbH, Germany) for 15 min at 30 hertz. To remove soluble particles and to obtain the lysate of *Mycobacterium* as antigen source, the lysate was centrifuged for 5 min at 13000 g.

3.1.2 Virological material

VCSM13 Interference-Resistant Helper Phage was provided by Agilent Technologies (cat. # 200251).

3.2 ANTIBIOTICS, ENZYMES, REACTANTS

Table 3: Antibiotics, enzymes and reactants

Antibiotics, enzymes, reactants	Provider and cat. nomber	Final concentration
The antibiotic ampicillin	Sigma (cat. # A0166)	100 µg ml^{-1}
The antibiotic carbenicillin	Duchefa Biochemie B.V. (cat. # C0109)	100 µg ml^{-1}
The antibiotic kanamycin	Duchefa Biochemie B.V. (cat. # K0126)	50 µg ml^{-1}
The antibiotic tetracycline	Sigma (cat. # T7660)	10 µg ml^{-1}
The enzyme Super Taq polymerase of 5 U $µl^{-1}$	Sphaero Q (cat. # TP05c)	used in accordance with the manufacturer's instructions
The enzyme *Sfi*I of 20 U $µl^{-1}$	New England Biolabs (cat. # R0123)	used in accordance with the manufacturer's instructions
The enzyme *Pst*I of 20 U $µl^{-1}$	New England Biolabs (cat. # R0140)	used in accordance with the manufacturer's instructions
The enzyme *Bst*EII (Eco91I) of 10 U $µl^{-1}$	New England Biolabs (cat. # R0140)	used in accordance with the manufacturer's instructions

to be continued …

… continued

The enzyme alkaline calf intestinal phosphatase (CIP) of 10000 U μl^{-1}	New England Biolabs (cat. # M0290)	used in accordance with the manufacturer's instructions
The enzyme T4 DNA Ligase	New England Biolabs (cat. # M0202)	used in accordance with the manufacturer's instructions
The enzyme trypsin (from bovine pancreas)	Sigma (cat. # T9935)	used in accordance with the manufacturer's instructions
Albumin from bovine serum (BSA)	Sigma (cat. # A7906)	used in accordance with the manufacturer's instructions

3.3 MEDIA AND GROWTH CONDITIONS

Strains of *Escherichia coli* were cultivated over night at 37 ˚C in:

- LB medium (Luria-Bertani) (Miller, 1972): yeast extract 5 g l^{-1} (Sigma-Aldrich, cat. #Y1625), tryptone 10 g l^{-1} (Sigma-Aldrich, cat. #169922), sodium chloride 5 g l^{-1}, stirred to dissolve.
- SB medium (Super Broth): yeast extract 30 g l^{-1}, tryptone g l^{-1}, MOPS (3 (N-Morpholino) propanesulfonic acid) 10 g l^{-1}, stirred to dissolve, titrated to pH 7.0.
- LB Top Agar: Bacto agar 7 g l^{-1} (DIFCO, cat. #214030), LB medium 25 g l^{-1}, stirred to dissolve. Autoclaved stored at 4 ˚C, melted in microwave before use.
- 2x YT (2x Yeast extract and Tryptone): tryptone 16 g l^{-1}, yeast extract 10 g l^{-1}, sodium chloride 5 g l^{-1}, stirred to dissolve.

All the media were prepared with distilled water and sterilized at 121 ˚C for 20 minutes. Antibiotics were added to the medium due to antibiotic based selection of the bacteria. Agar (15 g l^{-1}) was added to the medium when needed to prepare agar plates.

For the prolonged conservation of bacteria cultures they were stored and saved in microcentrifuge tubes with 20% of glycerol at -70˚C.

3.4 CLONING OF LN23 AND LN50 SEQUENCES

In a previous study Trilling *et al.* (2011) bulk-ligated V_HH sequences into a *Pst*I and *Not*I digested PRI-VSV expression vector. This is a strong expression vector for expression in the periplasm, based on the backbone of the pRSET-A vector (Invitrogen, The Netherlands).

To obtain V_HH sequences for error-prone PCR, the PRI expression vector was digested using the two unique restriction sites: *Pst*I and *Bst*EII. Primers reconstructing the the *Pst*I and *Bst*EII sites were designed containing additional *Sfi*I sites at both ends for cloning V_HH sequences into pComb3XSS vector (provided by The Scripps Research institute upon material transfer agreement).

3.4.1 Plasmid DNA isolation and double digestion with *Pst*I and *Bst*EII restriction enzymes

3.4.1.1 Plasmid DNA isolation

Plasmid DNA extraction was performed using the QIAGEN Plasmid Mini Kit (QIAGEN, cat. #12123) according to manufacturer's instructions. For the small-scale preparation (minipreparation), a volume of 3 ml of an overnight culture (*E. coli* cells) was used. Plasmid DNA was suspended in 30 µL sterile water. The DNA concentration was measured by NanoDrop ND-1000 Spectrophotometer.

Table 4: Plasmid DNA concentration measured by NanoDrop ND-1000 Spectrophotometer

DNA concentration (PRI expression vector containing the LN23 V_HH fragment)	DNA concentration (PRI expression vector containing the LN50 V_HH fragment)
462.2 ng μL^{-1}	434.2 ng μL^{-1}

3.4.1.2 Double digestion of the vector with *Pst*I and *Bst*EII restriction enzymes

Step 1: Digestion of the PRI-VSV expression vectors with *Bst*EII

Table 5: Reaction mixture for the *Bst*EII digestion of the PRI-VSV expression vectors

LN 23		LN 50	
DNA	15.0 µL	DNA	15.0 µL
Buffer 0	2.0 µL	Buffer 0	2.0 µL
*Bst*EII	1.5 µL	*Bst*EII	1.5 µL
Sterile water	1.5 µL	Sterile water	1.5 µL
Total	20.0 µL	Total	20.0 µL

The reaction mixture was incubated 1h at 37ºC.

Step 2: Purification of the DNA (PRI-VSV expression vectors digested with *Bst*EII) was performed with the JETquick PCR purification spin kit (Genomed, cat.# 410250). The DNA was eluted with 30 µL of sterile water (incubated 1 min prior to centrifugation).

Step 3: Digestion of the DNA extracted in step 2 with *Pst*I

Table 6: Reaction mixture for the *Pst*I digestion of the PRI-VSV expression vectors

LN 23		LN 50	
Extracted DNA from step 2	27.0 µL	Extracted DNA from step 2	27.0 µL
Buffer 3	4.0 µL	Buffer 3	4.0 µL
*Pst*I	1.5 µL	*Pst*I	1.5 µL
10 x Bovine serum albumine (BSA)	4.0 µL	10 x BSA	4.0 µL
Sterile water	3.5 µL	Sterile water	3.5 µL
Total	40.0 µL	Total	40.0 µL

The reaction mixture was incubated 1h at 37ºC.

3.4.1.3 Agarose gel electrophoresis

Gel electrophoresis in 1% agarose gel stained with ethidium bromide was used to evaluate the size of DNA fragments and for isolation of restriction fragments or PCR products in 1x

Tris/Borate/EDTA (TBE) buffer. The samples were mixed with 5x loading buffer Orange G (Sigma, cat.# O3756). The size of fragments was estimated by comparison with the electrophoretic mobility of a commercial 1kb Plus DNA Ladder (Invitrogen, cat.# 10787-026).

3.4.1.4 Extraction of LN23 V_HH sequence and LN50 V_HH sequence from agarose gel

The gel extraction was performed with JETquick gel extraction spin kit. The DNA was eluted with 30 µL TE buffer (DNA hydration buffer) preheated at 70 ºC. The DNA concentration was measured by NanoDrop ND-1000 Spectrophotometer.

Table 7: V_HH DNA concentration measured by NanoDrop ND-1000 Spectrophotometer

DNA concentration (LN23 V_HH fragment)	DNA concentration (LN50 V_HH fragment)
10.0 ng μL^{-1}	23.0 ng μL^{-1}

3.5 INSERTING MUTATIONS BY ERROR-PRONE PCR

3.5.1 Primer modeling

When designing primers for the PCR, the following facts were taken into consideration. First, by cutting out the V_HH sequence from the PRI expression vector using *Bst*EII and *Pst*I enzymes, also a part of the V_HH sequence was lost (6 N-terminal and the 44 C-terminal amino acids). These deleted amino acids belong to the V_HH backbone and are conserved in most V_HH fragments. Primers were designed to reconstruct *Pst*I and *Bst*EII restriction sites to enable back ligation later on. Second, addition of recognition sites for *Sfi*I at both ends of the V_HH sequence was necessary to ligate the V_HH sequence into vector pComb3XSS. A vector carrying plasmid properties (i.e., carries antibiotic resistance and enables replication of dsDNA) as well as features of phage vectors (i.e., allows for production and packing of ssDNA into virions) (Bratkovič, 2010, Smith and Scott, 1993). To enable insertion of V_HH sequences into this vector, additional base pairs at both ends of the primers were added encoding *Sfi*I restriction sites.

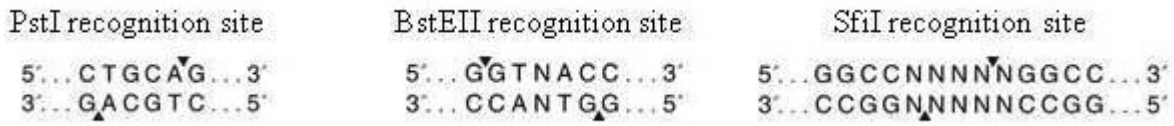

Figure 5: The recognition sites for *Pst*I, *Bst*EII and *Sfi*I enzymes

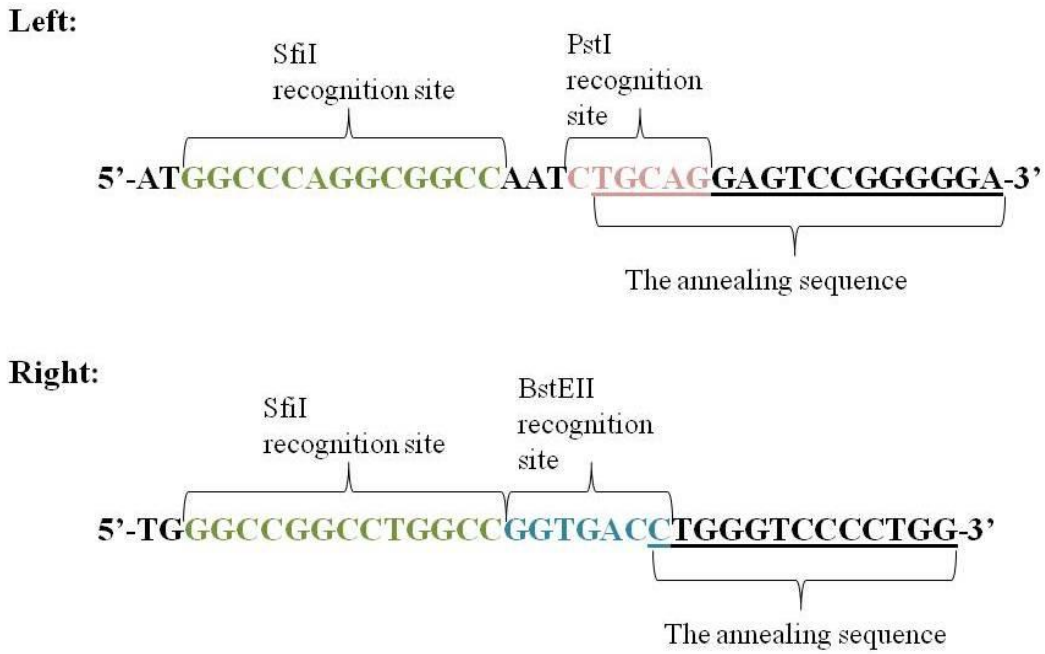

Figure 6: Designed primers

3.5.2 Normal PCR amplification of extracted V_HH fragments

The polymerase chain reaction (PCR) technique was developed by Kary Mullis in the 1980s (Bartlett and Stirling, 2003). PCR is based on using the ability of DNA polymerase to synthesize new strand of DNA complementary to the offered template strand. Because DNA polymerase can add a nucleotide only onto a preexisting 3'-OH group, it needs a primer to which it can add the first nucleotide. DNA is amplified *in vitro* by a series of polymerization cycles consisting of three temperature-dependent steps: DNA denaturation, primer-template annealing, and DNA synthesis by a thermostable DNA polymerase. To successfully amplify the desired sequence, several experiments are required to determine optimal conditions for PCR, even if good primers are chosen. The purity and yield of the reaction products depend on several parameters, one of which is the annealing temperature (T_a). At both sub- and super-optimal T_a values, non-specific products may be formed, and the yield of products is reduced. That is why the optimization of the T_a in necessary for every primer-template pair (Rychlik *et al.*, 1990).
The optimal T_a (60 ºC) was determined experimentally performing a variety of normal PCR using the designed primers at different T_a.

The reaction mixture is described in detail in Table 8.

The temperature program was:

- 2 min at 94 ºC
- 10 cycles of:
  - denaturation 1 min at 94 ºC
  - annealing 1 min at 60 ºC
  - extension 3 min at 72 ºC
- final extension 5 min at 72 ºC.

Table 8: The PCR amplification reaction mixture

	Stock solution	Final concentration
10x super taq buffer	10 x	1 x
dNTP	10 mM	80 µM
Upstream primer	5 µM	0.1 µM
Downstream primer	5 µM	0.1 µM
Super taq	5 U μL^{-1}	0.2 U μL^{-1}
DNA template	LN23 10 ng μL^{-1}	
	or LN50 23 ng μL^{-1}	0.4 ng μL^{-1}
Sterile water		
		Final volume: 25 µL

The gel extraction was performed with JETquick gel extraction spin kit. The DNA was eluted with 30 µL of sterile water and incubated 1 min before centrifugation.

The DNA concentration was measured by NanoDrop ND-1000 Spectrophotometer.

Table 9: V_HH DNA concentration after PCR amplification measured by NanoDrop ND-1000 Spectrophotometer

DNA concentration (LN23 V_HH fragment)	DNA concentration (LN50 V_HH fragment)
252.2 ng μL^{-1}	360.3 ng μL^{-1}

3.5.3 Error-prone PCR

The error-prone PCR protocol developed by McCullum *et al.* (2010) was modified. Adjustments were made in the optimization of error-prone PCR conditions. Changes are shown in detail in Table 10.

Changes in the error-prone PCR protocol compared to a standard PCR protocol include: increased concentration of $MgCl_2$ ions, increased concentration and unbalanced ratio of nucleotides and supplementation of the reaction with $MnCl_2$ ions.

McCullum *et al*. (2010) performed several serial dilution and amplification steps in which a portion of the amplified material (approximately 10 %) was successively transferred to a new tube after every fourth amplification cycle to serve as template material in a new PCR reaction. When 16 serial dilution steps were used, this technique produced an average error rate of approximately 3.5 % per nucleotide per PCR reaction. The serial dilution steps enable the control over the level of mutagenesis incorporated in the pool. As the percentage of mutations in the sequence increases by each cycle, 30 continuous cycles were performed instead of 16 dilution steps described by McCullum *et al*. (2010). To generate a mutagenic library containing a range of single-nucleotide point mutations, two different error-prone reactions were performed. The "error-prone PCR" contained an increased concentration of $MgCl_2$ ions and an increased concentration and an unbalanced ratio of nucleotides, whereas the "error-prone PCR +" was additionally supplemented with $MnCl_2$ ions. The reaction mixtures are described in detail in Table 10 and 10.

We expected a higher rate of mutations in the reaction mixture supplemented with $MnCl_2$.

The mutational rate was calculated with the following formula:

$$no.\ of\ mutations \ \ x \ \ (no.\ of\ total\ checked\ sequences\ x\ no.\ of\ bp)^{-1} \quad \ldots(1)$$

The temperature program was:

- 2 min at 94 ºC
- 30 cycles of:
  - denaturation 1 min at 94 ºC
  - annealing 1 min at 60 ºC
  - extension 3 min at 72 ºC
- final extension 5 min at 72 ºC.

Table 10: The error-prone PCR reaction mixture

	Stock solution	Final concentration
10x super taq buffer	10 x	1x
dGTP	10 mM	0.2 mM
dATP	10 mM	0.2 mM
dCTP	10 mM	1.0 mM
dTTP	10 mM	1.0 mM
Upstream primer	5 µM	0.5 mM
Downstream primer	5 µM	0.5 mM
Super taq	5 U μL^{-1}	0.05 U μL^{-1}
$MgCl_2$	1 M	5 mM
DNA template	LN23 (252.2 ng μL^{-1}) or LN50 (360.3 ng μL^{-1})	4 ng μL^{-1}
Sterile water		
		Final volume: 100 µL

Table 11: The error-prone PCR+ reaction mixture

	Stock solution	Final concentration
10x super taq buffer	10 x	1x
dGTP	10 mM	0.2 mM
dATP	10 mM	0.2 mM
dCTP	10 mM	1.0 mM
dTTP	10 mM	1.0 mM
Upstream primer	5 µM	0.5 mM
Downstream primer	5 µM	0.5 mM
Super taq	5 U μL^{-1}	0.05 U μL^{-1}
$MgCl_2$	1 M	5 mM
$MnCl_2$	10 mM	0.5 mM
DNA template	LN23 (252.2 ng μL^{-1}) or LN50 (360.3 ng μL^{-1})	4 ng μL^{-1}
Sterile water		
		Final volume: 100 µL

3.5.3.1 Agarose gel electrophoresis

Electrophoresis with ethidium bromide stained 1 % agarose gel in 1x Tris/Borate/EDTA (TBE) buffer was used to evaluatethe size of isolated error-prone PCR products. PCR

products (4 µL) were mixed with loading buffer Orange G (Sigma, cat.# O3756) (1 µL of 5x loading buffer) and loaded on the gel. The size of the PCR products was estimated by comparison with the electrophoretic mobility of a commercial 1kb Plus DNA Ladder (Invitrogen, cat.# 10787-026).

The gel extraction was performed with JETquick gel extraction spin kit / 250 (Genomed, cat. #420250). The DNA was eluted with 30 µL of sterile water and incubated 1 min before centrifugation.

The DNA concentration was measured by NanoDrop ND-1000 Spectrophotometer.

Table 12: V_HH DNA concentration after error-prone PCR and error-prone PCR+ amplifications measured by NanoDrop ND-1000 Spectrophotometer

DNA concentration (LN23)	DNA concentration (LN50)	DNA concentration (LN23+)	DNA concentration (LN50+)
346.3 ng μL^{-1}	302.1 ng μL^{-1}	265.9 ng μL^{-1}	354.7 ng μL^{-1}

3.5.4 Digestion of the pComb3XSS vector and error-prone PCR products with *Sfi*I enzyme

Digestion of pComb3XSS vector and error-prone PCR products with *Sfi*I enzyme was performed according to the protocol of restriction-digest of "overlap Fab PCR products" and "pComb3HSS or pComb3XSS vector" described by Barbas *et al.* (2001).

The content of the digest reaction mixtures are described in detail in Table 13 and Table 14. Both digestions were incubated for 1 hour and 30 minutes at 50 ºC.

Table 13: Digest mixtures for error-prone PCR and error-prone PCR+ products

	Stock solution	Final concentration
Error-prone PCR product	346.3 ng μL^{-1} (LN23) or 302.1 ng μL^{-1} (LN50) or 265.9 ng μL^{-1} (LN23+) or 354.7 ng μL^{-1} (LN50+)	50 ng μL^{-1}
*Sfi*I	20000 U mL^{-1}	533.3 U mL^{-1}
BSA	10 mg mL^{-1}	100 µg mL^{-1}
NEBuffer 4	10 x	1 x
Sterile water		
		Final volume: 300 µL

Table 14: Digest mixtures for pComb3XSS vector

	Stock solution	Final concentration
pComb3XSS	µg	100 ng μL^{-1}
*Sfi*I	20000 U mL^{-1}	400 U mL^{-1}
BSA	10 mg mL^{-1}	100 µg mL^{-1}
NEBuffer 4	10 x	1 x
Sterile water		
		Final volume: 300 µL

To concentrate the digested products, DNA ethanol precipitation was performed as described by Barbas *et al.* (2001).

Agarose gel electrophoresis was performed as described in section 3.4.1.3.

The size of the whole pComb3XSS vector was 4973 bp. After cutting with *Sfi*I enzyme, the vector resulted in two fragments, a 3301 bp long backbone fragment and a 1672 bp long stuffer fragment. Both bands were cut out of the gel. The vector backbone fragment (3301 bp) was used for the library ligation and stuffer fragment (1672 bp) was used as a control (for re-ligation in the vector backbone fragment).

Gel extraction was performed with QIAquick gel extraction kit (QIAGEN, cat. #28704). DNA was eluted with 30 µL of sterile water.

DNA concentration was measured by NanoDrop ND-1000 Spectrophotometer.

Table 15: V_HHs, backbone vector and stuffer fragment concentrations after the digestion with *Sfi*I enzyme measured by NanoDrop ND-1000 Spectrophotometer

DNA concentration (LN23)	DNA concentration (LN50)	DNA concentration (LN23+)	DNA concentration (LN50+)	Backbone vector	Stuffer fragment
142.3 ng μL^{-1}	107.6 ng μL^{-1}	43.0 ng μL^{-1}	126.0 ng μL^{-1}	64.4 ng μL^{-1}	25.1 ng μL^{-1}

The backbone vector fragment (3301 bp) was treated with Calf Intestinal Alkaline Phosphatase (CIP), to prevent self-ligation of the vector. Alkaline Phosphatase catalyzes the removal of 5´-phosphate groups from DNA, RNA, ribo- and deoxyribonucleoside triphosphates. Since CIP-treated fragments lack the 5´-phosphoryl termini required by ligases, they cannot self-ligate (Sambrook at al., 2001).

Table 16: Reaction mixture for CIP treatment of backbone vector fragment

	Volume
Backbone vector fragment (64.4 ng μL^{-1})	29 µL
NEBuffer 3 10 x	4 µL
CIP (10000 U μL^{-1})	1 µL
MQ	16 µL
	Final volume: 50 µL

3.6 PHAGE DISPLAY

3.6.1 Generation of the library

*Sfi*I digested error-prone PCR fragments (LN23, LN50, LN23+ and LN50+) were ligated into *Sfi*I digested backbone vector pComb3XSS to generate a library. First, a test ligation was performed following step 8 of protocol 9.1 described by Barbas *et al.* (2001) in Phage

Display, chapter 9, p. 9.20 – 9.22. Then, library ligation and library transformation into *E. coli* was performed following the protocol 10.3 "Library Ligation and Transformation" described by Barbas *et al.* (2001) in Phage Display, chapter 10, p. 9.21 – 9.22 with one modification.

1x phosphate buffered saline (PBS) pH 8.0 was used instead of tris buffered saline (TBS).

Bacteria *E. coli* (XL1-blue Electroporation-competent cells) have been used.

The total number of transformants has been calculated from the number of colony forming units (cfu) using the following formula:

$$Total\ no.\ of\ transformants = cfu \times culture\ volume \times (plating\ volume)^{-1} \quad \ldots(2)$$

3.7 SEQUENCING

Plasmid DNA extraction was performed using the QIAGEN plasmid mini kit (QIAGEN, cat. #12123) according to manufacturer's instructions. For small-scale preparation (minipreparation), a volume of 3 ml of an overnight culture (*E. coli* XL1-blue) was used. Plasmid DNA was suspended in sterile water. The DNA concentration was measured by NanoDrop ND-1000 Spectrophotometer.

Fragment sequencing was carried out by Greenomics (Wageningen, The Netherlands) using primers specific for the pComb3XSS vector ompseq (5'-AAGACAGCTATCGCGATTGCAG-3') and gback (5'-GCCCCCTTATTAGCGTTTGCCATC-3') provided by BioLegio.

3.8 SELECTION FROM ANTIBODY LIBRARIES

3.8.1 Preparation of helper phage

The preparation of the helper phage was performed according to the protocol 10.2 "Preparation of the Helper Phage" described by Barbas *et al.* (2001) in Phage Display, chapter 10, p. 10.5 – 10.6.

VCSM13 Interference-Resistant Helper Phage and bacteria *E. coli* (XL1-blue Electroporation-competent cells) have been used.

3.8.2 Library reamplification

The library reamplification was performed as described in the protocol 10.4 "Library Reamplification" (Barbas *et al.*, 2001), with some modifications.

VCSM13 Interference-Resistant Helper Phage and bacteria *E. coli* (XL1-blue Electroporation-competent cells) have been used.

1x PBS pH 8.0 was used instead of TBS.

Concentration of carbenicillin was 50 mg mL^{-1} instead of 100 mg mL^{-1}.

3.8.3 Library panning on immobilized antigens

Library panning on immobilized antigens was performed as described in protocol 10.5 of Barbas *et al.* (2001) in Phage Display, chapter 10, p. 10.12 – 10.15, with some modifications.

During these experiments the VCSM13 Interference-Resistant Helper Phage and the bacteria *E. coli* (XL1-blue Electroporation-competent cells) have been used.

1x PBS pH 8.0 was used instead of TBS.

The concentration of carbenicillin was 50 mg mL^{-1} instead of 100 mg mL^{-1}.

Flat-bottom enzyme-linked immunosorbent assay (ELISA) plates, medium-binding (GreinerBioOne, cat. #655001), were used.

The input tittering for each panning round was calculated from the number of cfu on agar plates before each panning round, the culture volume, the plating volume, the volume in 96 well plate per library and the dilution of the helper phage used.

$$(Cfu \times culture\ V \times helper\ phage\ dilution \times V\ in\ wells\ per\ library) \times (plating\ V)^{-1} \quad \ldots(3)$$

The output tittering was calculated for each panning round from the number of cfu on agar plates after each panning round, the culture volume and the plating volume.

$$(Cfu \times culture\ V) \times (plating\ V)^{-1} \quad \ldots(4)$$

3.8.3.1 First panning round

The following modifications were performed: The first selection was carried out by panning of V_HH-displayed phage libraries against the *M. tuberculosis* lysate. 4 wells of microtiter ELISA plates (GreinerBioOne, cat. #655001) per library were coated overnight at 4°C with 100 µL of 10 µg mL^{-1} lysate. In the second step of the protocol, the plates were blocked with 2 % milk in PBS for 1 hour and 40 minutes at room temperature. In step five eluated phages were stored at 4°C overnight. The next day, eluated phages were used to infect *E. coli* XL1-blue. The phage populations were amplified and rescued by VCSM13 helper phage to generate phages displaying V_HH. These phages were reamplified (as already described in section 3.8.2) and then used in the next round of panning.

3.8.3.2 Second panning round

For the second round of panning we coated 4 wells per library with 100 µL of 10 µg mL^{-1} *M. tuberculosis* lysate.

The eluted phages were then used to reamplify the library for the next panning round (like described in section 3.8.2).

3.8.3.3 Third panning round

For the third round of panning we coated 1 well per library with 100 µL of 10 µg mL^{-1} *M. tuberculosis* lysate.

In this last panning round binding competition was introduced by adding orriginal V_HH fragments (LN23 or LN50 – depending on the library) at a final concentration of 25 µg mL^{-1}.

3.9 RECLONING OF SELECTED V_HHs FOR EXPRESSION

Plasmids from selected phage pools were extracted using QIAprep Spin Miniprep Kit (QIAGENE, cat. #27106). DNA concentrations were measured by NanoDrop ND-1000 Spectrophotometer. Names were given according to the library and to the 96 well plate loading position to distinguish between different recombinant V_HH fragments (example: LNXX.YY, where XX refers to the library [LN23, LN23+, LN50 or LN50+] and YY to the location in the 96 well plate).

Table 17: Plasmids from selected phage pools DNA concentrations measured by NanoDrop ND-1000 Spectrophotometer

Sample	DNA concentration
LN50.A1	231.71 ng μL^{-1}
LN50.A11	103.61 ng μL^{-1}
LN50+.C1	245.43 ng μL^{-1}
LN50+.C2	98.84 ng μL^{-1}
LN50+.C3	314.47 ng μL^{-1}
LN50+.D4	123.82 ng μL^{-1}
LN50+.D11	131.91 ng μL^{-1}
LN23.E1	320.65 ng μL^{-1}
LN23.E9	222.03 ng μL^{-1}
LN23.E11	131.77 ng μL^{-1}
LN23.F3	164.07 ng μL^{-1}
LN50.A1	231.71 ng μL^{-1}
LN50.A11	103.61 ng μL^{-1}
LN50+.C1	245.43 ng μL^{-1}
LN50+.C2	98.84 ng μL^{-1}
LN50+.C3	314.47 ng μL^{-1}
LN50+.D4	123.82 ng μL^{-1}
LN50+.D11	131.91 ng μL^{-1}

to be continued …

… continued

LN23.E1	320.65 ng μL^{-1}
LN23.E9	222.03 ng μL^{-1}
LN23.E11	131.77 ng μL^{-1}
LN23.F3	164.07 ng μL^{-1}

Isolation of the V_HH sequences from pComb3XX vector was carried out by digestion with *Pst*I and *Bst*EII enzymes. Cut out V_HH sequences were purified using agarose gel electrophoresis and the Jetquick gel extraction spin kit (Genomed, cat. #420250). *Pst*I and *Bst*EII digested V_HH fragments were bulk-ligated into a *Pst*I and *Bst*EII digested PRI-VSV expression vector, like described by Trilling *et al.* (2011).
Only LN50+.C1 was not digestible. This is probably due to a mutation in the restriction site of at least one of the enzymes used.

3.10 EXPRESSION AND PURIFICATION OF RECOMBINANT V_HH FRAGMENTS

Expression and purification of recombinant V_HH fragments was carried out as described by Trilling *et al.* (2011).

PRI-VSV expression vector was extracted from *E. coli* XL1-blue and transformed to *E. coli* BL21-Al™ (One Shot® Chemically competent cells). Expression of recombinant V_HH fragments was induced in the presence of L-arabinose.

V_HH fragments were purified with Ni-NTA metal-affinity chromatography using Ni-NTA Superflow resin (QUIAGEN, cat. #30430) and eluated recombinant V_HH fragments were dialyzed against PBS. The Ni-NTA purification system is designed for purification of recombinant fusion proteins that are tagged with six tandem histidine residues.

Protein concentrations were determined using Bradford test. The method is based on the reaction of proteins with the dye Coomassie Brilliant Blue. The product of the reaction is a colored complex with the absorbance maximum at 595 nm (Bradford, 1976). The absorbance was measured at 590 nm in TECAN SpectraFluor Microplate reader. For the calibration graph BSA of a known concentration (0.136 mg mL^{-1}) was used in several dilutions. The unknown concentrations of tested V_HH proteins were determined from the calibration graph.

Successful expression and purification was verified by Western blot analysis on sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) also described by Trilling *et al.* (2011).

3.11 DIRECT ELISA

The direct ELISAs were performed as described by Trilling *et al.* (2011) with some modifications.

Wells of flat-bottom ELISA plates were coated with the 16 kDa heat shock protein in whole *E. coli* cell lysate solution[1] at a concentration of 0.02 μg mL^{-1} in PBS.

The absorbance was measured at 415 nm in TECAN SpectraFluor Microplate Reader.

In the first ELISA binding of 14 randomly from phage pool selected V_HH fragments was tested. Binding to the antigen was tested at two different V_HH concentrations, 10 μg mL^{-1} and 5 μg mL^{-1}.

In the second ELISA binding of 9 V_HH fragments that showed better binding than the wild type V_HHs LN23 and LN50 in the first ELISA, were tested. Binding was tested at 4 different V_HH concentrations: 10 μg mL^{-1}, 1 μg mL^{-1}, 0.1 μg mL^{-1} and 0.01 μg mL^{-1}.

[1] The *E.coli* BL21-Al™ strain expressing the 16 kDa heat shock protein of *M. tuberculosis* was used. The 16 kDa heat shock protein is the antigen of tuberculosis specific V_HH used in this study. Expression of recombinant 16 kDa heat shock protein was induced followed by lysis of *E. coli* cells, yielding 16 kDa heat shock protein in whole cell lysate solution.

4 RESULTS

4.1 ERROR-PRONE PCR

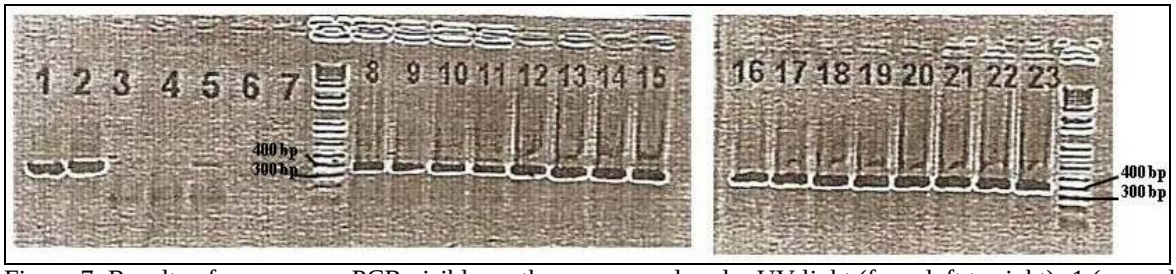

Figure 7: Results of error-prone PCR visible on the agarose gel under UV light (from left to right): 1 (normal PCR with LN23 as template), 2 (normal PCR with LN50 as template), 3 (normal PCR without template (negative control)), 4 (error-prone PCR+ without template (negative control)), 5 (error-prone PCR without template (negative control)), 6 (1 µL of LN23 template in sterile water), 7 (1 µL of LN50 template in sterile water), 8, 9, 10, 11 (all error-prone PCR+ with LN23 as template), 12, 13, 14, 15 (all error-prone PCR+ with LN50 as template), 16, 17, 18, 19 (all error-prone PCR with LN23 as template), 20, 21, 22, 23 (all error-prone PCR with LN50 as template)

Comparing sequences from mutated pools with the original one we noticed that we successfully managed to introduce mutations into V_HHs sequences.

For the error-prone PCR method used without the addition of $MnCl_2$, the rate of producing mutation was 3.8×10^{-3} errors/bp. For the method with the addition of $MnCl_2$, the rate of producing mutations was 5.2×10^{-3} errors/bp. In both cases there were some more transitions (purine to purine substitutions) than transversions (purine to pyrimidine or pyrimidine to purine substitutions). In the pool with the addition of $MnCl_2$, we had also 2 deletions (not in the same sequence) and in both cases it resulted in the reading frame shift.

4.2 PHAGE DISPLAY

4.2.1 Library ligation

The total number of transformants has been calculated from the number of colony forming units (cfu). For LN23+ there were 209 cfu, for LN23 258 cfu, for LN50+ 212 cfu and for LN50 145 cfu.

Table 18: Total number of transformants per library

Type of V_HH insert in bacteria	Total no. of transformants
LN23+	3.1×10^7
LN23	3.9×10^7
LN50+	3.2×10^7
LN50	2.2×10^7

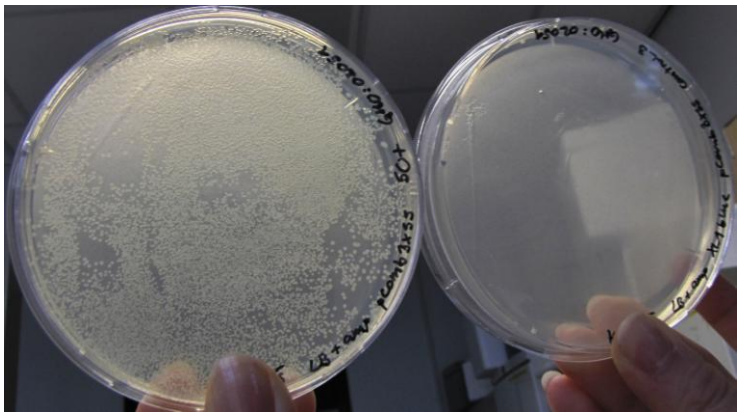
Figure 8: Positive (left) vs negative (right) control

4.2.2 The titer (the infectivity) of the VCSM13 helper phage

Determining the titer (the infectivity) of the VCSM13 helper phage we considered that 50 plaques on the plate derived from the 10^{-8} dilution corresponds to a titer of 5×10^{12} plaque-forming units (pfu) per mL.

On the plate with the 10^{-7} dilution 58 plaques have formed. The corresponding titer of the helper phage is therefore 5.8×10^{11} pfu per mL.

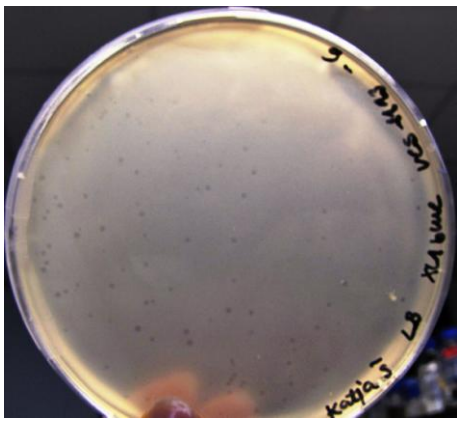
Figure 9: Plaques visible on LB agar plate due to VCSM13 phage infection

4.3 LIBRARY PANNING ON IMMOBILIZED ANTIGEN

After three rounds of panning, we titrated the input sample and the output sample for each library to monitor the success of the selection.

4.3.1 Panning of the LN23 library

Table 19: The input and the output in each panning round for the LN23 library

	1st panning round		2nd panning round		3rd panning round	
	Input	Output	Input	Output	Input	Output
CFU	1.68×10^{13}	8.96×10^{6}	6.94×10^{11}	9.28×10^{6}	3.57×10^{10}	8.00×10^{4}

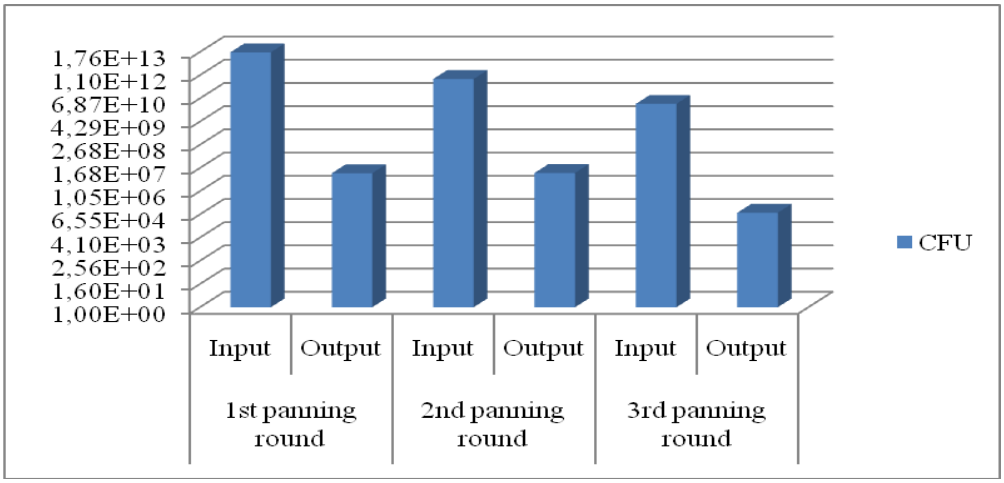

Figure 10: The input and the output in each panning round for the LN23 library

Table 20: The input vs. output ratio in trough panning rounds for the LN23 library

	1st panning round	2nd panning round	3rd panning round
In/out	1.88×10^{6}	7.48×10^{4}	4.46×10^{5}

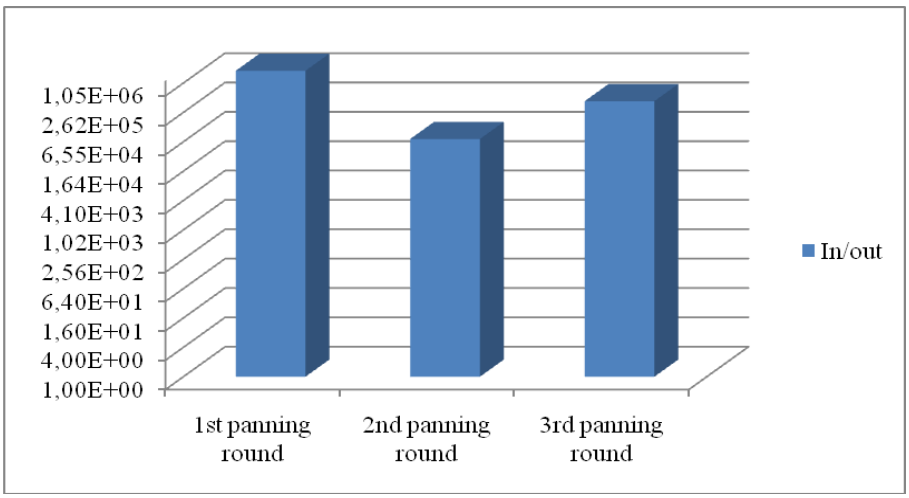

Figure 11: The input vs. output ratio trough panning rounds for the LN23 library

4.3.2 Panning of the LN50 library

Table 21: The input and the output in each panning round for the LN50 library

	1st panning round		2nd panning round		3rd panning round	
	Input	Output	Input	Output	Input	Output
CFU	2.30×10^{13}	8.88×10^{6}	8.98×10^{11}	1.34×10^{7}	5.10×10^{10}	1.92×10^{5}

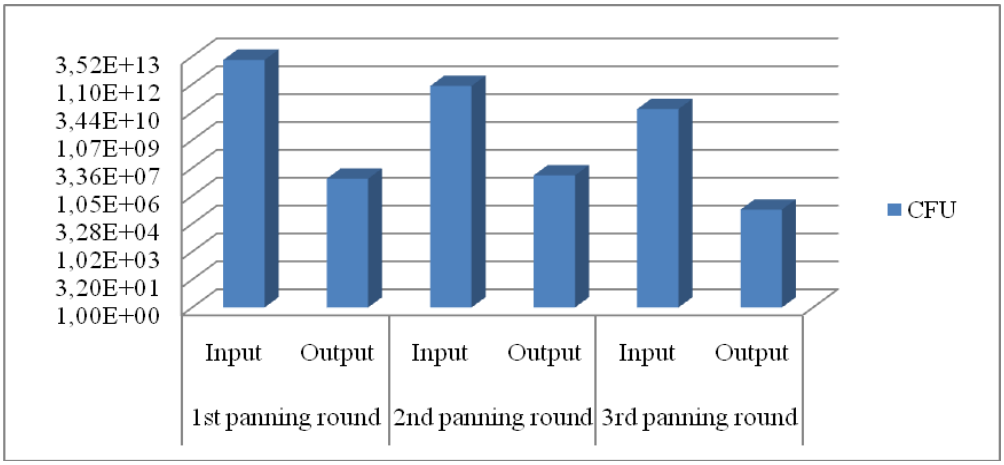

Figure 12: The input and the output in each panning round for the LN50 library

Table 22: The input vs. output ratio trough panning rounds for the LN50 library

	1st panning round	2nd panning round	3rd panning round
In/out	2.59×10^{6}	6.70×10^{4}	2.66×10^{5}

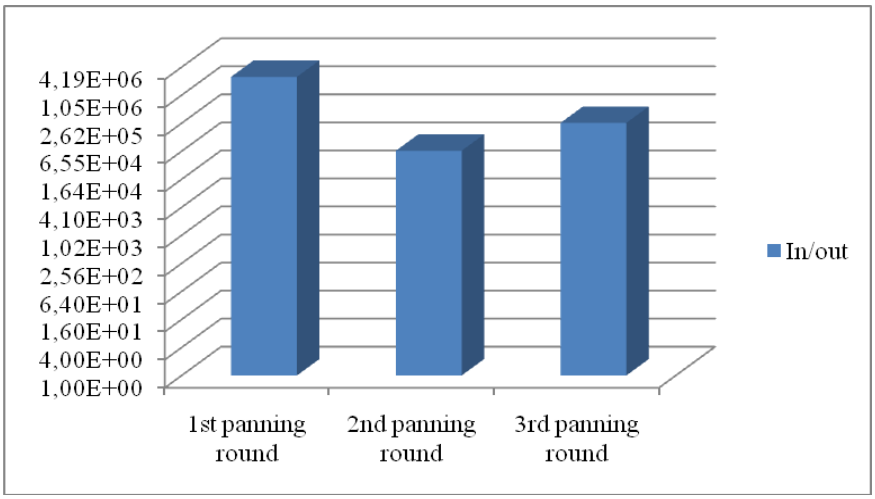

Figure 13: The input vs. output ratio trough panning rounds for the LN50 library

4.3.3 Panning of the LN23+ library

Table 23: The input and the output in each panning round for the LN23+ library

	1st panning round		2nd panning round		3rd panning round	
	Input	Output	Input	Output	Input	Output
CFU	7.14×10^{11}	1.12×10^{6}	1.47×10^{12}	3.20×10^{6}	5.10×10^{10}	1.60×10^{4}

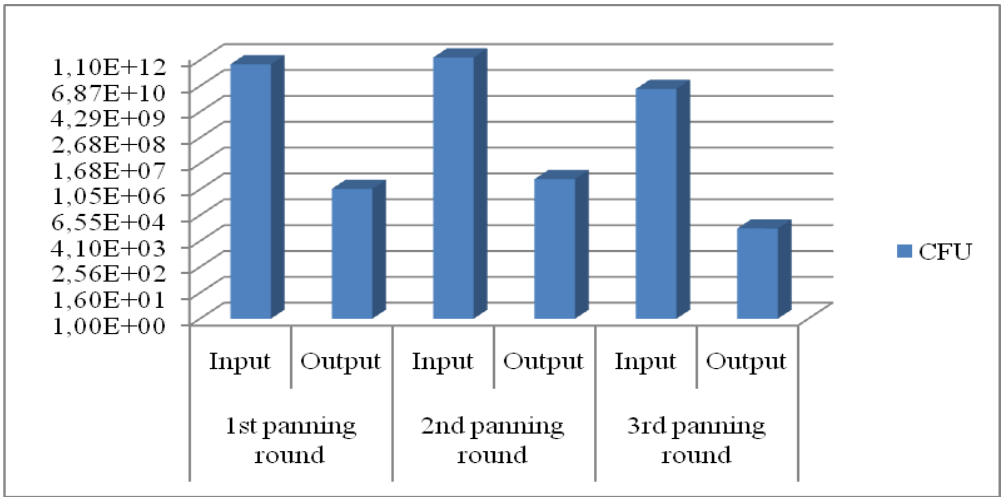

Figure 14: The input and the output in each panning round for the LN23+ library

Table 24: The input vs. output ratio trough panning rounds for the LN23+ library

	1st panning round	2nd panning round	3rd panning round
In/out	6.38×10^{5}	4.59×10^{5}	3.19×10^{6}

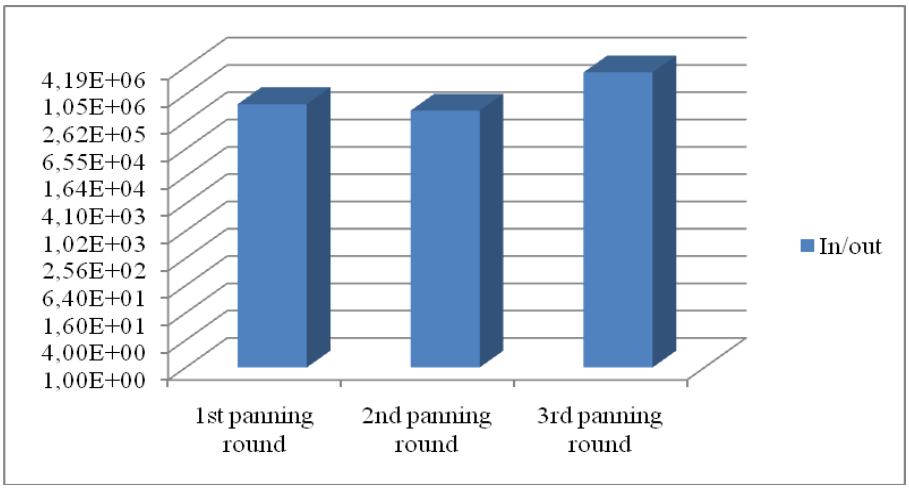

Figure 15: The input vs. output ratio trough panning rounds for the LN23+ library

4.3.4 Panning of the LN50+ library

Table 25: The input and the output in each panning round for the LN50+ library

	1st panning round		2nd panning round		3rd panning round	
	Input	Output	Input	Output	Input	Output
CFU	7.28×10^{13}	1.70×10^{7}	1.02×10^{12}	2.61×10^{7}	4.59×10^{10}	2.40×10^{4}

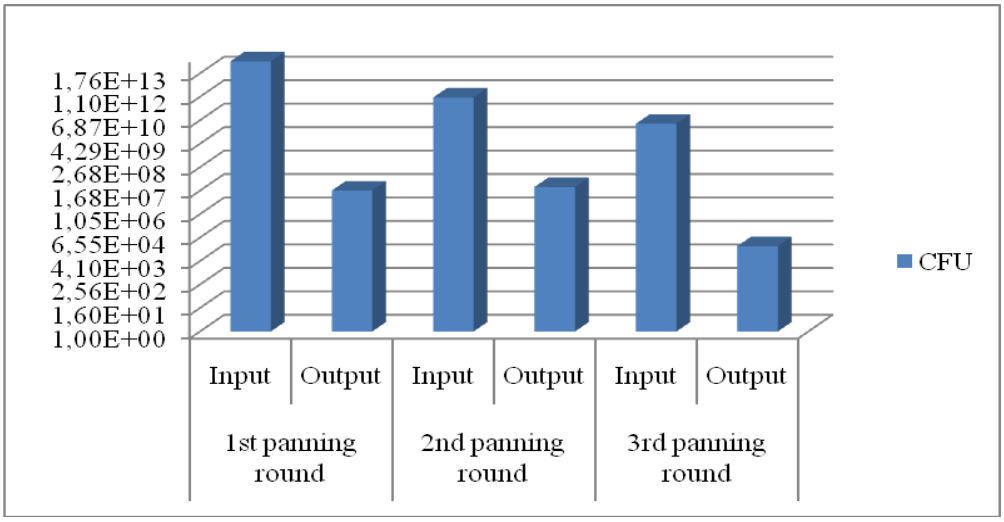

Figure 16: The input and the output in each panning round for the LN50+ library

Table 26: The input vs. output ratio trough panning rounds for the LN50+ library

	1st panning round	2nd panning round	3rd panning round
In/out	4.28×10^{6}	3.91×10^{4}	1.91×10^{6}

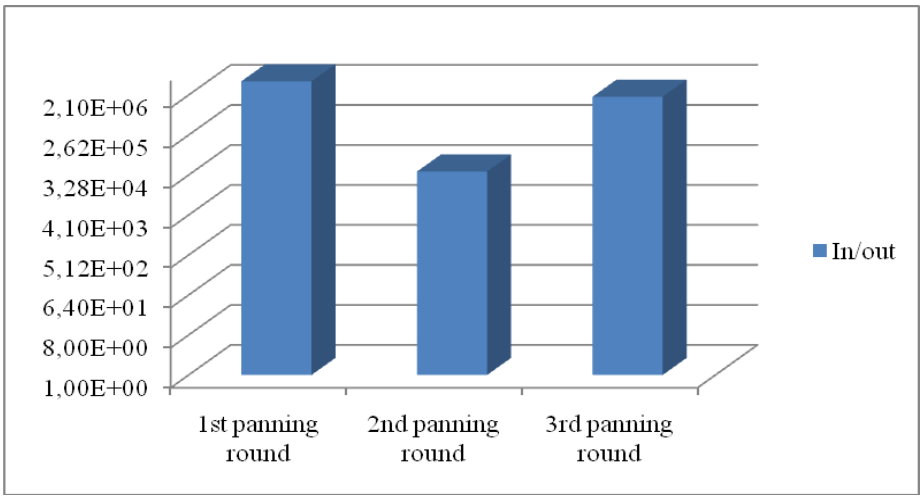

Figure 17: The input vs. output ratio trough panning rounds for the LN50+ library

4.4 EXPRESSION AND PURIFICATION OF RECOMBINANT V_HH FRAGMENTS

4.4.1 Determined protein concentration with Bradford test

Table 27: Protein concentration determined with Bradford test

Sample	Protein concentration
LN50.A1	1187.0 µg mL^{-1}
LN50.A11	287.4 µg mL^{-1}
LN50+.C2	80.4 µg mL^{-1}
LN50+.C3	258.9 µg mL^{-1}
LN50+.D4	229.8 µg mL^{-1}
LN50+.D11	1478.4 µg mL^{-1}
LN23.E1	10.3 µg mL^{-1}
LN23.E9	1167.3 µg mL^{-1}
LN23.E11	82.1 µg mL^{-1}
LN23.F3	86.1 µg mL^{-1}
LN23.F12	203.0 µg mL^{-1}
LN23+.G1	459.6 µg mL^{-1}
LN23+.G2	272.6 µg mL^{-1}
LN23+.H2	566.7 µg mL^{-1}
LN23+.H4	267.4 µg mL^{-1}
Pure LN23	384.9 µg mL^{-1}
Pure LN50	363.2 µg mL^{-1}

LN23.E1 (10.3 µg mL^{-1}), LN23.E11 (82.1 µg mL^{-1}) and LN23.F3 (86.1 µg mL^{-1}) showed low concentrations.

4.4.2 Western blot

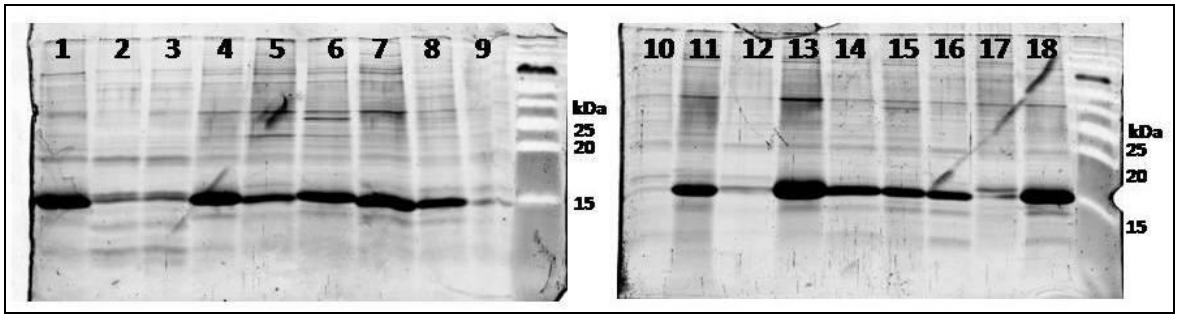

Figure 18: Results of western blot on polyacrilamide gel (from left to right): 1 (original VHH LN23 as positive control), 2 (original VHH LN23 with low concentration (2.3 µg mL^{-1}) as low conc. control), 3 (original VHH LN50 with low concentration (3.3 µg mL^{-1}) as low conc. control), 4 (LN23+.H4), 5 (LN23+.H2), 6 (LN23+.G2), 7 (LN23+.G1), 8 (LN23.F12), 9 (LN23.F3), 10 (LN23.E11), 11 (LN23.E9), 12 (LN23.E1), 13 (LN50+.D11), 14 (LN50+.D4), 15 (LN50+.C3), 16 (LN50+.C2), 17 (LN50.A11), 18 (LN50.A1).

On the polyacrilamide gel V_HH LN23.E1, LN23.E11 and LN23.F3 showed poor

expression, and LN50.A11 did not yield in a good quantity after purification. LN50.A11 showed normal concentration (287 µg mL^{-1}) in Bradford test, but on the gel we noticed that the V_HH is not the main-protein. LN23.E1 was present in too low concentration to take it along in the following ELISA experiment.

4.5 DIRECT ELISA

4.5.1 First ELISA

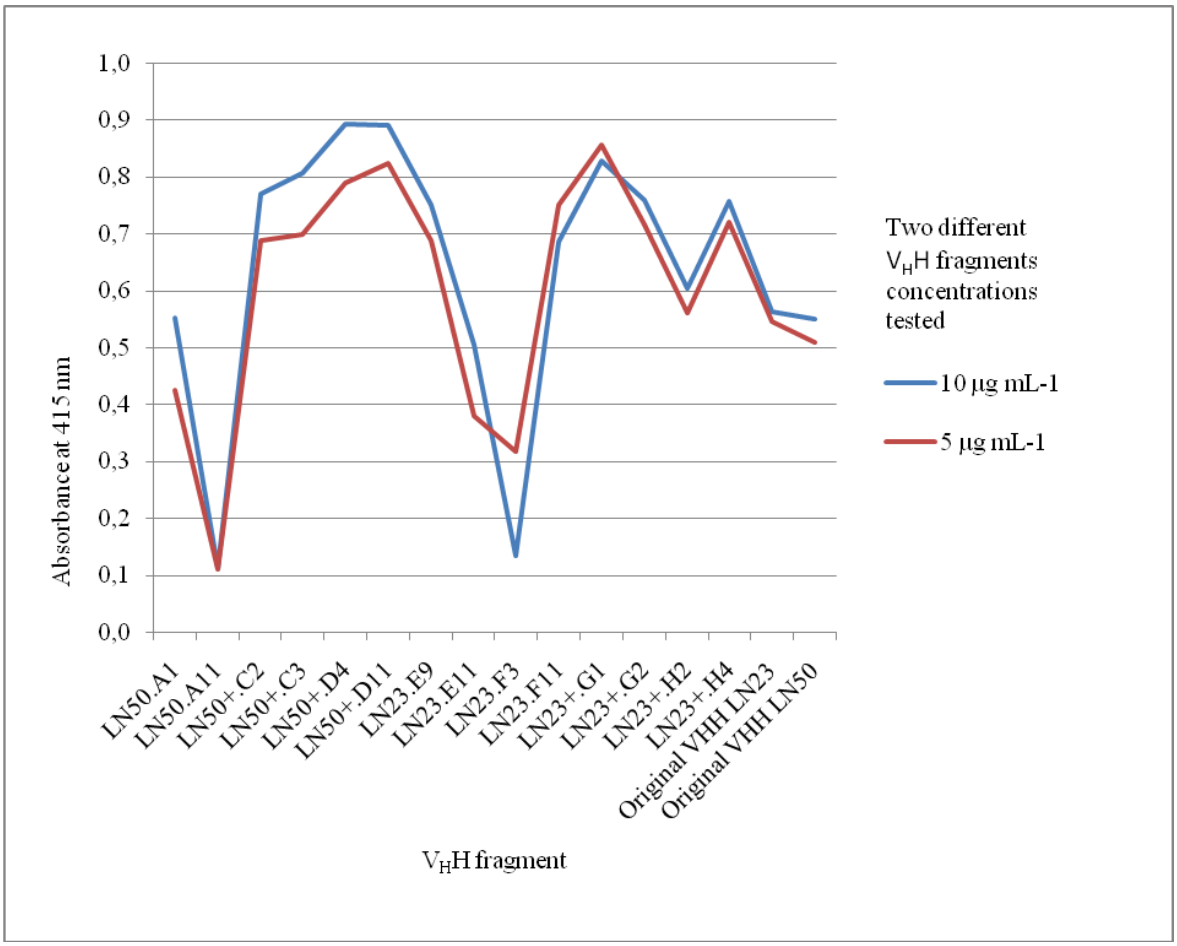

Figure 19: ELISA of 16 randomly selected V_HH fragments

From the first ELISA we see that LN50.A11, LN23.E11 and LN23.F3 show weaker binding to the antigen than original (wild type) V_HH fragments LN23 and LN50. The other 9 V_HH fragments are showing higher affinity for the antigen, that is why we have further on performing a second ELISA with 4 different V_HH fragments concentrations, to confirm the better binding of this 9 recombinant fragments.

4.5.2 Second ELISA

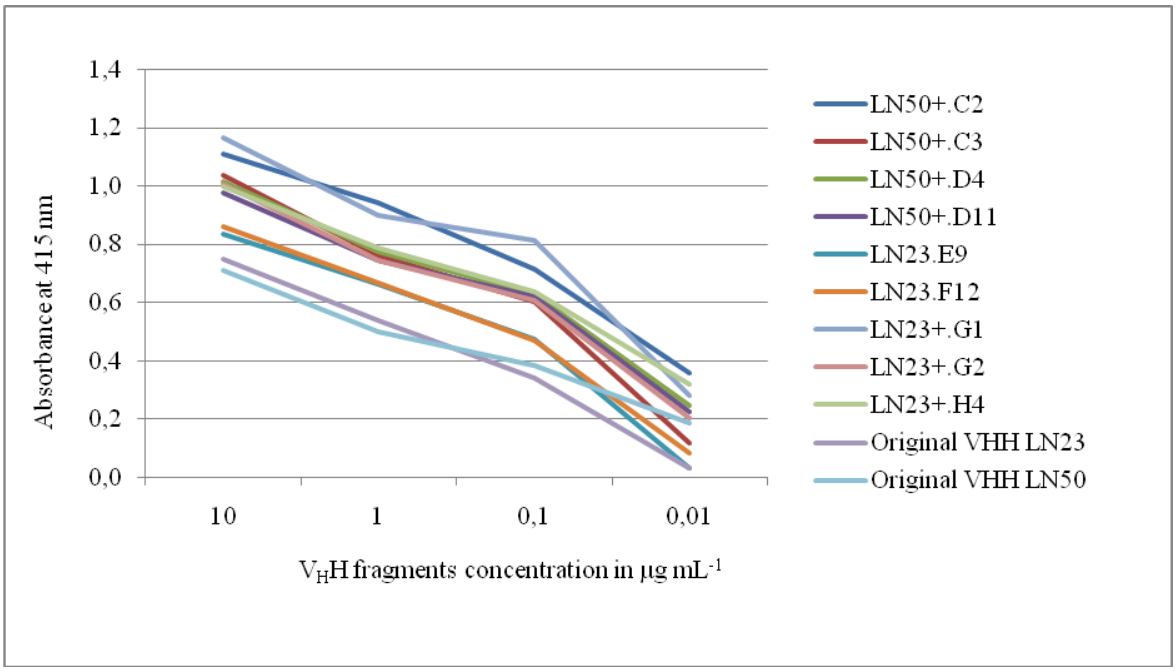

Figure 20: ELISA of 9 V_HH fragments that showed higher affinity for the antigen already in the first ELISA

With this second ELISA we confirmed that the 9 V_HH fragments that showed higher affinity for the antigen already in the first ELISA, are really better binding than the original wild type LN23 and LN50.

5 DISCUSSION AND CONCLUSIONS

5.1 DISCUSSION

5.1.1 Rate of producing mutations

Comparing sequences from mutated pools with the original two we noticed that we successfully managed to introduce mutations into the V_HH fragments sequences varying the conditions of the polymerase chain reaction resulting in the error-prone PCR. For the two reactions used the rate of producing mutations was 3.8×10^{-3} errors/bp and 5.2×10^{-3} errors/bp. Comparing this results with the normally present error rate of the Taq polymerase showed in Table 2 we can conclude, that we successfully determined conditions for the error-prone reactions.

With the addition of $MnCl_2$ in the second error-prone PCR reaction, we managed to vary slightly the mutational rate between the two reaction mixtures used. This resulted in approximately 1.4 times more mutations in sequences gained from the error-prone PCR where $MnCl_2$ was added.

In both cases there were some more transitions (purine to purine substitutions) than transversions (purine to pyrimidine or pyrimidine to purine substitutions), but this was to expect since transitions are normally more frequent in mutational processes. In the pool with the addition of $MnCl_2$, we had also 2 deletions (not in the same sequence) and in both cases it resulted in the reading frame shift and consequently in a totally different protein sequence, which was unwanted as we wanted to insert just some nucleotide substitutions to gain the same protein just with a higher affinity for its antigen.

5.1.2 Phage display and affinity selection

A major factor determining the quality of a library is its complexity (i.e. the number of different antibodies in the library). The greater the complexity, the more likely one is to select antibodies of the required affinity and/or specificity. But there is no way to determine the absolute complexity of an antibody library although we know that it cannot be higher than the no. of independent transformants after library ligation and transformation (Barbas *et al*., 2001). During the experimental work a V_HH phage display library in the reasonable size of approx. 3×10^7 transformants has been constructed. For antibody libraries derived from immune animals, reasonable library sizes are in the range of 10^7 to 10^8 independent transformants (Barbas *et al*., 2001).

Library panning consisted of three rounds of binding to *M. tuberculosis* lyzate immobilized to the well of an ELISA plate. During each round specific binding clones were selected and amplified and then used in the following selection round. After rounds of panning, we titrated the input and the output samples for each library to monitor the success of the

selection. The input of each round is usually in the range of 10^{12} phage, and the output is usually in the range of 10^5 to 10^8 phage, depending on the number of washing steps and the degree of enrichment occurring at a given round (Barbas *et al*., 2001). The input in our case was decreasing in each panning round. In the first round it was approx. 10^{13}, in the second 10^{11} to 10^{12} and in the third approx. 10^{10}. This is probably because of the need of library reamplification after each panning round. Phage preparations should be used for library selection only if they have been prepared the same day, because proteases present in trace levels cleave the displayed antibodies; therefore library reamplification was necessary. Because the rate of production of antibody-displaying phage is influenced by variations in antibody sequence, such that phage which display different antibodies are produced at different rates, it is assumed that reamplification of an existing antibody library reduces its complexity (Barbas *et al*., 2001).

Typically there is a 10- to 100-fold increase in output after selection round three or four (Barbas *et al*., 2001). In our case output was increasing after round two, but then after round three we have a decrease in output. This is because of the increased selection pressure in round three imposed by adding original (wild type) V_HH for binding competition. So in the first and second round of panning the selection pressure was the affinity for the target antigen and proteins that have lost the affinity in the process of mutation were washed away during washing steps. The only binding competition present in first two rounds was the degree of affinity between different V_HH clones. Differently in the last panning round we increased the stringency (the degree to which proteins with higher affinity are favored over proteins with lower affinity) by adding wild type V_HH fragments. The increased stringency usually entails decreased yield (the fraction of particles with a given affinity that survive selection) (Smith and Petrenko, 1997), so the decrease in output after round three was to expect.

5.1.3 Affinity determination

The binding of the mutated recombinant V_HH fragments to the antigen has been tested using the ELISA. From the first performed ELISA we have seen that three of fourteen tested recombinant V_HH fragments are less good binding to the antigen than wild type V_HH fragents. These results were also confirmed with low concentrations observed in the Bradford test for two of these three antibody fragments. For the third V_HH we noticed on the SDS-PAGE that it is not the main-protein. This suggests that the mutations introduced in the mentioned three sequences in two cases lowered the affinity of V_HH fragments for the antigen and in the case of the third V_HH the sequence has been changed in the way to result in a completely different protein. We think that maybe a deletion or insertion of a base pair occurred and this resulted in the frame shift. We encountered 2 similar cases already randomly sequencing mutated V_HH fragments after the error-prone PCR procedure and it is possible, that the mentioned protein matches the sequenced fragments with the frame shift.

On the other hand two V_HH fragments showed satisfying concentration on SDS-PAGE and Bradford test but still didn't seem to perform better than the wild type V_HH fragments. This could be due to mutations, that didn't affect the binding of the antibody fragments to the 16 kDa protein or there were no mutations at all.

Other 9 V_HH fragments showed higher affinity for the antigen comparing to the original V_HH fragments, which we confirmed also in a second ELISA using four different V_HH concentrations. The better binding of this 9 recombinant fragments is due to mutations that altered the V_HH sequences in the way to change their binding properties.

5.2 CONCLUSIONS

We successfully reached the main objective of this graduation thesis, to affinity maturate the single N-terminal domain of heavy chain antibodies (V_HH) derived from *Vicugna alpacos* using the method error-prone PCR to introduce mutations in sequences and then display the V_HH fragments on the surface of phages to subsequently select them by panning on the antigen.

To determine exactly how much stronger do selected V_HH fragments bind to the antigen in comparison to original V_HH fragments LN23 and LN50, further investigations need to be done. With Surface Plasmon resonance analysis the dissociation equilibrium (binding) constant (K_D) could be determined. Trilling *et al*. (2011) already used this technique to determine K_D of wild type of V_HH fragments. The K_D determined for LN23 was 2.4×10^{-9} M and for LN50 2.2×10^{-9} M. From this information we can only predict that the binding constants for affinity maturated V_HH fragments that bind better are higher than 2.4×10^{-9} M.

Sequencing better binding V_HH fragments would be also an interesting option for further research. Analyzing sequences would bring us the information about what mutations are bringing higher affinity properties to this kind of antibody fragments. This could be useful to then trying to engineer an even better binding V_HH fragment using site-directed mutagenic strategies.

6 SUMMARY (POVZETEK)

6.1 SUMMARY

In 1989 a group of biologists led by Raymond Hamers at the Free University of Brussels investigated the immune system of dromedaries. In addition to the expected four-chain antibodies, they identified simpler antibodies consisting only of two heavy chains – heavy chain antibodies. This discovery was published in *Nature* in 1993 (Hamers-Casterman *et al.,* 1993). The discovery that camelids produce functional antibodies devoid of light chain formed a further breakthrough because their single N-terminal domain (V_HH, also referred to as Nanobody® by Ablynx – the developper) binds antigen without requiring domain pairing.

In a previous study Trilling *et al.* (2011) selected V_HH fragments by phage display from a library generated from lymphocyte RNA from a 3- year old female *Vicugna alpacos* immunized with *Mycobacterium tuberculosis* lysate, and characterized. All characterized V_HH fragments bound to the same target – the 16 kDa *M. tuberculosis* antigen.

The object in the present graduation thesis was to produce recombinant V_HH fragments with a higher affinity for the immunodominant 16kDa heat shock protein of *M. tuberculosis* using an *in vitro* mutagenesis method called error-prone PCR.

With *in vitro* mutagenesis we tried to mimic the natural affinity maturation process that takes place during the secondary immune response. In this process B cells produce antibodies with increased affinity for antigen during the course of an immune response. Like the natural prototype, the *in vitro* affinity maturation is based on the principles of mutation and selection. It has successfully been used to optimize antibodies, antibody fragments or other peptide molecules. Techniques such as random mutagenesis, bacterial mutator strains passaging, site-directed mutagenesis, mutational hotspots targeting, parsimonious mutagenesis, antibody shuffling (chain, DNA and staggered extension process) have been used with various degrees of success to affinity mature or modify different kinds of antibodies (Sheedy *et al.*, 2007).

Error-prone PCR is a normal PCR that is typically performed using conditions that reduce the fidelity of Taq DNA polymerase during DNA synthesis to introduce a low level of point mutations randomly over a gene sequence. For our purpose we decided to use the error-prone PCR protocol developed by McCullum *et al.* (2010).

In a previous study Trilling *et al.* (2011) had bulk-ligated isolated V_HH sequences into a *Pst*I and *Not*I digested PRI-VSV expression vector, a strong expression vector for expression in the periplasm based on the backbone of the pRSET-A vector. To obtain V_HH sequences for error-prone PCR, the PRI expression vector was digested using the two unique restriction sites: *Pst*I and *Bst*EII. Primers reconstructing the the *Pst*I and *Bst*EII sites were designed containing additional *Sfi*I sites at both ends for cloning V_HH sequences into pComb3XSS vector.

The used error-prone PCR protocol differed from the standard PCR protocol in including: increased concentration of $MgCl_2$ ions, increased concentration and unbalanced ratio of nucleotides and the reaction was supplemented with $MnCl_2$ ions. For the error-prone PCR reactions used the rates of producing mutations resulted in 3.8 x 10^{-3} and 5.2 x 10^{-3} errors/bp.

To select V_HH antibody fragments with the affinity for the 16 kDa heat shock protein from *M. tuberculosis*, V_HH fragments were displayed on phage surface. For this propose amplified V_HH fragments from error-prone PCR were ligated into the pComb3XSS phagemid vector. This is a type of cloning vector developed as a hybrid of the filamentous phage M13 and plasmids to produce a vector that have plasmid properties and with features of phage vectors. The phagemids containing DNA fragments were introduced into a bacterial host (electrocompetent *E. coli* cells) by electroporation. Later on (after every panning round) the bacterial host containing the phagemid has been infected with a helper phage VCSM13 which provided the necessary viral components (absent in the phagemid) as well as a defective origin of DNA replication. This origin of DNA replication is sufficiently active to permit propagation of the phage, but it is much weaker than the origin contained in phagemid vectors. The infection of phagemid-containing bacterial cells with helper phage resulted in the packaging of only the phagemid. In other words, phagemids replicate as plasmids in *E. coli*, and they can also be packaged as recombinant M13 phage in the presence of helper phage (Bratkovič, 2010; Smith and Scott, 1993).

The selection was carried out by panning of the V_HH-displayed phage library, containing approximately 10^7 individual clones, against *M. tuberculosis* lysate. In the first and second round of panning the selection pressure was the affinity for the target antigen and proteins that have lost the affinity in the process of mutation were washed away during washing steps. The only binding competition present in first two rounds was the degree of affinity between different V_HH clones. The selection pressure was increased in round three by adding pure wild type V_HH for binding competition.

After three rounds of panning, plasmids from selected phage pools were extracted and bulk-ligated into a PRI-VSV expression vector. Constructs were afterwards introduced into *E.coli* BL-21-Al which expressed recombinant V_HH fragments in the presence of L-arabinose. V_HH fragments were purified using Ni-NTA metal-affinity chromatography and eluates were dialyzed against PBS. Protein concentrations were determined using Bradford test. Successful expression and purification was verified by Western blot analysis on sodium dodecylsulphate polyacrylamide gel electrophoresis.

In the enzyme-linked immunosorbent assay wells of flat-bottom ELISA plates were coated with the 16 kDa heat shock protein in whole *E. coli* lysate solution. In the first ELISA we tested the binding of 14 V_HH fragments that were selected randomly from phage pools. The binding to the antigen was tested at two different V_HH concentrations, 10 μg mL^{-1} and 5 μg mL^{-1}. In the second ELISA we tested the binding of 9 V_HHs from the first ELISA, that seemed to have a better binding that wild type V_HH fragments. The binding was tested at 4 different V_HH concentrations: 10 μg mL^{-1}, 1 μg mL^{-1}, 0.1 μg mL^{-1} and 0.01 μg mL^{-1}. 9 of the tested V_HH fragments resulted in a higher affinity for the antigen than the original (wild type) fragments.

6.2 POVZETEK

Stoletja je tuberkuloza predstavljala velik zdravstveni problem po celem svetu. Leta 2010 je bilo odkritih 8,8 milijonov novih primerov obolelih in 1,4 milijonov smrtnih primerov zaradi okužbe z bakterijo *Mycobacterium tuberculosis*, povzročiteljem tuberkuloze (Global tuberculosis…, 2011).

Preživetje bolnikov je pogosto odvisno od hitre in točne diagnoze, kar je hkrati ključ do uspešnega nadzorovanja bolezni. Trenutne diagnostične metode za odkrivanje tuberkuloze temeljijo na dokazovanju bakterijske DNA, biokemijskih in seroloških pristopih (Ferrara, et al. 2009), vendar pa nobena od teh metod še ni primerna za hitro in poceni diagnosticiranje bolezenskega stanja v obliki dostopnega laboratorijskega testiranja ob preiskovancu (ang. point of care test).

Naprava z visoko občutljivostjo - biosenzor z vgrajenimi protitelesi za lovljenje in/ali detekcijo bakterije oz. njenih proteinov (antigenov), bi lahko predstavljala novo orodje, za klasifikacijo tuberkuloze. Taki biosenzorji bi lahko služili kot laboratorij na čipu (ang. lab on a chip) za hitro, poceni in natančno diagnostiko tuberkuloze.

Rekombinantni fragmenti protiteles lame, ki so sestavljena le iz dveh težkoh verig (V_HH) bi lahko bili primerni za uporabo v takih biosenzorjih zaradi njihove velikosti, ki znaša le 15 kDa (Harmsen and De Haard 2007). Trilling *et al*. (2011) so dokazali, da so protitelesa te vrste sposobna razlikovanja *M. tuberculosis* od drugih vrst iz rodu *Mycobacterium*. Vsi selekcionirani fragmenti V_HH iz njihovega poskusa so prepoznali vrstno-specifičen 16 kDa protein iz *M. tuberculosis*.

Raymond Hamers je leta 1989 na Svobodni Univerzi v Bruslju vodil skupino biologov, ki so raziskovali imunski sistem dromedar. Poleg pričakovanih protiteles iz štirih polipeptidnih verig so odkrili še preprostejša protitelesa zgrajena le iz dveh težkih verig. Odkritje je bilo objavljeno v reviji Nature leta 1993 (Hamers-Casterman *et al.*, 1993). Odkritje, da pripadniki družine *Camelidae* proizvajajo funkcionalna protitelesa brez lahkih verig je bilo izjemnega pomena saj ima njihov variabilni fragment (težke verige) V_HH vso sposobnost vezave antigena kot jo imajo imunoglobulini z lahko in težko verigo v variabilnem delu.

Rekombinantna lamina protitelesa (V_HH), narejena na osnovi protiteles z le težko vergo, so kompaktna (15 kDa) saj so zgrajena le iz enojne imunoglobulinske domene. V primerjavi z drugimi rekombinantnimi protitelesi, kot npr. enoverižnimi Fvs, kažejo V_HH (imenovani tudi Nonobodies®) opazno fizikalno-kemijsko stabilnost (Beekwilder *et al*., 2008) in topnost, medtem ko sta vezavna specifičnost in afiniteta podobni lastnostim konvencionalnih protiteles (Dolk *et al*., 2005). Prenesejo ekstremne pH vrednosti in so sposobna vezave na antigen tudi ob prisotnosti visokih koncentracij kaotropnih snovi (Dumoulin *et al*., 2002). Lamina protitelesa so torej boljša oz. primernejša v primerjavi s konvencionalnimi v smislu stroškov produkcije, specifičnosti, afinitete in še posebej v smislu stabilnosti v diagnostičnih pogojih. So torej odlična izbira za uporabo v biosenzorjih, saj regeneracija le teh (elucija vezanega antigena) temelji na inkubaciji v za

proteine močno denaturirajočih pogojih (Wesolowski *et al*., 2009).

V ta namen so Trilling *et al*. (2011) v predhodni študiji imunizirali 3 leta staro *Vicugna alpacos* z lizatom *M. tuberculosis*. Iz knjižnice protiteles, ki so jih pri tem dobili iz limfocitne RNA, so bili izolirani fragmenti V_HH z metodo predstavitve na fagu (angl. phage display), in okarakterizirani. Vsi ti protitelesni fragmenti V_HH (čeprav z različno sekvenco) vežejo isti antigen – imunodominantni protein z molsko maso 16 kDa iz *M. tuberculosis*.

Kljub že dokazani specifičnosti protiteles do povzročitelja tuberkuloze, pa je želja po še višji afiniteti do antigena povsem upravičena, saj bi s tem povečali občutljivost testa.

Cilj diplomskega dela je bilo raziskovanje možnosti *in vitro* afinitetnega zorenja že izoliranih fragmentov V_HH, ki so sicer afinitetno zorenje že prestala *in vivo* po večkratni imunizaciji lame. Z mutageno metodo imenovano »error-prone« verižna reakcija s polimerazo (error-prone PCR) smo poizkušali pridobiti rekombinantne V_HH fragmente z višjo afiniteto do imunodominantnega stresnega proteina z molsko maso 16 kDa (angl. heat shock protein) iz *M. tuberculosis*. Glede na to, da sta mutacija in selekcija temelja evolucije smo domnevali, da je s posnemanjem naravnega poteka zorenja protiteles mogoče dobiti protitelesa z boljšimi lastnostmi. Ker pa so izbrani lamini fragmenti V_HH že šli skozi proces afinitetnega zorenja *in vivo*, smo dopuščali možnost, da tekom mutagenega procesa morda ne bomo pridobili fragmentov z višjo afiniteto do antigena v primerjavi z nemutiranimi fragmenti V_HH.

Z *in vitro* mutagenezo skušamo posnemati naravni proces afinitetnega zorenja protiteles, ki poteka med sekundarnim imunskim odzivom. V tem procesu celice B tekom imunskega odgovora proizvajajo protitelesa z višjo afiniteto do antigena. Tudi zorenje afinitete *in vitro*, kot *in vivo*, temeljita na principu mutacij in selekcije. Slednje je uspešno pri optimizaciji protiteles, njihovih fragmentov ali drugih proteinskih molekul. Tehnike kot so naključna mutageneza, točkovno usmerjena mutageneza, pasaže bakterijskih mutator sevov, premeščanje (DNA, verig) protiteles, StEP in druge so se izkazale z različno stopnjo uspeha pri afinitetnem zorenju protiteles ali modifikaciji različnih tipov protiteles (Sheedy *et al.*, 2007).

V predhodni študiji so Trilling *et al.* (2011) ligirali izolirane sekvence V_HH v ekspresijski vektor PRI-VSV restrikcijsko razrezan z encimoma *Pst*I in *Not*I. Ekspresijski vektor PRI-VSV je močan ekspresijski vektor za izražanje v periplazmi. Da smo pridobili sekvence V_HH za »error-prone« PCR, smo vektor razrezali restriktazama *Pst*I in *Bst*EII. Sledila je rekonstrukcija *Pst*I in *Bst*EII restrikcijskih mest na koncih sekvenc in dodajanje restrikcijskih mest za restriktazo *Sfi*I na oba konca, za kasnejše lažje kloniranje sekvenc v fagni vektor pComb3XSS. V ta namen smo zasnovali začetne oligonukleotide za pomnoževanje nukleotidnih zaporedij in sicer tako, da so poleg prilegajočega zaporedja na drugem koncu vsebovali še zaporedje z omenjenimi restrikcijskimi mesti.

Sledilo je pomnoževanje fragmentov z verižno reakcijo s polimerazo ob uporabi oligonukleotidnih začetnikov (5'-ATGGCCCAGGCGGCCAATCTGCAGGAGTCCGGGGGA-3' in 5'-TGGGCCGGCCTGGCCGGTGACCTGGGTCCCCTGG-3'). Kary Mullis je metodo PCR

razvil leta 1980 (Bartlett and Stirling, 2003). Temelji na sposobnosti DNA polimeraze, da sintetizira komplementarno verigo DNA matrični verigi. Vendar pa DNA polimeraza lahko doda nukleotid le na že obstoječo prosto 3'-OH skupino in zato potrebuje začetnik nukleotid, kamor lahko doda prvi nukleotid.

»Error-prone« PCR je podobna normalni PCR, le da se uporabijo pogoji, ki zmanjšajo zanesljivost (učinkovitost) delovanja Taq DNK polimeraze med sintezo DNK. Na ta način se v sekvenco naključno uvede majhno število točkovnih mutacij. V tej diplomski nalogi smo se ob določevanju reakcijskih pogojev nanašali na protokol, ki so ga razvili McCullum *et al.* (2010). Protokol za »error-prone« PCR se od protokola za standardno PCR razlikuje v višji koncentraciji $MgCl_2$ ionov, višji koncentraciji nukleotidov, v neenakomernem razmerju posameznih nukleotidov in dodatku $MnCl_2$ ionov.

Za vstavljanje naključnih točkovnih mutacij v sekvence fragmentov V_HH smo uporabili dve različni reakcijski mešanici za »error-prone« PCR. Razlika je bila le v vsebnosti $MnCl_2$ ionov (ena reakcijska mešanica jih je vsebovala, druga pa ne). Obe reakciji sta bili sestavljeni iz 30 zaporednih ciklov. Temperatura prileganja začetnih nukleotidov pa je bila 60 ^{0}C. Višjo raven mutacij smo pričakovali za reakcijo z dodatkom $MnCl_2$ ionov.

Da bi ugotovili stopnjo izzvanih mutacij za posamezno »error-prone« PCR tehniko, smo pridobljene mutirane sekvence najprej ekstrahirali iz reakcijskih mešanic. Na agarozni gelski elektroforezi smo preverili, ali smo pri posamezni reakciji dobili produkte želene velikosti (okoli 363 bp). Naključna nukleotidna zaporedja V_HH fragmentov, ki smo jih dobili z »error-prone« PCR, in pComb3XSS vektor smo najprej cepili z restriktazo *Sfi*I. Vektor smo nato še obdelali z encimom alkalna fosfataza. Slednja je katalizirala odstranitev fosfatne skupine s 5'- konca DNA verig vektorja. S tem smo preprečili, da bi se vektor povezal nazaj z ligazo. Ligacija pComb3XSS in V_HH fragmentov je potekala v prosotnosti encima DNA-ligaza, ki katalizira tvorbo fosfodiesterske vezi med 5'-fosfatom enega nukleotida in 3'-hidroksilno skupino drugega.

Vektor z insertom smo nato z metodo elektroporacije vnesli v elektrokompetentne *E. coli* celice. Te smo za tem namnožili na LB agarnih ploščah z vsebnostjo antibiotika ampicilin. Naslednji dan smo naključno vzorčili posamezne nastale kolonije in pripravili kulture za čez noč iz katerih smo dan kasneje izolirali vektorje in jim določili nukleotidno zaporedje. Določanje nukleotidnega zaporedja fragmentom je bilo izvedeno s strani Greenomics (Wageningen, Nizozemska) ob uporabi nukleotidnih začetnikov specifičnih za pComb3XSS, ompseq (5'-AAGACAGCTATCGCGATTGCAG-3') in gback (5'-GCCCCCTTATTAGCGTTTGCCATC-3').

Določena nukleotidna zaporedja posameznih klonov se nahajajo v aneksu A. Po pregledu zaporedij in primerjanju z zaporedji nemutiranih tipov V_HH fragmentov LN23 in LN50 smo na podlagi identificiranih mutacij določili stopnjo izzvanih mutacij. Za uporabljeni metodi »error-prone« PCR se je izkazalo, da sta bili stopnji izzvanih mutacij $3{,}8 \times 10^{-3}$ in $5{,}2 \times 10^{-3}$ mutacij na bazni par. Primerjanje dobljenih rezultatov z normalno prisotno stopnjo napak polimeraze Taq (glej tabelo 2) lahko zaključimo, da smo uspešno določili pogoje za »error-prone« PCR in izvali željeno število mutacij. Z dodatkom $MnCl_2$ eni izmed reakcijskih mešanic smo uspeli nekoliko spremeniti stopnjo mutacij glede na reakcijske mešanice kjer teh ionov nismo uporabili. In sicer smo ugotovili, da je v

nukleotidnih zaporednjih ob uporabi MnCl2 prišlo do 1,4 krat več mutacij. V obeh primerih (ob uporabi MnCl2 in brez) je bilo več tranzicij (substitucij purina z purinom) kakor transverzij (substitucij purina s pirimidinom in obratno), kar je bilo za pričakovati, saj so tranzicije v mutagenih procesih bolj pogoste mutacije od transverzij. V reakcijah kjer smo dodali MnCl2 smo imeli med mutacijami tudi dve deleciji baznega para, vendar ne v istem nukleotidnem zaporedju. V obeh primerih je prišlo do premika bralnega okvirja in posledično do formacije popolnoma drugačne proteinske molekule. Tovrstne mutacije so bile z našega stališča nezaželjene, saj smo želeli uvesti le nekaj nukleotidnih substitucij (in s tem spremeniti afiniteto proteina do antigena), še vedno pa ohraniti osnovni protein.

V literaturi najdemo kar nekaj opisov metod, ki za selekcijo proteinov, ki imajo določeno želeno lastnost, omenjene proteine predstavijo na površini bakteriofagov, bakterij, celic kvasovk, ribosomov itd. Z izrazom "predstavitev na površini" ali "predstavitev na fagih" pogosto označujemo tudi selekcijsko tehniko in ne samo to, da je rekombinanti protein izražen na površini. Postopek selekcije se imenuje izpiranje (Angl. panning).

Vendar pa sistemi za predstavitev peptidov ali proteinov najpogosteje izkoriščajo bakteriofage. Bakteriofage je prvi opisal Frederick Twort leta 1915, kasneje leta 1917 pa še Felix d'Hérelle. D'Hérelle jih je poimenoval bakteriofagi zaradi njihove sposobnosti liziranja bakterij na površini agarne plošče (fag v grščini pomeni "jesti"). Redkeje se uporabljajo evkariontski virusi, bakterijske celice in evkariontske celice kot npr. kvasovke ali sesalske celice. Nekoliko pogosteje so v ta namen v uporabi ribosomi. Vsem sistemom pa je skupno to, da se na njihovi površini (bodisi površini celic, virusov ali rubosomov) predstavi protein, ki nas zanima.

Največje prednosti predstavitve na fagu so preprostost, stabilnost fagnih delcev (ki omogoča selekcijo na celični površini) in trdnost tehnike (Dufner at al., 2006). Ima pa tudi ta tehnika določene omejitve. Ker povezovanje genotipa in fenotipa (npr. sinteza proteinov in združevanje fagnih delov) poteka v bakterijskem gostitelju, je potrebno DNA najprej prenesti v gostitelja. Velikost knjižnice je zaradi tega omejena že z učinkovitostjo transformacije bakterij (Dufner *et al.*, 2006). Ko so se znanstveniki začeli zavedati teže te omejitve, so se začele pojavljati ideje o novih metodah, ki bi uporabljale isti princip, le da bi se izognili potrebi po transformaciji gostiteljskih celic. Leta 1994 so Mattheakis *et al.* predstavili knjižnico sintetičnih peptidov predstavljenih na površini ribosomov. Je najbolj razširjena alternativna metoda predstavitvi na bakteriofagih (Dufner *et al.*, 2006). Slabša alternativa bi bila še predstavitev protiteles na površini celic kvasovk, kar je kot prvim uspelo Boder in Wittrupu leta 1997. Najbolj pogosto uporabljena kvasovka je pri tem *Saccharomyces cerevisiae* (Tanaka *et al.*, 2012). Prednost tehnike bi bilo to, da so kvasovke evkarionti in nudijo post translacijsko modifikacijo (Boder in Wittrup, 1997). Vendar pa je slabost to, da pride pri kvasovkah do drugačne glikozilacije v primerjavi s celicami sesalcev, poleg tega pa nastale knjižnice niso zadovoljive velikosti v primerjavi s knjižnicami sorodnih tehnik (Boder *et al.*, 2000). V uporabi pa so še nekatere druge predstavitvene tehnologije, npr. mRNA predstavitev, predstavitev na površini bakterijskih celic, predstavitev na površini sesalskih celic in druge.

Za selekcijo fragmentov V_HH z afiniteto do 16 kDa stresnega proteina (angl. heat shock protein) iz *M. tuberculosis* smo fragmente predstavili na površini fagov z metodo predstavitve na fagu. Fagna predstavitev je metoda namenjena študiji protein-protein,

protein-peptid in protein-DNA interakcij, ki uporablja bakteriofage, da poveže proteine z njihovim genetskim zapisom. Z drugimi besedami: fagna predstavitev opisuje predstavitev tujih (poli)peptidov na površini faga. Predstavitev peptidov in proteinov na površini bakterijskih virusov je že leta 1985 opisal Smith (Bratkovič, 2010; Smith, 1985). Na površini lahko izrazimo veliko število mutant nekega zaporedja, nato pa selekcioniramo tiste, ki specifično interagirajo z neko substanco (drugo makromolekulo ali antigenom ipd.). Tako lahko preučujemo interakcije med proteini, študiramo afiniteto in specifičnost povezav. Predstavitev željene beljakovine na površini faga lahko dosežemo tako, da gen, ki kodira to beljakovino, vstavimo v gen bakteriofaga, ki kodira strukturni protein kapside. Ker ima fag kapsido zgrajeno iz številnih proteinov, je mogoče predstaviti željeno beljakovino na katerem koli od teh.

pComb3XSS je vrsta konstrukta – fagni vektor, ustvarjen kot hibrid med nitastim bakteriofagom M13 in plazmidi. Ima lastnosti plazmida in bakteriofaga. Fagni vektorji z integriranimi DNA fragmenti so bili z elektroporacijo vstavljeni v *E. coli* elektrokompetentne celice.

Kasneje (po vsaki stopnji selekcije – angl. panning) so bile gostiteljke celice okužene z VCSM13 fagi, ki so prispevali potrebne viroidne komponente (ki jih v fagnem vektorju ni) kakor tudi defektno mesto za začetek podvojevanja (ORI, angl. origin of replication). To mesto za začetek replikacije DNA je aktivno do te mere, da dovoljuje razmnoževanje fagov je pa hkrati mnogo bolj šibko kakor ORI, ki se nahaja v fagemidnih vektorjih. Infekcija bakterijskih celic, ki vsebujejo fagemid, s pomožnim fagom ima za rezultat pakiranje samo fagnega vektorja. Z drugimi besedami, fagni vektorji se razmnožujejo v *E. coli* kot plazmidi, ob prisotnosti pomožnega faga pa so lahko tudi pakirani kot rekombinantni M13 fagi (Bratkovič, 2010; Smith and Scott, 1993). Kot rezultat smo dobili raznoliko populacijo rekombinantnih virusnih delcev, ki na svoji površini izražajo naključne proteine V_HH v obliki fuzijskih proteinov s plaščnim proteinom faga.

Pred tem smo tudi določili bakteriofagni titer oz. število bakteriofagov. Kulturo gostiteljskega seva smo okužili z bakteriofagi in namnožili na LB agarnem gojišču. Naslednji dan smo prešteli prozorne plake na konfluentno preraslem gojišču. Titer bakteriofagov je znašal 5,8 x 10^{11} pfu na mL.

Afinitetna selekcija proteinov V_HH z bakteriofagno predstavitveno knjižnico je potekala v treh stopnjah. Kot vir antigena smo uporabili lizat *M. tuberculosis*, ki je bil vezan na dno mikrotiterske plošče. Lizat smo izpostavili knjižnici fragmentov V_HH predstavljenih na površini fagov, ki je vsebovala okoli 10^7 individualnih klonov. Nevezane bakteriofage smo po vsaki stopnji izločili s spiranjem. V prvi in drugi stopnji je selekcijski pritisk temeljil na afiniteti do tarčnega antigena in proteini, ki so med mutagenim procesom izgubili afiniteto, so bili izločeni iz knjižnice med postopki spiranja. Edina vezavna kompeticija prisotna v prvih dveh stopnja selekcije je bila stopnja afinitete med različnimi V_HH kloni. Selekcijski pritisk smo povečali v tretji stopnji z dodatkom čistega nemutiranega V_HH z namenom vezavne kompeticije. Vezane bakteriofage smo po vsaki stopnji eluirali z dodatkom tripsina. Del eluata smo po vsaki stopnji uporabili, da smo jih pomnožili z okužbo gostiteljske bakterijske kulture. Fagni pripravki za knjižnično selekcijo se lahko uporabijo le, če so bili pripravljeni istega dne. Prisotne proteaze namreč cepijo protitelesa predstavljena na fagni površini in zato je bilo potrebno knjižnico pomnožiti pred vsako

selekcijsko stopnjo. Po in pred vsako selekcijsko stopnjo smo določili tudi titer (število) bakteriofagov, ki so vstopali in izstopali iz določene selekcijske stopnje. S tem smo sledili uspehu selekcije. Število fagnih klonov, ki vstopajo v posamezno selekcijsko stopnjo se ponavadi giblje okoli 10^{12} klonov, število klonov ob izstopu pa okoli 10^5 do 10^8 (Barbas *et al*., 2001). V našem primeru se je število fagnih klonov ob vstopu zmanjševalo. V prvi selekcijski stopnji je bilo okoli 10^{13} vseh fagnih klonov, v drugi 10^{11} do 10^{12} in v tretji okoli 10^{10}. Temu je najverjetneje tako zaradi potrebe po pomnožitvi knjižnice po vsaki selekcijski stopnji, ta pa naj bi zmanjševala kompleksnost obstoječe knjižnice (Barbas *et al*., 2001).

Po navadi lahko opazimo 10- do 100-kratno povečanje števila za antigen specifičnih fagnih klonov ob izstopu iz tretje selekcijske stopnje. Z drugimi besedami, razmerje med številom klonov, ki vstopajo v določeno selekcijsko stopnjo in število klonov, ki iz nje izstopajo, naj bi se zmanjševalo skozi selekcijske stopnje (Barbas *et al*., 2001). V našem primeru lahko to opazimo po drugi selekcijski stopnji. Po tretji pa smo opazili manjše število fagnih klonov ob koncu selekcije. Temu je tako zato, ker smo v zadnji stopnji povečali selekcijski pritisk z dodatkom čistih nemutiranih V_HH in s tem ustvarili še dodatno vezavno kompeticijo.

Po treh stopnjah selekcije so bili naključno izbrani fagni vektorji ekstrahirani in cepljeni z restriktazama *Pst*I in *Bst*EII in vstavljeni v ravno tako restrikcijsko razrezan ekspresijski vektor PRI-VSV. Konstrukti so bili nato vstavljeni v *E.coli* BL-21-Al, ki je ob prisotnosti L-arabinoze proizvedla rekombinantne proteine V_HH. Čiščenje fragmentov je potekalo na kovinski afinitetni kromatografiji Ni-NTA. Pufersko raztopino eluatov pa smo zamenjali z dializo proti PBS pufru.

Koncentracijo proteinov smo določili z metodo po Bradfordu. Metoda temelji na reakciji beljakovin z barvilom Coomassie Brilliant Blue pri čemer nastane obarvan produkt z absorpcijskim maksimumom pri 595 nm (Bradford, 1976). Za standard smo uporabili goveji serumski albumin. Koncentracijo proučevanega proteina smo odčitali iz umeritvene krivulje narejene iz raztopin govejega serumskega albumina z različnimi koncentracijami, pri čemer smo upoštevali, da je absorbanca obarvanega produkta reakcijske raztopine proporcionalna koncentraciji proteinov V_HH v vzorcu. Za tri proteine smo izmerili nizko koncentracijo, kar nakazuje na nizko stopnjo izražanja proteina.

Uspešnost izražanja in očiščenja smo preverili tudi s hibridizacijo odtisa western. Poleg nizke koncentracije treh proteinov, katerim smo to pripisali že po določanju koncentracije z metodo po Bradfordu, smo opazili tudi, da pri enem proteinu ne gre za protein V_HH.

Da bi preverili ali imajo izolirani fragmenti V_HH višjo afiniteto do antigena kakor nemutirani proteini, smo izvedli encimsko imunski test. S tem smo kvalitativno ocenili afiniteto vezave naključno izbranih fragmentov. Vdolbinice mikrotiterske plošče smo prevlekli s stresnim proteinom z molsko maso 16 kDa v lizatu celih *E.coli* celic. Najprej smo izvedli encimsko imunski test kjer smo testirali 14 naključno izbranih proteinov V_HH. Vezavo na antigen smo testirali ob dveh različnih koncentracijah proteinov V_HH, 10 µg mL^{-1} in 5 µg mL^{-1}. Trije rekombinantni fragmenti V_HH so se izkazali s slabšo afiniteto do antigena kakor nemutirani V_HH. Dvema od teh fragmentov smo že ob izvedbi metode po Bradfordu določili nizko koncentracijo. Predvidevamo, da so mutacije v sekvencah

omenjenih dveh proteinov znižale afiniteto do antigena. Na hibridizaciji odtisa western pa smo za tretji protein opazili, da gre za drugačen protein (in ne V_HH). Predvidevamo, da je moralo priti do mutacij (insercij ali delecij), ki so premaknile bralni okvir, kar je imelo za posledico popolnoma drugačen protein. V primeru dveh drugih proteinov pa se afiniteta do antigena ni bistveno spremenila, koncentracija določena po metodi po Bradfordu pa je bila primerljiva s koncentracijami fragmentov, ki so kazali višjo afiniteto. Vzrok za to so lahko mutacije, ki niso imele vpliva na afiniteto, obstaja pa tudi možnost, da do mutacije v omenjenih proteinih sploh ni prišlo in gre za nemutiran tip fragmentov V_HH. Ostalih devet proteinov, ki so kazali višjo afiniteto, smo ponovno testirali z encimsko imunskim testom, le da smo tokrat testirali pri štirih različnih koncentracijah proteinov V_HH, 10 µg mL^{-1}, 1 µg mL^{-1}, 0.1 µg mL^{-1} in 0.01 µg mL^{-1}. S slednjim testom smo potrdili višjo afiniteto omenjenih devetih proteinov V_HH v primerjavi z nemutiranimi V_HH. Pri devetih proteinih, kjer smo dokazali višjo afiniteto do stresnega proteina z molsko maso 16 kDa je torej moralo pritit do mutacij, ki so bistveno vplivale na njihovo afiniteto in jo zvišale.

Da pa bi določili kako močno se izbrani fragmenti V_HH vežejo na antigen v primerjavi z nemutiranimi V_HH, bodo potrebne nadaljne raziskave. Z analizo resonance površinskih plazmonov bi lahko določili disociacijsko ravnotežno (afinitetno) konstanto (K_D). Trilling *et al*. (2011) so z omenjeno analitsko metodo že določili K_D nemutiranih tipov fragmentov V_HH. K_D določena za fragment V_HH LN23 je bila 2,4 x 10^{-9} M, za LN50 pa 2,2 x 10^{-9} M. Iz tega lahko predvidimo, da bi afinitetne konstante uspešno afinitetno zorjenih protitelesnih fragmentov V_HH morala presegati 2,4 x 10^{-9} M.

Tudi določanje nukleotidnega zaporedja fagmentov V_HH, ki so se izkazali z višjo afiniteto, bi bilo lahko predmet nadaljnih raziskav. Z analizo nukleotidnih zaporedij bi lahko katere mutacije in v katerem delu zaporedja so bile ključne za zvišanje afinitete do antigena. Pridobljeno znanje bi lahko uporabili z namenom pridobivanja protitelesnih fragmentov V_HH s še višjo afiniteto, morda tokrat z uporabo metod usmerjene mutagenze.

7 REFERENCES

Balint R.F., Larrick J.W. 1993. Antibody engineering by parsimonious mutagenesis. Gene, 137, 1: 109-118

Barbas C.F., Burton D.R., Scott J.K., Silverman G.J. 2001. Phage display: A laboratory manual. New York, Cold Spring Harbor Laboratory Press: 736 p.

Bartlett J.M.S., Stirling D. 2003. A short history of polimerase chain reaction. Methods in Molecular Biology, PCR protocols. 2nd edition. Totowa, Humana Press Inc.: 546 p.

Beekwilder J., Van Houwelingen A., Van Beckhoven J., Speksnijder A. 2008. Stable recombinant alpaca antibodies for detection of Tulip virus X. European Journal of Plant Pathology, 121, 4: 477-485

Boder E.T., Midelfort K.S., Wittrup K.D. 2000. Directed evolution of antibody fragments with monovalent femtomolar antigen-binding affinity. Proceedings of the National Academy of Sciences of the United States of America, 97: 10701-10705

Boder E.T., Wittrup K.D. 1997. Yeast surface display for screening combinatorial polypeptide libraries. Nature Biotechnology, 15: 553-560

Bradford M. 1976. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. Analitical Biochemistry, 72: 248-254

Bratkovič T. 2010. Progress in phage display: evolution of the technique and its applications. Cellular and Molecular Life Sciences, 67: 749-767

Chames P., Coulon S., Baty D. 1998. Improving the affinity and the fine specificity of an anti-cortisol antibody by parsimonious mutagenesis and phage display. The Journal of Immunology, 161, 10: 5421-5429

Chowdhury P.S., Pastan I. 1999. Improving antibody affinity by mimicking somatic hypermutation *in vitro*. Nature Biotechnology, 17, 6: 568-572

Cirino P.C., Mayer K.M., Umeno D. 2003. Generating mutant libraries using error-prone PCR. In: Methods in Molecular Biology, vol. 231. Directed Evolution Library Creation: Methods and Protocols. Arnold F.H., Georgiou G. (eds.). Totowa, Humana Press Inc.: 3-9

Coia G., Hudson P.J., Irving R.A. 2001. Protein affinity maturation *in vivo* using *E. coli* mutator cells. Journal of Immunological Methods, 251, 1-2: 187-193

Dolk E., Van der Vaart M., Hulsik D.L., Vriend G., De Haard H., Spinelli S., Cambillau C., Frenken L.,Verrips T. 2005. Isolation of Llama Antibody Fragments for Prevention of Dandruff by Phage Display in Shampoo. Applied and Environmental Microbiology, 71, 1: 442-450

Dumoulin M., Conrath K., Van Meirhaeghe A., Meersman F., Heremans K.,. Frenken L.G.J, Muyldermans S., Wyns L., Matagne A. 2002. Single-domain antibody fragments with high conformational stability. Protein Science, 11, 3: 500-515

Dufner P., Jermutus L., Minter R.R. 2006. Harnessing phage and ribosome display for antibody optimisation. Trends in Biotechnology, 24, 11: 523-529

Ferrara G., Losi M., Fabbri L. M., Migliori G. B., Richeldi L., Casali L. 2009. Exploring the immune response against *Mycobacterium tuberculosis* for a better diagnosis of the infection. Archivum Immunologiae et Therapiae Experimentalis, 57, 6: 425-433

Global tuberculosis control, WHO report 2011. 2011. Geneva, World Health Organisation: 246 p.

Hamers-Casterman C., Atarhouch T., Muyldermans S., Robinson G., Hamers C., Songa E.B., Bendahman N., Hamers R. 1993. Naturally occurring antibodies devoid of light chains. Nature, 363: 446-448

Harmsen M.M., De Haard H.J. 2007. Properties, production, and applications of camelid single-domain antibody fragments. Applied Microbiology and Biotechnology, 77: 13-22

Hayden M.S., Gilliland L.K., Ledbetter J.A. 1997. Antibody engineering. Current Opinion in Immunology, 9, 2: 201-212

Irving R.A., Kortt A.A., Hudson P.J. 1996. Affinity maturation of recombinant antibodies using *E. coli* mutator cells. Immunotechnology, 2, 2: 127-143

Jirholt P., Ohlin M., Borrebaeck C.K., Soderlind E. 1998. Exploiting sequence space: shuffling *in vivo* formed complementary determining regions into a master framework. Gene, 215: 471-476

Kang A.S., Jones T.M., Burton D.R. 1991. Antibody redesign by chain shuffling from random combinatorial immunoglobulin libraries. Immunology, 88: 11120-11123

Kehoe J.W., Kay B.K. 2005. Filamentous phage display in the new millennium. Chemical Review, 105, 11: 4056-4072

Khalissa D., Hengliang S., Liang L., Xingzhi W., Xiaojuan Z. 2009. Nanobodies - the new concept in antibody engineering. African Journal of Biotechnology, 8, 12: 2645-2652

Mattheakis L.C., Bhatt R.R., Dower W.J. 1994. An *in vitro* polysome display system for identifying ligands from very large libraries. Proceedings of the National Academy of Sciences of the United States of America, 91: 9022-9026

McCullum E.O., Williams B.A., Zhang J., Chaput J.C. 2010. Random mutagenesis by error-prone PCR. In : *In vitro* mutagenesis protocols. 3rd edition. Methods in molecular biology, 634. Braman J. (ed.). La Jolla, Humana Press: 103-109

Ness J.E., Kim S., Gottman A., Pak R., Krebber A., Borchert T.V., Govindarajan S., Mundorff E.C., Minshull J. 2002. Synthetic shuffling expands functional protein diversity by allowing amino acids to recombine independently. Nature Biotechnology, 20, 12: 1251-1255

Park S.G., Lee J.S., Je E.Y., Kim I.J., Chung J.H., Choi I.H. 2000. Affinity maturation of natural antibody using a chain shuffling technique and the expression of recombinant antibodies in *Escherichia coli*. Biochemical and Biophysical Research Communications, 275, 2: 553-557

pComb3X Family. The Scripps Research Institute. http://www.scripps.edu/mb/barbas/content/pcomb_images/updatepcomb_image.htm#Section%201 (15. Jul. 2012)

pComb3X Maps. The Scripps Research Institute. http://www.scripps.edu/mb/barbas/content/pcomb_images/pcomb_images_files/pComb_Maps/pComb3X_Maps.pdf (15. Jul. 2012)

Pitchard L., Corne D., Kell D., Rowland J., Winson M. 2005. A general model of error-prone PCR. Journal of Theoretical Biology, 234: 497-509

Rychlik W., Spencer W.J., Rhoads R.E. 1990. Optimization of the annealing temperature for DNA amplification *in vitro*. Nucleic Acids Research, 18, 21: 6409-6412

Sambrook J., Russel D.W. 2001. Molecular Cloning: A Laboratory Manual. 3rd edition. Cold Spring Harbor Laboratory Press: 544 p.

Smith G.P., Scott J.K. 1993. Libraries of peptides and proteins displayed on filamentous phage. Methods in Enzymology, 217: 228-257

Sheedy C., Mackenzie R.C., Hall J.C. 2007. Isolation and affinity maturation of hapten-specific antibodies. Biotechnology Advances, 25, 4: 333-352

Smith G.P. 1985. Filamentous fusion phage: Novel expression vectors that display cloned antigens on the virion surface. Science, 228, 4705: 1315-1317

Smith G.P., Petrenko V.A. 1997. Phage display. Chemical Reviews, 97, 2: 391-410

Stanfield R.L., Dooley H., Verdino P., Flajnik M.F., Wilson I.A. 2007. Maturation of Shark Single-Domain (IgNAR) Antibodies: Evidence for Induced-Fit Binding. Journal of Molecular Biology, 367, 2: 358-372

Stemmer W.P. 1994. DNA shuffling by random fragmentation and reassembly: *In vitro*recombination for molecular evolution. Genetics, 91, 22: 10747-10751

Tanaka T., Yamada R., Ogino C., Kondo A. 2012. Recent developments in yeast cell surface display toward extended applications in biotechnology. Applied Microbiology and Biotechnology, 95, 3: 577-91

Taq DNA polymerase (native and recombinant). Invitrogen, life technologies corporation. http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/PCR/pcr-enzymes-master-mixes/taq-dna-polymerase-enzymes/taq_dna_polymerase.html (29. jul. 2012)

Trilling A.K., De Ronde H., Noteboom L., Van Houwelingen A., Roelse M., Srivastava S.K., Haasnoot W., Jongsma M.A., Kolk A., Zuilhof H., Beekwilder J. 2011. A broad set of different llama antibodies specific for a 16 kDa heat shock protein of *Mycobacterium tuberculosis*. PLoS ONE, 6, 10: 1-10

Van der Linden R.H.J., Frenken L.G.J., De Geus B., Harmsen M.M., Ruuls R.C., Stok W., De Ron L., Wilson S., Davis P., Verrips C.T. 1999. Comparison of physical chemical properties of llama V_HH antibody fragments and mouse monoclonal antibodies. Biochimica et Biophysica Acta, 1431: 37-46

Wesolowski J., Alzogaray V., Reyelt J., Unger M., Juarez K., Urrutia M., Cauerh V.A., Danquah W., Rissiek B., Scheuplein F., Schwarz N., Adriouch S., Boyer O., Seman M., Licea A., Serreze D.V., Goldbaum F.A., Haag F., Koch-Nolte F. 2009. Single domain antibodies: promising experimental and therapeutic tools in infection and immunity. Medical Microbiology and Immunology, 198, 3: 157–174

Zahao H., Giver L., Shao Z., Affholter J.A., Arnold F.H. 1998. Molecular evolution by staggered extension process (StEP) *in vitro* recombination. Nature Biotechnology, 16: 258-261

ACKNOWLEDGMENTS

It is a pleasure to thank those who made this thesis possible in the first place by starting this project and accepting me to their laboratory: Anke Kristin Trilling and my co-mentor Dr. Jules Beekwilder. Especial thanks goes to Anke, which has made her support available in a number of ways helping me with research and knowledge. Thank you for all you thought me and for supporting me and always letting me know that I have so much more to learn.

I am grateful to prof. Dr. Mojca Narat for accepting the mentorship for my graduation thesis and for all helpful advices and coments.

I would like to thank prof. Dr. Peter Dovč for reviewing my thesis and for all useful coments.

I am also thankful to the whole team in the Bioscience department at PRI in Wageningen for the warm welcome and the wonderful working atmosphere. It was an honor for me to work by your side.

I would like to show my gratitude especially to Dr. Katja Cankar for all the help when Anke was not available and for your friendship.

I owe my deepest gratitude to my husband for supporting me in all possible ways and for all the patients.

To my parents, for all the support and for helping me in making my dreams come true.

To my grandparents, for believing in me unconditionally during my whole study.

ANNEXES

Annex A:

Sequences of original LN23 and LN50 V_HH fragments

>LN23 (333 bp)
CTGCAGGAGTCCGGGGGAGGCTTGGTGCAGCCTGGGGGGTCTCTGAGACTCTC
CTGTGCAGCCTCTGGGCTCATCTTCAGATTCTCTGCCATGAGCTGGTACCGACA
GGGTCCAGGGAAGGAGCGCGAGTTGGTCGCAGGTATTACTAGCAATGGTGGTA
CCACAAACTATGCAGACTTCGCGAAGGGCCGATTCACCATCTCCAGAGACAAT
GCCAAGAACACGGTGGATCTACAAATGAACAGCCTGAAACCTGAGGACACGG
CCGTGTATTACTGTCATGCAGGGCTCCGCATGGATTCGCGAGACTGGGGCCAG
GGGACCCAGGTCACC

>LN50 (358 bp)
CTGCAGGAGTCCGGGGGAGGATTGGTGCAGGCTGGGGGCTCTCTGACGCTCTC
CTGTACAGCCTCTGGGCGCTCCTTCAGCAATCATGCCATGGGCTGGTTCCGCCA
GGCTCCAGGGAAAGAGCGTGGGTTTGTAGCGGCTGTTGGTTGGATTGCTGGTA
ATACATATTATGCGGACTCTGTGAAGGGCCGCTTCACCATCTCTAGAGACAAC
GCCAAGAACACGCTGTCTCTGCAAATGAACAACCTGAAACCTGAGGACACGGC
CGTGTATTACTGTGCAGCACGGAACGCATTGCGACCAGTTAGTAATAACCTGT
CGGACTACACGTACTGGGGCCAGGGGACCCAGGTCACCG

Annex B:

Sequences of LN23 and LN50 V_HH fragments with mutations determined during this work

>LN23.4 (333 bp)
CTGCAGGAGTCCGGGGGAGGCTTGGTGCAGCCTGGGGGGTTTCTGAGACTCTC
CTGTGCAGCCTCTGGGCTCATCTTCAGATTCTCTGCCATGAGCTGGTACCGACA
GGGTCCAGGGAAGGAGCGCGAGTTGGTCGCAGGTATTACTAGCAATGGTGGTA
CCACAAACTATGCAGACTTCGAGAAGGGCCGATTCACCATCTCCAGAGACAAT
GCCAAGAACACGGTGGACCTACAAATGAACAGCCTGAAACCTGAGGACTCGG
CCGTGTATTACTGTCTTGCAGGGCTCCGCATGAATTCGCGAGACTGGGGCCAG
GGGACCCAGGTCACC

>LN23.9 (333 bp)
CTGCAGGAGTCCGGGGGAGGCTTGGTGCAGCCTGGGGGGTCTCTGAGACTCTC
CTGTGCAGCCTCGGGGCTCATCTTCAGATTCTCTGCCATGAGCTGGTACCGACA
GGGTCCAGGGAAGGAGCGCGAGTTGGTCGCAGGTATTACTAGCAATGGTGGTA
CCACAAACTATGCAGACTTCGCGAAGGGCCGATTCACCATCTCCAGAGACAAT
GCCAAGAACACGGTGGATCTACAAATGAACAGCCTGAAACCTGAGGACACGG
CCGTGTATTACTGTCATGCAGGGCTCCGCATGGATTCGCGAGACTGGGGCCAG
GGGACCCAGGTCACC

>LN23.11 (333 bp)
CTGCAGGAGTCCGGGGGAGGCTTGGTGCAGCCTGGGGGGTCTCTGAGACTCTC
CTGTGCAGCCTCTGGGCTCATCTTCAGATTCTCTGCCATGAGCTGGTACCGACA
GGGTCCAGGGAAGGAGCGCGAGTTGGTCGCAGGTATTACTAGCAATGGTGGTA
CCACAAACTATGCAGACTTCGCGAAGGGCCGATTCACCATCTCCAGAGACAAT
GCCAAGAACACGGTGGATCTACAAATGAACAGCCTGAAACCTGAGGACACGG
CCGTGTATTACTGTCATGCAGGGCTCCGCATGGATTCGCGAGACTGGGGCCAG
GGGACCCAGGTCACC

>LN23.13 (333 bp)
CTGCAGGAGTCCGGGGGAGGCTTGGTGCAGCCTGGGGGGTCTCTGTGACTCTC
CTGTGCAGCCTCTGGGCTCATCTTCAGATTCTCTGCCATGAGCTGGTACCGACA
GGGTCCAGGGAAGGAGCGCGAGTTGGTCGCAGGTATTACTAGCAATGGTGGTA
CCACAAACTATGCAGACTTCGCGAAGGGCCGATTCACCATCTCCAGAGACAAT
GCCAAGAACACGGTGGATCTACAAATGAACAGCCTGAAACCTGAGGACACGG
CCGTGTATTACTGTCATGCAGGGCTCCGCATGGATTCGCGAGACTGGGGCCAG
GGGACCCAGGTCACC

>LN23.15 (333 bp)
CTGCAGGAGTCCGGGGGAGGCTTGGTGCAGCCTGGGGGGTCTCTGAGACTCTC
CTGTACAGCCTCTGGGCTCATCTTCAGATTCTCTGCCATGAGCTGGTACCGACA
GGGTCCAGGGAAGGAGCGCGAGTTGGTCGCAGGTATTACTAGCAATGGTGGTA
CCACAAACTATGCAGACTTCGCGAAGGGCCGATTCACCATCTCCAGAGACAAT
GCCAAGAACACGGTGGATCTACAAATGAACAGCCTGAAACCTGAGGACACGG

CCGTGTATTACTGTCATGCAGGGCTCCGCATGGATTCGCGAGACTGGGGCCAG
GGGACCCAGGTTACC

>LN23.17 (333 bp)
CTGCAGGAGTCCGGGGGAGGCTTGGTGCAGCCTGGGGGGTCTCTGAGACTCTC
CTGTGCAGCCTCTGGGCTCATCTTCAGATTCTCCGCCATGAGCTGGTACCGACA
GGGTCCAGGGAAGGAGCGCGAGTTGGTCGCAGGTATTACTAGCAATGGTGGTA
CCACAAACTATGCAGACTTCGCGAAGGGCCGATTCACCATCTCCAGAGACAAT
GCCAAGAACACGGTGGATCTACAATTGAACAGCCTGAAACCTGAGGACACGG
CCGTGTATTACTGTCATGCAGGGCTCCGCATGGATTCGCGAGACTGGGGCCAG
GGGACCCAGGTCACC

>LN23.18 (333 bp)
CTGCAGGAGTCCGGGGGAGGCTTGGTGCAGCCTGGGGGGTCTCTGAGACTCTC
CTGTGCAGCCTCTGGGCTCATCTTCAGATTCTCTGTCATGAGCTGGTACCGACA
GGGTCCAGGGAAGGAGCGCGAGTTGGTCGCAGGTATTACTAGCAATGGTGGTA
CCACAGACTATGCAGACTTCGCGAAGGGCCGATTCACCACCTCCAGAGACAAT
GCCAAGAACACGGTGGATCTACAGATGAACAGCCTGAAACCTGAGGACACGG
CCGTGTATTACTGTCATGCAGGGCTCCGCATGGATTCGCGAGACTGGGGCCAG
GGGACCCAGGTCACC

>LN23.20 (333 bp)
CTGCAGGAGTCCGGGGGAGGCTTGGTGCAGCCTGGGGGGTCTCTGAGACTCTC
CTGTCCAGCCTCTGGGCTCATCTTCAGATTCTCTGCCATGAGCTGGTACCGTCA
GGGTCCAGGGAAGGAGCTCGAGTTGGTCGCAGGTATTACTAGCAATGGTGGTA
CCACAAACCATGCAGACTTCGCGAAGGGCCGATTCACCATCTCCAGAGACAAT
GCCAAGAACACGGTGGATCTACAAATGAACAGCCTGAAACCTGAGGACACGG
CCGTGTATTACTGTCATGCAGGGCTCCGCATGGATTCGCGAGACTGGGGCCAG
GGGACCCAGGTCACC

>LN23.21 (333 bp)
CTGCAGGAGTCCGGGGGAGGCTTGGTGCAGCCTGGGGGGTCTCTGAGACTCTC
CTGTGCAGCCTCTGGGCTCATCTTCAGATTCTCTGCCATGAGCTGGTACCGACA
GGGTCCAGGGAAGGAGCGCGAGTTGGTCGCAGGTATTACTAGCAATGGTGGTA
CCACAAACTATGCAGACTTCGCGAAGGGCCGATTCACCATCTCCAGAGACAAT
GCCAAGAACACGGTGGATCTACAAATGAACAGCCTGAAACCTGAGGACACGG
CCGTGTATTACTGTCATGCAGGGCTCCGCATGGATTCGCGAGACTGGGGCCAG
GGGACCCAGGTCACC

>LN23.22 (333 bp)
CTGCAGGAGTCCGGGGGAGGCTTGGTGCAGCCTGGGGGGTCTCTGAGACTCTC
CTGTGCAGCCTCTGGGCTCATCTTCAGATTCTCTGCCATGAGCTGGTACTGACA
GGGTCCAGGGATGGAGCGCGAGTTGGTCGCAGGTATTACTAGCAATGGTGGTA
CCACAAACTATGCAGACTTCGCGAAGGGCCGATTCACCATCTCCAGAGACAAT
GCCAAGAACACGGTGGATCTACAAATGAACAGCCTGAAACCTGAGGACACGG
CCGTGTATTACTGTCATGCAGGGCTCCGCATGGATTCGCGAGACTGGGGCCAG
GGGACCCAGGTCACC

>LN23.23 (333 bp)
CTGCAGGAGTCCGGGGGAGGCTTGGTGCAGCCTGGGGGGTCTCTGAGACTCTC
CTGTGCAGCCTCTGGGCTCATCTTCAGATTCTCTGCCATGAGCTGGTACCGACA
GGGTCCAGGTAAGGAGCGCGAGTTGGTCGCAGGTATTACTAGCAATGGTGGTA
CCACAAACTATGCAGACTTCGCGAAGGGTCGATTCACCATCTCCAGAGACAAT
GCCAAGAACACGGTGGATCTACAAATGAACAGCCTGAAACCTGAGGACACGG
CCGTGTATTACTGTCATGCAGGGCTCCGCATGGATTCGCGAGACTGGGGCCAG
GGGACCCAGGTCACC

>LN23.24 (333 bp)
CTGCAGGAGTCCGGGGGAGGCTTGGTGCAGCCTGGGGGGTCTCTGAGACTCTC
CTGTGCAGCCTCTGGGCTCATCTTCAGATTCTCTGCCATGAGCTGGTACCGACA
GGGTCCAGGGAAGGAGCGCGAGTTGGTCGCAGGTATTACTAGCAATGGTGGTA
CCACAAACTATGCAGACTTCGCGAAGGGCCGATTCACCATCTCCAGAGACAAT
GCCAAGAACACGGTGGATCTACAAATGAACAGCCTGAAACCTGAGGACACGG
CCGTGTATTACTGTCATGCAGGGCTCCGCATGGATTCGCGAGACTGGGGCCAG
GGGACCCAGGTCACC

>LN23.25 (333 bp)
CTGCAGGAGTCCGGGGGAGGCTTGGTGCAGCCTGGGGGGTCTCTGAGACTCTC
CTGTGCAGCCTCTGGGCTCATCTTCAGATTCTCTGCCATGAACTGGTACCGTCA
GGGTCCAGGGAAGGAGCGCGAGTTGGTCGCAGGTATTACTAGCAATGGTGGTA
CCACAAACTATGCAGACTTCGCGAAGGGCCGATTCACCATCTCCAGAGACAAT
GCCAAGAACACGGTGGATCTACAAATGAACAGCCTGAAACCTGAGGACACGG
CCGTGTATTACTGTCATGCAGGGCTCCGCATGGATTCGCGAGACTGGGGCCAG
GGGACCCAGGTCACC

>LN50.1 (358 bp)
CTGCAGGAGTCCGGGGGAGGATTGGTGCAGGCTGGGGGCTCTCTGACGCTCTC
CTGTACAGCCTCTGGGCGCTCCTTCAGCAATCATGCCATGGGCTGGTTCCGCCA
GGCTCCAGGGAAAGAGCGTGAGTTTGTAGCGGCTGTTGGTTGGATTGCTGGTA
ATACATATTATGCGGACTCTGTGAAGGGCCGCTTCACCATCTCTAGAGACAAC
GCCAAGAACACGCTGTCTCTGCAAATGAACAACCTGAAACCTGATGACACGGC
CGTGTATTACTGTGCAGCACGGAACGCATTGCGACCAGTTAGTAATAACCTGT
CGGACTACACGTACTGGGGCCAGGGGACCCAGGTCACCG

>LN50.2 (358 bp)
CTGCAGGAGTCCGGGGGAGGATTGGTGCAGGCTGGGGGCTCTCTGACGCTCTC
CTGTACAGCCTCTGGGCGCTCCTTCAGCAATCATGCCATGGGCTGGTTCCGCCA
GGCTCCAGGGAAAGAGCGTGGGTTTGTAGCGGCTGTTGGTTGGATTGCTGGTA
ATACATATTATGCGGACTCTGTGAAGGGCCGCTTCACCATCTCTAGAGACAAC
GCCAAGAACACGCTGTCTCTGCAAATGAACAACCTGAAACCTGAGGACACGGC
CGTGTATTACTGTGCAGCACGGAACGCATTGCGACCAGTTAATAATAACCTGT
CGGACTACACGTACTGGGGCCAGGGGACCCAGGTCACCG

>LN50.3 (358 bp)

CTGCAGGAGTCCGGGGGAGGATTGGTGCAGGCTGGGGGCTCTCTGACGCTCTC
CTGTACAGCCTCTGGGCGCTCCTTCAGCAATCATGCCATGGGCTGGTTCCGCCA
GGCTCCAGGGAAAGAGCGTGAGTTTGTAGCGGCTGTTGGTTGGATTGCTGGTA
ATACATATTATGCGGACTCTGTGAAGGGCCGCTTCACCATCTCTAGAGACAAC
GCCAAGAACACGCTGTCTCTGCAAATGAACAACCTGAAACCTGAGGACACGGC
CGTGTATTACTGTGCAGCACGGAACGCATTGCGACCAGTTAGTAATAACCTGT
CGGACTACACGTACTGGGGCCAGGGGACCCAGGTCACCG

>LN50.4 (358 bp)
CTGCAGGAGTCCGGGGGAGGATTGGTGCAGGCTGGGGGCTCTCTGACGCTCTC
CTGTACAGCCTCTGGGCGCTCCTTCAGCAATCATGCCATGGGCTGGTTCCGCCA
GGCTCCAGGGAAAGAGCGTGGGTATGTAGCGGCTGTTGGTTGGATTGCTGGTA
ATACATATTATGCGGACTCTGTGAAGGGCCGCTTCACCATCTCTAGAGACAAC
GCCAAGAACACGCTGTCTCTGCAAATGAACAACCTGAAACCTGAGGACACGGC
CGTGTATTACTGTGCAGCACGGAACGCATTGCGACCCGTTAGTAATAACCTGT
CGGACTACACGTACTGGGGCCAGGGGACCCAGGTCACCG

>LN50.5 (358 bp)
CTGCAGGAGTCCGGGGGAGGATTGGTGCAGGCTGGGGGCTCTCTGACGCTCTC
CTGTACAGCCTCTGGGCGCTCCTTCAGCAATCATGCCATGGGCTGGTTCCGCCA
GGCTCCAGGGAAAGAGCGTGGGTTAGTAGCGGCTGTTGGTTGGATTGCTGGTA
ATACATATTATGCGGACTCTGTGAAGGGCCGCTTCACCATCTCTAGAGACAAC
GCCAAGAACACGCTGTCTCTGCAAATGAACAACCTGAAACCCGAGGACACGG
CCGTGTATTACTGTGCAGCACGGAACGCATTGCGACCAGTTAGTAATAACCTG
TCGGACTACACGTACTGGGGCCAGGGGACCCAGGTCACCG

>LN50.6 (358 bp)
CTGCAGGAGTCCGGGGGAGGATTGGTGCAGGCTGGGGGCTCTCTGACGCTCTC
CTGTACAGCCTCTGGGCGCTCCTTCAGCAATCATGCCATGGGCTGGTTCCGCCA
GGCTCCAGGGAAAGAGCGTGGGTTTGTAGCGGCTGTTGGTTGGATTGCTGGTA
ATACATATTATGCGGACTCTGTGAAGGGCCGCTTCACCATCTCTAGAGACAAC
GCCAAGAACACGCTGTCTCTGCAAATGAACAACCTGAAACCTGAGGACACGGC
CGTGTATTACTGTGCAGCACGGAACGCATTGCGACCAGTTAGTAATAACCTGT
CGGACTACACGTACTGGGGCCAGGGGACCCAGGTCACCG

>LN50.7 (358 bp)
CTGCAGGAGTCCGGGGGAGGATTGGTGCAGGCTGGGGGCTCTCTGACGCTCTC
CTGTACAGCCTCTGGGCGCTCCTTCAGCAATCATGCCATGGGCTGGTTCCGCCA
GGCTCCAGGGAAAGAGCGTGGGTTTGTAGCGGCTGTTGGTTGGATTGCTGGTA
ATACATATTATGCGGACTCTGTGAAGGGCCGCTTCACCATCTCTAGAGACAAC
GCCAAGAACACGCTGTCTCTGCAAATGAACAACCTGAAACCTGAGGACACGGC
CGTGTATTACTGTGCAGCACGGAACGCATTGCGACCAGTTAGTAATAACCTGT
CGGACTACACGTACTGGGGCCAGGGGACCCAGGTCACCG

>LN50.8 (358 bp)
CTGCAGGAGTCCGGGGGAGGATTGGTGCAGGCTGGGGGCTCTCTGACGCTCTC
CTGTACAGCCTCTGGGCGCTCCTTCAGCAATCATGCCATGGGCTGGTTCCGCCA

GGCTCCAGGGAAAGAGCGTGAGTTTGTAGCGGCTGTTGGTTGGATTGCTGGTA
ATACATATTATGCGGACTCTGTGAAGGGCCGCTTCACCATCTCTAGAGACAAC
GCCAAGAACACGCTGTCTCTGCAAATGAACAACCTGAAACCTGAGGACACGGC
CGTGTATTACTGTGCAGCACGGAACGCATTGCGACCAGTTAGTAATAACCTGT
CGGACTACACGTACTGGGGCCAGGGGACCCAGGTCACCG

>LN50.9 (358 bp)
CTGCAGGAGTCCGGGGGAGGATTGGTGCAGGCTGGGGGCTCTCTGACGCTCTC
CTGTACAGCCTCTGGGCGCTCCTTCAGCAATCATGCCATGGGCTGGTTCCGCCA
GGCTCCAGGGAAAGAGCGTGGGTTTGTAGCGGCTGTTGGTTGGATTGCTGGTA
ATACATATTATGCGGACTCTGTGAAGGGCCGCTTCACCATCTCTAGAGACAAC
GCCAAGAACACGCTGTCTCTGCAAATGAACAACCTGAAACCTGAGGACACGGC
CGTGTATTACTGTGCAGCACGGAACGCATTGCGACCAGTTAGTAATAACCTGT
CGGACTACACGTACTGGGGCCAGGGGACCCAGGTCACCG

>LN50.10 (358 bp)
CTGCAGGAGTCCGGGGGAGGATTGGTGCAGGCTGGGGGCTCTCTGACGCTCTC
CTGTACAGCCTCTGGGCGCTCCTTCAGCAATCATGCCATGGGCTGGTTCCGCCA
GGCTCCAGGGAAAGAGCGTGGGTTTGTAGCGGCTGTTGGTTGGATTGCTGGTA
ATACATATTATGCGGACACTGTGAAGGGCCGCTTCACCATCTCTAGAGACAAC
GCCAAGAACACGCTGTCTCTGCAAATGAACAACCTGAAACCTGAGGACACGGC
CGTGTATTACTGTGCAGCACGGAACGCATTGCGACCAGTTAGTAATAACCTGT
CGGACTACACGTACTGGGGCCAGGGGACCCAGGTCACCG

>LN50.12 (358 bp)
CTGCAGGAGTCCGGGGGAGGATTGGTGCAGGCTGGGGGCTCTCTGACGCTCTC
CTGTACAGCCTCTGGGCGCTCCTTCAGCAATCATGCCATGGGCTGGTTCCGCCA
GGCTCCAGGGAAAGAGCGTGTGTTTGTAGCGGCTGTTGGTTGGATTGCTGGTA
ATACATATTATGCGGACTCTGTGAAGGGCCGCTTCACCATCTCTAGAGACAAC
GCCAAGAACACGCTGTCTCTGCAAATGAACAACCTGAAACCTGAGGACACGGC
CGTGTATTACTGTGCAGCACGGAACGCATTGCGACCAGTTAGTAATAACCTGT
CGGGCTACACGTACTGGGGCCAGGGGACCCAGGTCACCG

>LN50.13 (358 bp)
CTGCAGGAGTCCGGGGGAGGATTGGTGCAGGCTGGGGGCTCTCTGACGCTCTC
CTGTACAGCCTCTGGGCGCTCCTTCAGCAATCATGCCATGGGCTGGTTCCGCCA
GGCTCCAGGGAAAGAGCGTGGGTTTGTAGCGGCTGTTGGTTGGATTGCTGGTA
ATACATATTATGCGGACTCTGTGAAGGGCCGCTTCACCATCTCTAGAGACAAC
GCCAAGAACACGCTGTCTCTGCAAATGAGCAACCTGAAACCTGAGGACACGGC
CGTGTATTACTGTGCAGCACGGAACGCATTGCGACCAGTTAGTAATAACCTGT
CGGACTACACGTACTGGGGCCAGGGGACCCAGGTCACCG

>LN50.14 (358 bp)
CTGCAGGAGTCCGGGGGAGGATTGGTGCAGGCTGGGGGCTCTCTGACACTCTC
CTGTACAGCCTCTGGGCGCTCCTTCAGCAATCATGCCATGGGCTGGTTCCGCCA
GGCTCCAGGGAAAGAGCGTGGGTTTGTAGCGGCTGTTGGTTGGATTGCTGGTA
ATACATATTATGCGGACTCTGTGAAGGGCCGCTTCACCATCTCTAGAGACAAC

GCCAAGAACACGCTGTCTCTGCAAATGAACAACCTGAAACCTGAGGACACGGC
CGTGTATTACTGTGCAGCACGGAACGCATTGCGACCAGTTAGTAATAACCTGT
CGGACTACACGTACTGGGGCCAGGGGACCCAGGTCACCG

>LN50.15 (358 bp)
CTGCAGGAGTCCGGGGGAGGATTGGTGCAGGCTGGGAGCTCTCTGACGCTCTC
CTGTACAGCCTCTGGGCGCTCCTTCAGCAATCATGCCATGGGCTGGTTCCGCCA
GGCTCCAGGGAAAGAGCGAGGGTTTGTAGCGGCTGTTGGTTGGATTGCTGGTA
ATACATATTATGCGGACTCTGTGAAGGGCCGCTTCACCATCTCTAGAGACAAC
GCCAAGAACACGCTGTCTCTGCAAATGAACAACCTGAAACCTGAGGACACGGC
CGTGTATTACTGTGCAGCACGGAACGCATTGCGACCAGTTAGTAATAACCTGT
CGGACTACACGTACTGGGGCCAGGGGACCCAGGTCACCG

>LN50.16 (358 bp)
CTGCAGGAGTCCGGGGGAGGATTGGTGCAGGCTGGGGGCTCTCTGACGCTCTC
CTGTACAGCCTCTGGGCGCTCCTTCAGCAATCATGCCATGGGCTGGTTCCGCCA
GGCTCCAGGGAAAGAGCGTGGGTTTGTAGCGGCTGTTGGTTGGATTGCTGGTA
ATACATATTATGCGGACTCTGTGAAGGGCCGCTTCACCATCTCTAGAGACAAC
GCCAAGAACACGCTGTCTCTGCAAATGAACAACCTGAAACCTGAGGACACGGC
CGTGTATTACTGAGCAGCACGGAACGCATTGCGACCAGTTAGTAATAACCTGT
CGGACTACACGTACTGGGGCCAGGGGACCCAGGTCACCG

>LN50.17 (358 bp)
CTGCAGGAGTCCGGGGGAGGATTGGTGCAGGCTGGGGGCTCTCTGACGCTCTC
CTGTACAGCCTCTGGGCGCTCCTTCAGCAATCATGCCATGGGCTGGTTCCGCCA
GGCTCCAGGGAAAGAGCGTGGGTTTGTAGCGGCTGTTGGTTGGATTGCTGGTA
ATACATATTATGCGGACTCTGTGAAGGGCCGCTTCACCATCTCTAGAGACAAC
GCCAAGAACACGCTGTCTCTGCAAATGAACAACCTGAAACCTGAGGACACGGC
CGTGTATTACTGTGCAGCACGGAACGCATTGCGACCAGTTAGTAATAACCTGT
CGGACTACACGTACTGGGGCCAGGGGACCCAGGTCACCG

>LN50.18 (358 bp)
CTGCAGGAGTCCGGGGGAGGATTGGTGCAGGCTGGGGGCTCTCTGACGCTCTC
CTGTACAGCCTCTGGGCGCTCCTTCAGCAATCATGCCATGGGCTGGTTCCGCCA
GGCTCCAGGGAAAGAGCGTGGGTTTGTAGCGGCTGTTGGTTGGATTGCTGGTA
ATACATATTATGCGGACTCTGTGAAGGGCCGCTTCACCATCTCTAGAGACAAC
GCCAAGAACACGCTGTCTCTGCAAATGAACAACCTGAAACCTGAGGACACGGC
CGTGTATTACTGTGCAGCACGGAACGCATTGCGACCAGTTAGTAATAACCTGT
CGGACTACACGTACTGGGGCCAGGGGACCCAGGTCACCG

>LN50.19 (358 bp)
CTGCAGGAGTCCGGGGGAGGATTGGTGCAGGCTGGGGGCTCTCTGACGCTCTC
CTGTACAGCCTCTGGGCGCTCCTTCAGCAATCATGCCATGGGCTGGTTCCGCCA
GGCTCCAGGGAAAGAGCGTGGGTTTGTAGCGGCTGTTGGTTGGATTGCTGGTA
ATACATATTATGCGGACTCTGTGAAGGGCCGCTTCACCATCTCTAGAGACAAC
GCCAAGAACACGCTGTCTCTGCAAATGAACAACCTGAAACCTGAGGACACAGC

CGTGTATTACTGTGCAGCACGGAACGCATTGCGACCAGTTAGTAATAACCTGT
CGGACTACACGTACTGGGGCCAGGGGACCCAGGTCACCG

>LN50.20 (358 bp)
CTGCAGGAGTCCGGGGGAGGATTGGTGCAGGCTGGGGGCTCTCTGACGCTCTC
CTGTACAGCCTCTGGGCGCTCCTTCAGCAATCATGCCATGGGCTGGTTCCGCCA
GGCTCCAGGGAAAGAGCGTGGGTTTGTAGCGGCTGTTGGTTGGATTGCTGGTA
ATACATATTATGCGGACTCTGTGAAGGGCCGCTTCACCATCTCTAGAGACAAC
GCCAAGAACACGCTGTCTCTGCAAATGAACAACCTGAAACCTGAGGACACGGC
CGTGTATTACTGTGCAGCACGGAACGCATTGCGACCAGTTAGTAATAACCTGT
CGGACTACACGTACTGGGGCCAGGGGACCCAGGTCACCG

>LN50.21 (358 bp)
CTGCAGGAGTCCGGGGGAGGATTGGTGCAGGCTGGGGGCTCTCTGACGCTCTC
CTGCACAGCCTCTGGGCGCTCCTTCAGCAATCATGCCATGGGCTGGTTCCGCCA
GGCTCCAGGGAAAGAGCGTGAGTTTGTAGCGGCTGTTGGTTGGATTGCTGGTA
ATACATATTATGCGGACTCTGTGAAGGGCCGCTTCACCATCTCTAGAGACAAC
GCCAAGAACACGCTGTCTCTGCAAATGAACAACCTGAAACCTGAGGACACGGC
CGTGTATTACTGTGCAGCACGGAACGCATTGCGACCAGTTAGTAATAACCTGT
CGGACTACACGTACTGGGGCCAGGGGACCCAGGTCACCG

>LN50.22 (358 bp)
CTGCAGGAGTCCGGGGGAGGATTGGTGCAGGCTGGGGGCTCTCTGACGCTCTC
CTGTACAGCCTCTGGGCGCTCCTTCAGCAATCATGCCATGGGCTGGTTCCGCCA
GGCTCCAGGGAAAGAGCGTGAGTTTGTAGCGGCTGTTGGTTGGATTGCTGGTA
ATACATATTATGCGGACTCTGTGAAGGGCCGCTTCACCATCTCTAGAGACAAC
GCCAAGAACACGCTGTCTCTGCAAATGAACAACCTGAAACCTGAGGACACGGC
CGTGTATTACTGTGCAGCACGGAACGCATTGCGACCAGTTAGTAATAACCTGT
CGGACTACACGTACTGGGGCCAGGGGACCCAGGTCACCG

>LN50.23 (358 bp)
CTGCAGGAGTCCGGGGGAGGATTGGTGCAGGCTGGGGGCTCTCTGACGCTCTC
CTGTACCGCCTCTGGGCGCTCCTTCAGCAATCATGCCATGGGCTGGTTCCGCCA
GGCTCCAGGGAAAGAGCGTGGGTTTGTAGCGGCTGTTGGTTGGATTGCTGGTA
ATACATATTATGCGGACTCTGTGAAGGGCCGCTTCACCATCTCTAGAGACAAC
GCCAAGAACACGCTGTCTCTGCAAATGAACAACCTGAAACCTGAGGACACGGC
CGTGTATTACTGTGCAGCACGGAACGCATTGCGACCAGTTAGTAATAACCTGT
CGGACTACACGTACTGGGGCCAGGGGACCCAGGTCACCG

>LN50.24 (358 bp)
CTGCAGGAGTCCGGGGGAGGATTGGTGCAGGCTGGGGGCTCTCTGACGCTCTC
CTGTACAGCCTCTGGGCGCTCCTTCAGCAATCATGCCATGGGCTGGTTCCGCCA
GGCTCCAGGGAAAGAGCGTGGGTTTGTAGCGGCTGTTGGTTGGATTGCTGGTA
ATACAGATTATGCGGACTCTGTGAAGGGCCGCTACACCATCTCTAGAGACAAC
GCCAAGAACACGCTGTCTCTGCAAATGAACAACCTGAAACCTGAGGACACGGC
CGTGTATTACTGTGCAGCATGGAACGCATTGCGACCAGTTAGTAATAACCTGT
CGGACTACACGTACTGGGGCCAGGGGACCCAGGTCACCG

>LN50.25 (358 bp)
CTGCAGGAGTCCGGGGGAGGATTGGTGCAGGCTGGGGGCTCTCTGACGCTCTC
CTGTACAGCCTCTGGGCGCTCCTTCAGCAATCATGCCATGGGCTGGTTCCGCCA
GGCTCCAGGGAAAGAGCGTGGGTTTGTAGCGGCTGTTGGTTGGATTGCTGGTA
ATACATATTATGCGGACTCTGTGAAGGGCCGCTTCACCATCTCTAGAGACAAC
GCCAAGAACACGCTGTCTCTGCAAATGAACGACCTGAAACCTGAGGACACGGC
CGTGTATTACTGTGCAGCACGGAACGCATTGCGACCAGTTAGTAATAACCTGT
CGGACTACACGTACTGGGGCCAGGGGACCCAGGTCACCG

>LN50.26 (358 bp)
CTGCAGGAGTCCGGGGGAGGATTGGTGCAGGCTGGGGGCTCTCTGACGCTCTC
CTGTACAGCCTCTGGGCGCTCCTTCAGCAATCATGCCATGGGCTGGTTCCGCCA
GGCTCCAGGGAAAGAGCGTGGGTTTGTAGCGGCTGTTGGTTGGATTGCTGGTA
ATACATATTATGCGGACTCTGTGAAGGGCCGCTTCACCATCTCTAGAGACAAC
GCCAAGAACACGTTGTCTCTGCAAATGAACAACCTGAAACCTGAGGACACGGC
CGTGTATTACTGTGCAGCACGGAACGCATTGCGACCAGTTAGTAATAACCTGT
CGGACTACACGTACTGGGGCCAGGGGACCCAGGTCACCG

>LN50.27 (358 bp)
CTGCAGGAGTCCGGGGGAGGATTGGTGCAGGCTGGGGGCTCTCTGACGCTCTC
CTGTACAGCCTCTGGGCGCTCCTTCAGCAATCATGCCATGGGCTGGTTCCGCCA
GGCTCCAGGGAAAGAGCGTGGGTTTGTAGCGGCTGTTGGTTGGATTGCAGGTA
ATACATATTATGCGGACTCTGTGAAGGGCCGCTTCACCATCTCTAAAGACAAC
GCCAAGAACACGCTGTCTCTGCAAATGAACAACCTGAAACCTGAGGACACGGC
CGTGTATTACTGTGCAGCACGGAACGCATTGCGACCAGTTAGTAATAACCTGT
CGGACTACACGTACTGGGGCCAGGGGACCCAGGTCACCG

>LN23+.2 (333 bp)
CTGCAGGAGTCCGGGGGAGGCTTGGTGCAGCCTGGGGGGTCTCTGAGACTCTC
CTGTGCAGCCTCTGGGCTCATCTTCAGATTCTCTGCCATGAGCTGGTACCGACA
GGGTCCAGGGAAGGAGCGCGAGTTGGTCGCAGGTATTACTAGCAATGGTGGTA
CCACAAACTATGCAGACTTCGCGAAGGGCCGATTCACCATCTCCAGAGACAAT
GCCAAGAACACGGTGGATCTACAAATGAACAGCCTGAAACCTGAGGACACGG
CCGTGTATTACTGTCATGCAGGGCTCCGCATGGATTCGCGAGACTGGGGCCTG
GGGACCCAGGTCACC

>LN23+.3 (333 bp)
CTGCAGGAGTCCGGGGGAGGCTTGGTGCAGCCTGGGGGGTCTCTGAGACTCTC
CTGTGCAGCCTCTGGGCTCATCTTCAGATTCTCTGCCATGAGCTGGTACCGACA
GGGTCCAGGGAAGGAGCGCGAGTTGGTCGCAGGTATTACTAGCAATGGTGGTA
CCACAAACTATGCAGACTTCGCGAAGGGCCGATTCACCATCTCCAGAGACAAT
GCCAAGAACACGGTGGATCTACAAATGAACAGCCTGAAACCTGAGGACACGG
CCGTGTATTACTGTCATGCAGGGCTCCGCATGGATTCGCGAGACTGGGGCCTG
GGGACCCAGGTCACC

>LN23+.5 (333 bp)

CTGCAGGAGTCCGGGGGAGGCTTGGTGCAGCCTGGGGGGTCTCTGAGACTCTC
CTGTGCAGCCTCAGGGCTCATCTTCAGATTCTCTGCCATGAGCTGGTACCGACA
GGGTCCAGGGAAGGAGCGCGAGTTTGTCGCAGGTATTACTAGCAATGGTGGTA
CCACAAACTATGCAGACTTCGCGAAGGGCCGATTCACCATCTCCAGAGACAAT
GCCAAGAACACGGTGGATCTACAAATGAACAGCCTGAAACCTGAGGACACGG
CCGTGTATTACTGTCATGCAGGGCTCCGCATGGATTCGCGAGACTGGGGCCAG
GGGACCCAGGTCACC

>LN23+.7 (333 bp)
CTGCAGGAGTCCGGGGGAGGCTTGGTGCAGCCTGGGGGGTCTCTGAGACTCTC
CTGTGCAGCCTCTGGGCTCATCTTCAGATTCTCTGCCATGAGCTGGTACCGACA
GGGTCCAGGGAAGGAGCGCGAGTTGGTCGCAGGTACTACTAGCAATGGTGGT
ACCACAAACTTTGCAGACTTCGCGAAGGGCCGATTCACCATCTCCAGAGACAA
TGCCAAGAACACGGTGGATCTACAAATGAACAGCCTGAAACCTGAGGACACG
GCCGTGTATTACTGTCATGCAGGGCTCCGCATGGATTCGCGAGACTGGGGCCA
GGGGACCCAGGTCACC

>LN23+.8 (333 bp)
CTGCAGGAGTCCGGGGGAGGCTTGGTGCAGCCTGGGGGGTCTCTGAGACCCTC
CTGTGCAGCCTCTGGGCTCATCTTCAGATTCTCTGCCATGAGCTGGTACCGACA
GGGTCCAGGGAAGGAGCGCGAGTTGGTCGCAGGTATTCTAGCAATGGTGGTAC
CACAAACTATGCAGACTTCGCGAAGGGCCGATTCACCTTCTCCAGAGACAATG
CCAAGAACACGGTGGATCTACAAATGAACAGCCTGAAACCTGAGGACACGGC
CGTGTATTACTGTCATGCAGGGCTCCGCATGGATTCGCAAGACTGGGGCCAGG
GGACCCAGGTCACC

>LN23+.9 (333 bp)
CTGCAGGAGTCCGGGGGAGGCTTGGTGCAGCCTGGGGGGTCTCTGAGACTCTC
CTGTGCAGCCTCTGGGCTCATCTTCAGATTCTCTGCCATGAGCTGGTACCGACA
GGGTCCAGGGAAGGAGCGCGAGTTGGTCGCAGGTATTACTAGCAATGGTGGTA
CCACAAACTATGCAGTCTTCGCGAAGGGCCGATTCACCATCTCCAGAGACAAT
GCCAAGAACACGGTGGTTCTACAAATGAACAGCCTGAAACCTGAGGACACGG
CCGTGTATTACTGTCATGCAGGGCTCCGCATGGATTCGCGAGACTGGGGCCAG
GGGACCCAGGTCACC

>LN23+.10 (333 bp)
CTGCAGGAGTCCGGGGGAGGCTTGGTGCAGCCTGGGGGGTCTCTGAGACTCTC
CTGTGCAGCCTCTGGGCTCATCTTCAGATTCTCTGCCATGAGCTGGTACCGACA
GGGTCCAGGGAAGGAGCGCGAGTTGGTCGCAGGTATTACTAGCAATGGTGGTA
CCACAAACTATGCAGACTTCGCGAAGGGCCGATTCACCATCTCCAGAGACAAT
GCCAAGAACACGGTGGACCTACAAATGAACAGCCTGAAGCCTGAGGACACGG
CCGTGTATTACTGTCATGCAGGGCTCCGCATGGATTCGCGAGACTGGGGCCAG
GGGACCCAGGTCACC

>LN23+.11 (333 bp)
TTGCAGGAGTCCGGGGGAGGCTTGGTGCAGCCTGGGGGTCTCTGAGACTCTCC
TGTGCAGCCTCTGGGCTCATCTTCAGATTCTCTGCCATGAGCTGGTACCGACAG

GGTCCAGGGAAGGAGCGCGAGTTGGTCGCAGGTATTACTAGCAATGGTGGTAC
CACAAACTATGCAGACTTCGCGAAGGGCCGATTCACCATCTCCAGAGACAATG
CCAAGAACACGGTGGATCTACAAATGAACAGCCTGAAACCTGAGGACACGGC
CGTGTATTACTGTCATGCAGGGCTCCGCATGGATTCGCGAGACTGGGGCCAGG
GGACCCAGGTCACC

>LN23+.12 (333 bp)
CTGCAGGAGTCCGGGGGAGGCTTGGTGCAGCCTGGGGGGTCTCTGAGACTCTC
CTGTGCAGCCTCTGAGCTCATCTTCAGATTCTCTGCCATGAGCTGGTACCGACA
GGGTCCAGGGAAGGTGCGCGAGTTGGTCGCAGGTATTACTTGCAATGGTGGTA
CCACAAACTATGCAGACTTCGCGAAGGGCCGATTCACCATCTCCAGAGACAAT
GCCAAGAACACGGTGGATCTACAAATGAACAGCCTGAAACCTGAGGACACGG
CCGTGTATTACTGTCATGCAGGGCTCCGCATGGATTCGCGAGACTGGGGCCAG
GGGACCCAGGTCACC

>LN23+.13 (333 bp)
CTGCAGGAGTCCTGGGGAGGCTTGGTGCAGCCGGGGGGGTCCCTGAGACTCTC
CTGTGCAGCCTCTGGGCTCATCTTCAGATTCTCTGCCATGAGCTGGTACCGACA
GGGTCCAGGGAAGGAGTGCGAGTTGGTCGCAGGTATTACTAGCAATGGTGGTA
CCACAAACTATGCAGACTTCGCGAAGGGCCGATTCACCATCTCCAGAGACAAT
GCCAAGAACACGGTGGATCTACAAATGAACAGCTTGAAACCTGAGGACACGG
CCGTGTATTACTGTCATGCAGGGCTCCGCATGGATTCGCGAGACTGGGGCCAG
GGGACCCAGGTCACC

>LN50+.1 (358 bp)
CTGGAGGAGTCCGGGGGAGGATTGGTGCAGGCTGGGGGCTCTCTGACGCTCTC
CTGTACAGCCTCTGGGCGCTCCTTCAGCAATCATGCCATGGGCTGGTTCCGCCA
GGCTCCAGGGAAAGAGCGTGGGTTTGTAGCGGCTGTTGGTTGGATTGCTGGTA
ATACATATTATGCGGACTCTGTGAAGGGCCGCTTCACCATCTCTAGAGACAAC
GCCAAGAACACGCTGTCTCTGCAAATGAACAACCTGAAACCTGAGGACACGGC
CGTGTATTACTGTGCAGCACGGAACGCATTGCGACCAGTTAGTAATAACCTGT
CGGACTACACGTACTGGGGCCAGGGGACCCAGGTCACCG

>LN50+.2 (358 bp)
CTGCAGGAGTCCGGGGGAGGATTGGTGCAGGCTGGGGGCTCTCTGACGCTCTC
CTGTACAGCCTCTGGGCGCTCCTTCAGCAATCATGCCATGGGCTGGTTCCGCCA
GGCTCCAGGGAAAGAGCGTGGGTTTGTAGCGGCTGTTGGTTGGATTGCTGGTA
ATACATATTATGCGGACTCTGTGAAGGGCCGCTTCACCATCTCTAGAGACAAC
GCCAAGAACACGCTGTCTCTGCAAATGAACAACCTGAAACCTGAGGACACGGC
CGTGTATTACTGTGCAGCACGGAACGCATTGCGACCAGTTAGTAATAACCTGT
CGGACTACACGTACTGGGGCCAGGGGACCCAGGTCACCG

>LN50+.3 (358 bp)
CTGCAGGAGTCCGGGGGAGGATTGGTGCAGGCTGGGGGCTCTCTGACGCTCTC
CTGTACAGCCTCTGGGCGCTCCTTCAGCAAGCATGCCATGGGCTGGTTCCGCCA
GGCTCCAGGGAAAGAGCGTGGGTTTGTAGCGGCTGTTGGTTGGATTGCTGGTA
ATACATATTATGCGGACTCTGTGAAGGGCCGCTTCACCATCTCTAGAGACAAC

GCCAAGAACACGCTGTCTCTGCAAATGAACAACCTGAAACCTGAGGACACGGC
CGTGTATTACTGTGCAGCGCGGAACGCATTGCGACCAGTTAGTAATAACCTGT
CGGACTACACGTACTGGGGCCAGGGGACCCAGGTCACCG

>LN50+.4 (358 bp)
CTGCAGGAGTCCGGGGGAGGATTGGTGCAGGCTGGGGGCTCTCTGACGCTCTC
CTGTACAGCCTCTGGGCGCTCCTTCAGCAATCATGCCATGGGCTGGTTCCGCCA
GGCTCCAGGGAAAGAGCGTGGGTTTGTAGCGGCTGTTGGTTGGATTGCTGGTA
ATACATATTATGCGGACTCTGTGAAGGGCCGCTTCACCATCTCTAGAGACAAC
GCCAAGAACACGCTGTCTCTGCAAATGAACAACCTGAAACCTGAGGACACGGC
CGTGTATTACTGTGCAGCACGGAACGCATTGCGACCAGTTAGTAATAACCTGT
CGGACTACACGTACTGGGGCCAGGGGACCCAGGTCACCG

>LN50+.5 (358 bp)
CTGCAGGAGTCCGGGGGAGGATTGGTGCAGGCTGGGGGCTCTCTGACGCTCTC
CTGTACAGCCTCTGGGCGCTCCTTCAGCAATCATGCCATGGGCTGGTTCCGCCA
GGCTCCAGGGAAAGAGCGTGGGTTTGTAGCGGCTGTTGGTTGGATTGCTGGTA
ATACATATTATGCGGACTCTGTGAAGGGCCGCTTCACCATCTCTAGAGACAAC
GCCAAGAACACGCTGTCTCTGCAAATGAACAACCTGAAACTTGAGGACACGGC
CGTGTATTACTGTGCAGCACGGAACGCATTGCGACCAGTTAGTAATAACCTGT
CGGACTACACGTACTGGGGCCAGGGGACCCAGGTCACCG

>LN50+.6 (358 bp)
CTGCAGGAGTCCGGGGGAGGATTGGTGCAGGCTGGGGGCTCTCTGACGCTCTC
CTGTACAGCCTCTGGGCGCTCCTTCAGCAATCATGCCATGGGCTGGTTCCGCCA
GGCTCCAGGGAAAGAGCGTGGGTTTGTAGCGGCTGTTGGTTGGATTGCTGGTA
ATACATATTATGCGGACTCTGTGAAGGGCCGCTTCACCATCTCTAGAGACAAC
GCCAAGAACACGCTGTCTCTGCAAATGAACAACCTGAAACCTGAGGACACGGC
CGTGTATTACTGTGCAGCACGGAACGCATTGCGACCAGTTAGTAATAACCTGT
CGGACTACACGTACTGGGGCCAGGGGACCCAGGTCACCG

>LN50+.7 (358 bp)
TTGCAGGAGTCCGGGGGAGGATTGGTGCAGGCTGGGGGCTCTCTGACGCTCTC
CTGTACAGCCTCTGGGCGCTCCTTCAGCAATCATGCCATGGGCTGGTTCCGCCA
GGCTCCAGGGAAAGAGCGTGGGTTTGTAGCGGCTGTTGGTTGGATTGCTGGTA
ATACATATTATGCGGACTCTGTGAAGGGCCGCTTCACCATCTCTAGAGACAAC
GCCAAGAACACGCTGTCTCTGCAAATGAACAACCTGAAACCTGAGGACACGGC
CGTGTATTACTGTGCAGCACGGAACGCATTGCGACCAGTTAGTAATAACCTGT
CGGACTACACGTACTGGGGCCAGGGGACCCAGGTCACCG

>LN50+.8 (358 bp)
CTGCAGGAGTCCGGGGGAGGATTGGTGCAGGCTGGGGGCTCTCTGACGCTCTC
CTGTACAGCCTCTGGGCGCTCCTTCAGCAATCATGCCATGGGCTGGTTCCGCCA
GGCTCCAGGGAAAGAGCGTGAGTTTGTAGCGGCTGTTGGTTGGATTGCTGGTA
ATACATATTATGCGGACTCTGTGAAGGGCCGCTTCACCATCTCTAGAGACAAC
GCCAAGAACACGCTGTCTCTGCATATGAACAACCTGAAACCTGAGGACACGGC

CGTGTATTACTGTGCAGCACGGAACGCATTGCGACCAGTTAGTAATAACCTGT
CGGACTACACGTACTGGGGCCAGGGGACCCAGGTCACCG

>LN50+.9 (358 bp)
CTGCAGGAGTCCGGGGGAGGATTGGTGCAGGCTGGGGGCTCTCTGACGCTCTC
CTGTACAGCCTCTGGGCGCTCCTTCAGCAATCATGCCATGGGCTGGTTCCGCCA
GGCACCAGGGAAAGAGCGTGGGTTTGTGGCGGCTGTTGGTTGGATTGCTGGTA
ATACATATTATGCGGACTCTGTGAAGGGCCGCTTCACCATCTCTAGAGACAAC
GCCAAGAACACGCTGTCTCTGCAAATGAACAACCTGAAGCCTGAGGACACGGC
CGTGTATTACTGTGCAGCACGGAACGCATTGCGACCAGTTAGTAATAACCTGT
CGGACTACACGTACTGGGGCCAGGGGACCCAGGTCACCG

>LN50+.10 (358 bp)
CTGCAGGAGTCCGGGGGAGGATTGGTGCAGGCTGGGGGCTCTCTGACGCTCTC
CTGTACAGCCTCTGGGCGCTCCTTCAGCAATCATGCCATGGGCTGGTTCCGCCA
GGCTCCAGGGAAAGAGCGTGGGTTTGTAGCGGCTGTTGGTTGGATTGCTGGTA
ATACATATTATGCGGACTCTGTGAAGGGCCGCTTCACCATCTCTAGAGACAAC
GCCAAGAACACGCTGTCTCTGCAAATGAACAACCTGAAACCTGAGGACACGGC
CGTGTATTACTGTGCAGCACGGAACGCATTGCGACCAGTTAGTAATAACCTGT
CGGACTACACGTACTGGGGCCAGGGGACCCAGGTCACCG

>LN50+.11 (358 bp)
CTGCAGGAGTCCGGGGGAGGATTGGTGCAGGCTGGGGGCTCTCTGACGCTCTC
CTGTACAGCCTCTGGGCGCTCCTTCAGCAATCATGCCATGGGCTGGTTCCGCCA
GGCTCCAGGGAAAGAGCGTGGGTTTGTAGCGGCTGTTGGTTGGATTGCTGGTA
ATACATATTATGCGGACTCTGTGAAGGGCCGCTTCACCATCTCTAGAGACAAC
GCCAAGAACACGCTGACTCTGCAAATGAACAACCTGAAACCTGAGGACACGG
CCGTGTATTACTGTGCAGCACGGAACGCATTGCGACCAGTTAGTAATAACCCG
TCGGACTACACGTACTGGGGCCAGGGGACCCAGGTCACCG

>LN50+.12 (358 bp)
CTGCAGGAGTCCGGGGGAGGATTGGTGCAGGCTGGGGGCTCTCTGACGCTCTC
CTGTACAGCCTCTGGGCGCTCCTTCAGCAATCATGCCATGGGCTGGTTCCGCCA
GGCTCCAGGGAAAGAGCGTGAGTTTGTAGCGGCTGTTGGTTGGATTGCTGGTA
ATACATATTATGCGGACTCTGTGAAGGGCCGCTTCACCATCTCTAGAGACAAC
GCCAAGAACACGCTGTCTCTGCAAATGAACAACCTGAAACCTGAGGACACGGC
CGTGTATTACTGTGCAGCACGGAACGCATTGCGACCAGTTAGTAATAACCTGT
CGGACTACACGTACTGGGGCCAGGGGACCCAGGTCACCG

>LN50+.13 (358 bp)
CTGCAGGAGTCCGGGGGAGGATTGGTGCAGGCTGGGGGCTCTCTGACGCTCTC
CTGTACAGCCTCTGGGCGCTCCTTCAGCAATCATGCCATGGGCTGGTTCCGCCA
GGCTCCAGGGAAAGAGCGTGAGTTTGTAGCGGCTGTTGGTTGGATTGCTGGTA
ATACATATTATGCGGACTCTGTGAAGGGCCGCTTCACCATCTCTAGAGACAAC
GCCAAGAACACGCTGTCTCTGCAAATGAACAACCTGAAACCTGAGGACACGGC
CGTGTATTACTGTGCAGCACGGAACGCATTGCGACCAGTTAGTAATAACCTGT
CGGACTACACGTACTGGGGCCAGGGGACCCAGGTCACCG

>LN50+.14 (358 bp)
CTGCAGGAGTCCGGGGGAGGATTGGTGCAGGCTGGGGGCTCTCTGACGCTCAC
CTGTACAGCCTCTGGGCGCTCCTTCAGCAATCATGCCATGGGCTGGTTCCGCCA
GGCTCCAGGGAAAGAGCGTGAGTTTGTAGCGGCTGTTGGTTGGATTGCTGGTA
ATACATATTATGCGGACTCTGTGAAGGGCCGCTTCACCATCTCTAGAGACAAC
GCCAAGAACACGCTGTCTCTGCAAATGGACAACCTGAAACCTGAGGACACGGC
CGTGTATTACTGTGCAGCACGGAATGCATTGCGACCAGTTAGTAATAACCAGT
CGGACTACACGTACTGGGGCCAGGGGACCCAGGTCACCG

>LN50+.15 (358 bp)
CTGCAGGAGTCCGGGGGAGGATTGGTGCAGGCTGGGGGCTCTCTGACGCTCTC
CTGTACAGCCTCTGGGCGCTCCTTCAGCAATCATGCCATGGGCTGGTTCCGCCA
GGCTCCAGGGAAAGAGCGTGAGTTTGTAGCGGCTGTTGGTTGGATTGCTGGTA
ATACATATTATGCGGACTCTGTGAAGGGCCGCTTCACCATCTCTAGAGACAAC
GCCAAGAACACGCTGTCTCTGCATATGAACAACCTGAAACCTGAGGACACGGC
CGTGTATTACTGTGCAGCACGGAACGCATTGCGACCAGTTAGTAATAACCTGT
CGGACTACACGTACTGGGGCCAGGGGACCCAGGTCACCG

>LN50+.16 (358 bp)
CTGCAGGAGTCCGGGGGAGGATTGGTGCAGGCTGGGGGCTCTCTGACGCTCTC
CTGTACAGCCTCTGGGCGCTCCTTCAGCAATCATGCCATGGGCTGGTTCCGCCA
GGCTCCAGGGAAGGAGCGTGAGTTTGTAGCGGCTGTTGGTTGGATTGCTGGTA
ATACATATTATGCGGACTCTGTGAAGGGCCGCTTCACCATCTCTAGAGACAAC
GCCAAGAACACGCTGTCTCTGCAAATGAACAACCTGAGACCTGAGGACACGGC
CGTGTATTACTGTGCAGCACGGAACGCATTGCGACCAGTTAGTAATAACCTGT
CGGACTACACGTACTGGGGCCAGGGGACCCAGGTCACCG