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Foamy virus Bet fusion proteins for induction of neutralising antibodies against HIV-1

Master Thesis

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Statement

Declaration

Hereby I declare that this Master thesis is completely my own work and that I used no other sources and aids than those which are indicated or acknowledged.

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me and without their permanent support and financial help my scientific education would not have been possible.

Zusammenfassung

Seit der Entdeckung des HI Virus in den frühen 1980iger Jahren, spiegelt die von ihm ausgelöste AIDS Pandemie mit mehr als 30 Millionen Toten weltweit eine der ernstzunehmensten Krankheiten unserer Zeit wieder. Trotz intensiver Forschung und der stetig wachsenden Kenntnis des HIV Lebenszyklus ist heutzutage noch immer kein sicherer und preventiver HIV Impfstoff verfügbar. Die hohe Diverstät von HIV kompliziert die Herstellung eines universellen Impfstoffes gegen verschiedene Subtypen. Ein Ansatz in der HIV Impfstoffentwicklung konzentriert sich daher auf die Induzierung breitneutralisierender Antikörper gegen stark konservierte Bereiche des HI Virus. Zwei dieser breitneutralisierenden Antikörper, 2F5 und 4E10, richten sich gegen Epitope in der Membran-proximalen Region (MPER) des viralen transmembranen Hüllproteins gp41 und gehören zur Zeit mit einem Neutralisationspotential von 95% gegen die meist verbreitenden HIV Stämme zu den potentesten breitneutralisierenden Antikörper. Jedoch blieb die Induzierung derartiger Antikörper durch klassische Vakzinierungsstrategien bislang erfolglos. Als mögliche Gründe stehen die hohe konformelle Flexibilität des Zielepitops, dessen geringe Immunität sowie Immuntoleranzmechanismen zur Diskussion. In dieser Masterarbeit soll mit Hilfe Replikations-kompetenter Foamyviren eine neue Strategie der Antigenpräsentation verfolgt werden, um durch eine erhöhte Immunogenität der Antigene und dessen kontinuierlichen Präsentierung die Induktion neutralisierender 2F5 und 4E10 ähnlicher Antikörper zu ermöglichen. Für das Einfügen der HIV Domänen soll das akzessorische Bet Protein des Foamyvirus verwendet werden. Es konnte gezeigt werden, dass Bet im hohen Maße von infizierten Zellen exprimiert und an seine Umgebung sekretiert wird und somit als ideales Trägerprotein zur Antigenverbreitung dient. Im Gegensatz zu anderen vorherigen Strategien sollen die 2F5 und 4E10 Epitope durch eine zweite Domäne, die auf dem entgegengesetzt gelegenen N-Terminus des gp41 lokalisiert ist, stabilisiert werden. In früheren Studien wurde gezeigt, dass es durch die Stabilisierung dieser zweiten Domäne zu einer erhöhten Bindungsstärke von 2F5 an sein Epitop kommt. Die vorliegende Masterarbeit befasst sich daher mit der Herstellung rekombinanter Bet/HIV-1 Hybridantigenen, die die 2F5 und 4E10 Epitope sowie die Epitop-stabilisierende Domäne beinhalten. Diese Hybride werden auf ihre Antigenität sowie ihre Immunogenität in Immunisierungsstudien untersucht um festzustellen, ob HIV-spezifische Antikörper nach Verabreichung der HIV-Hybride induziert wurden und ob diese Antikörper womöglich bereits auch ohne die Präsentation der Antigen durch das Foamyvirus neutralisierend auf HIV wirken. Die Untersuchungen ergaben, dass alle Antigene, die die 2F5 und 4E10 Epitope enthielten, sowohl unter denaturierenden als auch unter physiologischen Bedingungen von den HIV-neutralisierenden Antikörpern erkannt wurden und somit als

funktionsfähige Antigen charakterisiert werden konnten. Die Immunisierungsstudien zeigten eine hohe Immunogenität für das Bet-Fusionsprotein, das eine Induzierung hoher Antikörpertiter gegen das vollständige Immunogen bewirkte. Auch die fusionierten HIV-Domänen wurden von dem Immunsystem erkannt und gp41-spezifische sowie 2F5- und 4E10-Epitop-spezifische Antikörper wurden gebildet. Diese Antikörper erkennen zwar ihre natürlichen Epitope, jedoch wiesen sie keine Neutralisierungsaktivität gegenüber dem HI Virus auf, was in einem Indikator-Zelllinien basierten Neutralisations-Assay gezeigt wurde. Zusammengefasst zeigt diese Masterarbeit die Eignung Bet Proteins als hoch immunogenen Fusionspartner für die Präsentation der HIV-Domänen. Aufgrund der vorliegenden Daten kann die hier vorgestellte Strategie weiter verfolgt und die Präsentation der Antigene mit Hilfe von Replikationskompetenten Foamyviren getestet werden.

Abstract

AIDS is with more than 33 million deaths worldwide one of the most serious diseases of our time. Despite intensive research, 30 years after HIV discovery no preventive vaccine is available. In regard to vaccine development, the broadly neutralising antibodies 2F5 and 4E10 have since been from particular interest. These antibodies target epitopes in the membrane proximal region of the transmembrane envelope protein gp41 of HIV-1 and are able to neutralise up to 95% of all major HIV-1 strains. However, although these antibodies were isolated from naturally infected individuals, all attempts to induce such antibodies by vaccination failed so far. As reasons, the conformational variablity of the target epitope, its low immunogenicity as well as immune tolerance mechanisms are discussed. This thesis was embedded in a project that aims to deliver the 2F5 and 4E10 epitopes by a novel approach using replication competent foamy viruses to increase antigen immunogenicity. In contrast to other strategies, the 2F5 and 4E10 epitopes should hereby be stabilised by a second domain derived from the opposite N-terminal part of gp41 which has been shown to result in increased epitope binding of 2F5. The HIV domain shall fused with the accessory Bet protein of foamy viruses which is highly expressed in infected cells and secreted to the environment representing an ideal carrier protein for immune stimulation. The aim of this thesis was to investigate whether such Bet fusion proteins with HIV-1 inserts are accessible for monoclonal antibodies 2F5 and 4E10, whether they are immunogenic when used in immunisation studies and, whether HIV specific neutralsising antibodies can be induced. For this, recombinant Bet and Bet/HIV proteins were produced in *E. coli* and based on refolding protocols purified to homogeneity. All proteins containing the 2F5 and 4E10 epitopes were recognised by these antibodies under denaturing as well as under physiological conditions. By immunisation of rats with these proteins it was found that the Bet carrier protein is a highly immunogenic fusion partner inducing high levels of antibodies in all rats. Importantly, also the fused HIV domains were recognised by the immune system and gp41 as well as 2F5 and 4E10 epitope specific antibodies generated. However, despite the fact that these antibodies targeted the HIV epitopes, they were not able to prevent HIV infection in an indicator cell line based neutralisation assay. In summary, this work demonstrates the suitability of Bet as highly immunogenic fusion partner for antigen delivery and provides promising data for testing the transfer of these Bet/HIV hybrid domains into replication competent vectors.

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Abbreviations

aa	amino acid
Amp	ampicillin
APOBEC	apolipoprotein B mRNA editing enzyme catalytic polypeptide 3
APS	ammoniumpersulfat
ATP	adenosin triphosphat
Ca	capsid
CHR	C-terminal region
CMC	critical micelle concentration
CT	cytoplasmatic tail
DDM	n-dodecyl-ß-D-maltosid
DTT	Dithiothreitol
NU	nucleocapsid
DNA	deoxyribonucleic acid
cDNA	copy DNA
dsDNA	double stranded DNA
bp	base pairs
ELISA	enzyme linked immuno sorbent assay
Env	Envelope
FV	foamy virus
FFV	feline foamy virus
FP	fusion peptide
FPPR	fusion peptide proximal region
HIV	human immunodeficiency virus
IN	integrase
IPTG	Isopropyl-β-D-thiogalactopyranosid
kb	kilo base
kDa	kilo Dalton
LTR	long term repeats
Ma	matrix
MPER	membrane proximal external region
MSD	membrane spanning domain
NC	nucleocapsid

Nef	negative factor
NHR	N-terminal helical region
NP-40	Non-idet P40
nm	nano meter
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline 0.02% Tween
PCR	polymerase chain reaction
PEI	Polyehylenimine
PVDF	polyvinylidene fluoride
PR	protease
Ref	regulator of expression virion proteins
RNA	ribonucleic acid
RT	reverse transcriptase
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamid gel electrophoresis
Tat	transactivating factor
TEMED	tetramethylethylenediamin
TM	transmembrane protein
t-RNA	transfer ribonucleic acid
Vif	viral infectifity factor
Vpr	viral protein R
Vpu	viral protein U

1. Introduction

1.1. The human immunodeficiency virus (HIV)

1.1.1 HIV- a lethal disease

Since its discovery in the early 1980s [1], HIV infection has become a serious global epidemic with more than 30 million deaths due to AIDS or AIDS related diseases and thus developed to one of the greatest challenges for human health [2]. According to the last survey made in 2009 by the World Health Organisation (WHO) 33.3 million people are currently living with HIV and 2.6 million were newly infected this year (http://www.who.int/hiv/data/en/index.html). The most devastating consequences of this disease have been seen in developing nations like Sub-Saharan Africa which alone accounts for 68% of all HIV infections worldwide and which has with 1.8 million the highest rate of new infections (Figure 1 and http://www.who.int/hiv/data/en/index.html). However due to its high prevalence also in the developed nations research to control HIV and prevent new infections has highest priority [3]. Despite great progress in the understanding of HIV's biology and the successful development of anti-HIV medications like highly active antiretroviral therapy (HAART), the currently available treatments achieve only a life-extending effect and are suffering from poor long-term efficiency and high costs [4, 5]. A protection against AIDS by an effective and save vaccine for several subtypes to stem the global epidemic is therefore of fundamental interest and urgently needed [6].

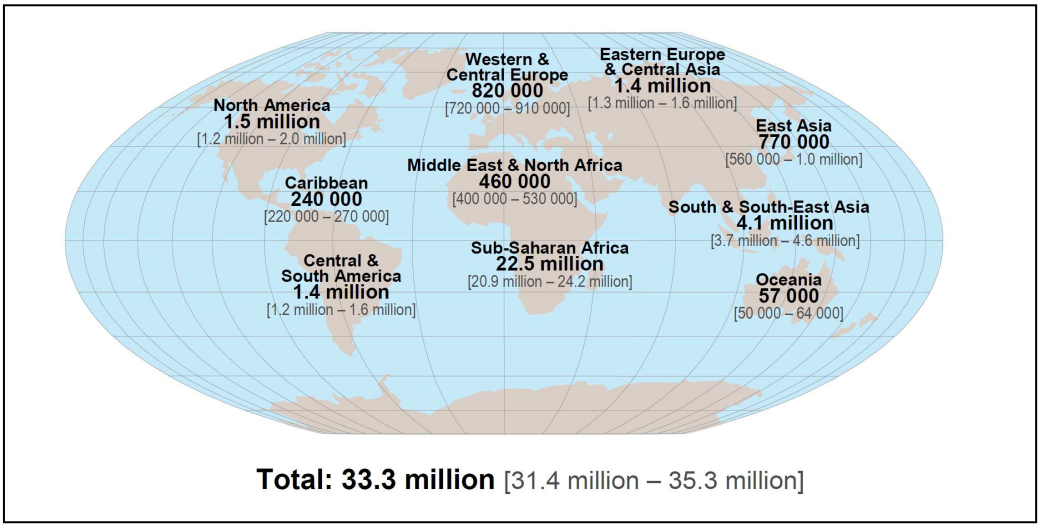

Figure 1: Estimations of adults and children living with HIV worldwide (http://www.who.int/hiv/data/en/index.html)

1.1.2 Classification and origin of HIV

The HIV subtypes 1 and 2 belong to the genus of the lentiviruses and are a member of the *Retroviridae* family. Retroviruses are characterised by a dimer of positive single stranded RNA as genome which is reverse transcribed into dsDNA after infection [7, 8]. In contrast to other retroviruses, HIV possesses as a lentivirus, the ability to infect non-dividing cells and causes a prolonged and slow process of an ultimately fatal diseases [4]. HIV-1 is worldwide the predominant virus type with the greatest diversity whereas occurrence of type 2 is mainly restricted to the area around West Africa [4]. By phylogenetical analysis, HIV-1 can be grouped into four major groups: Main (M), Outlier (O), New (N), and the recently identified group P [9]. The most common HIV-1 group M is further divided into nine distinct subtypes or clades named by letters (A, B, C, D, F, G, H, J and K) and sub-subtypes denoted with numbers (A1, A2, A3, A4, F1 and F5) which exhibit a less genetic distance between each other and arose from a distinct subtype. Additionally there exist numerous circulating recombinant forms which result from co-infection of individuals with various subtypes and further increase the complexity of classification [4, 10-12].

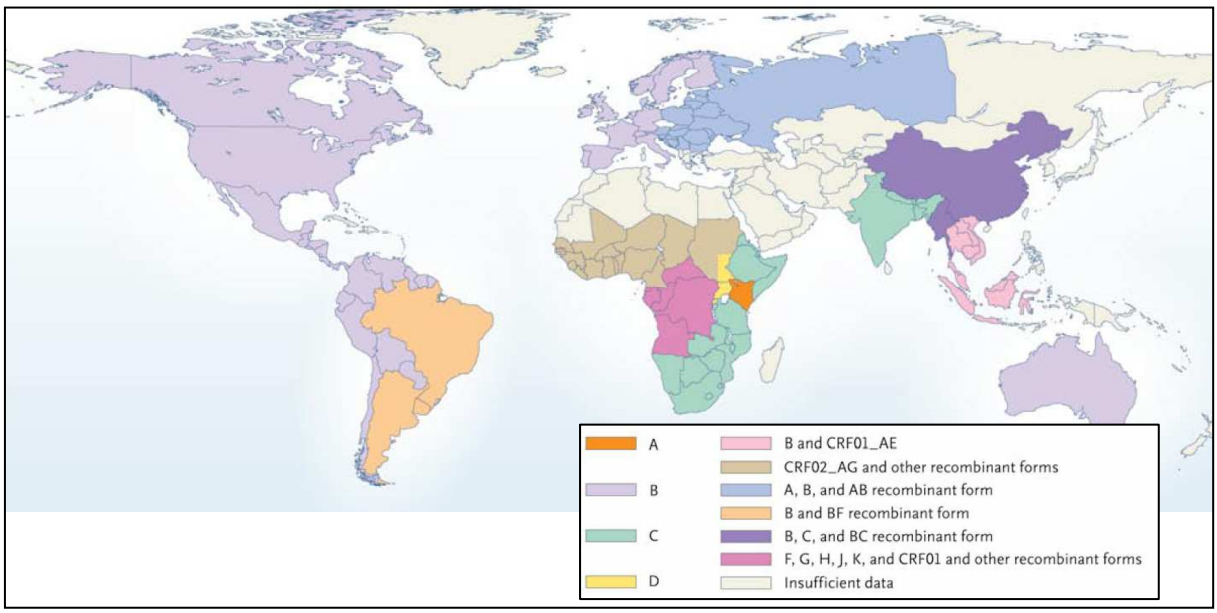

Figure 2: Current global distribution of HIV-1 M subtypes and recombinant forms [12]

The earliest HIV-1 infection can be traced back to a human blood sample from 1959 taken in the area near the Congo River [13]. With the advent of molecular sequencing techniques researchers' recognised that HIV-1 has extreme homology to a simian immunodeficiency

virus (SIVcpz), isolated from a subspecies of chimpanzee *Pan troglodytes troglodytes* living in West Central Africa [11, 12]. These chimpanzees harbour viruses with about 85% sequence identity to the virus found in humans and are therefore thought to be the reservoir of a HIV-1 progenitor that was transmitted to humans resulting in a zoonosis [12, 14]. Moreover, strains related to the M and N group have also been found later in many species of chimpanzees, further confirming this thesis [12].

1.1.3 Genomic organisation of HIV-1 and virion structure

HIV-1 belongs to the group of complex retroviruses and encodes in addition to the canonical Gag, Pol and Env proteins several accessory proteins such as Rev, Nef, Tat, Vif, Vpr and Vpu (Figure 3). Gag is the major structural precursor protein, which is further processed into the matrix (MA, p17), capsid (CA, p24) and nucleocapsid (NC, p7) proteins. The *pol* gene codes the viral enzymes protease (PR), reverse transcriptase (RT) and integrase (IN) which have important functions during viral replication [7, 15]. The *env* gene codes the gp160 precursor protein that is cleaved by a furin-like host protease into the surface protein (SU, gp120) and the transmembrane envelope protein (TM, gp41) which together form the viral spike on the virion surface [16]. Further important proteins during virus infection are the accessory proteins Rev, Tat, Nef, Vif, Vpr and Vpu whose functions are summarised in Table 1.

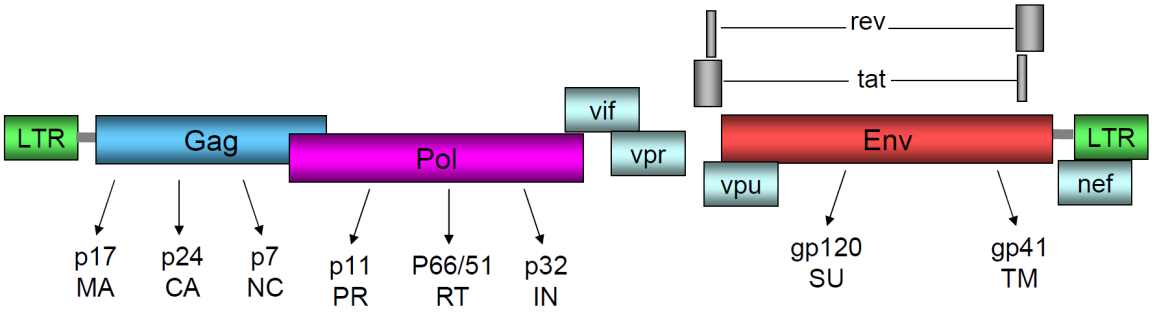

Figure 3: Genomic organisation of HIV-1 and viral proteins [4]
Two long term repeat sites (LTR) enclose the three main genes Gag (processed to matrix (MA, p17), capsid (CA, p24) and nucleocapsid (NC, p7) proteins), Pol (further processed to protease (PR), reverse transcriptase (RT) and integrase (IN) and Env (processed to the surface protein (SU, gp120) and the transmembrane envelope protein (TM, gp41). Furthermore encoded are the accessory proteins Vif, Vpr, Vpu, Nef, Rev and Tat.

Table 1: Overview of the functions of the accessory proteins of HIV-1

Viral accessory protein	Function	Reference
Rev (regulator of expression of virion proteins)	• Supports the sequence specific transport of unspliced and incompletly spliced viral mRNA from the nucleus into the cytoplasm. For this Rev binds specifically to the "Rev-response element (RRE) on the viral RNA	[17]
Tat (transactivating factor)	• Enhances transcriptional elongation of viral mRNA by binding to the trans-activating response element (TAR) of the stem-loop site on the newly synthesised mRNA	[18]
Nef (negative factor)	• Important for achieving and maintaining high viral loads. Enhances viral replication and stimulates a reduction of CD4 receptors on the surface of infected cells to prevent superinfection after budding	[19, 20],
Vif (viral infectifity factor)	• Seems to have a function during virus assembly and maturation • Avoids the packaging of cellular apolipoprotein B mRNA editing enzyme catalytic polypeptide 3 (APOBEC) proteins into the virus particle which would catalyses RNA mutations.	[21]
Vpr (viral protein R)	• Binds on the cellular nuclear complex and supports the transport of the pre-integrationcomplex into the nucleus	[22]
Vpu (Viral protein U)	• Protects newly synthesised gp160 Envelope molecules from binding to newly synthesised CD4 receptors in the endoplamatic reticulum .	[23], [24]

The HIV-1 virion (Figure 4) exhibits a size of about 100 to 120 nm in diameter and is surrounded by a lipid bilayer membrane derived from the infected host cell which includes major histocompatibility complex antigens, actin and ubiquitin [4, 7, 25]. In this lipid bilayer the viral receptor of the HI virus build by trimers of the envelope glycoprotein (Env, gp160) is embedded. The Env protein consists of two subunits, an extensively N-glycosylated surface protein (SU, gp120) and the membrane anchored transmembrane envelope protein (TM or gp41) [26, 27]. The center of the particle harbours the approximately 9.7 kb large genome of the virus which contains two copies of unspliced single stranded RNAs, stabilised as a ribonucleoprotein complex with about 2000 copies of the nucleocapsid protein p7 (NC), and the three major enzymes protease (PR), integrase

(IN) and reverse transcriptase (RT). These elements are surrounded by a core unit which is build by the capsid protein (CA, p24) followed by a second shell consisting of the structural matrix protein p17 (MA) [4, 7, 20, 28].

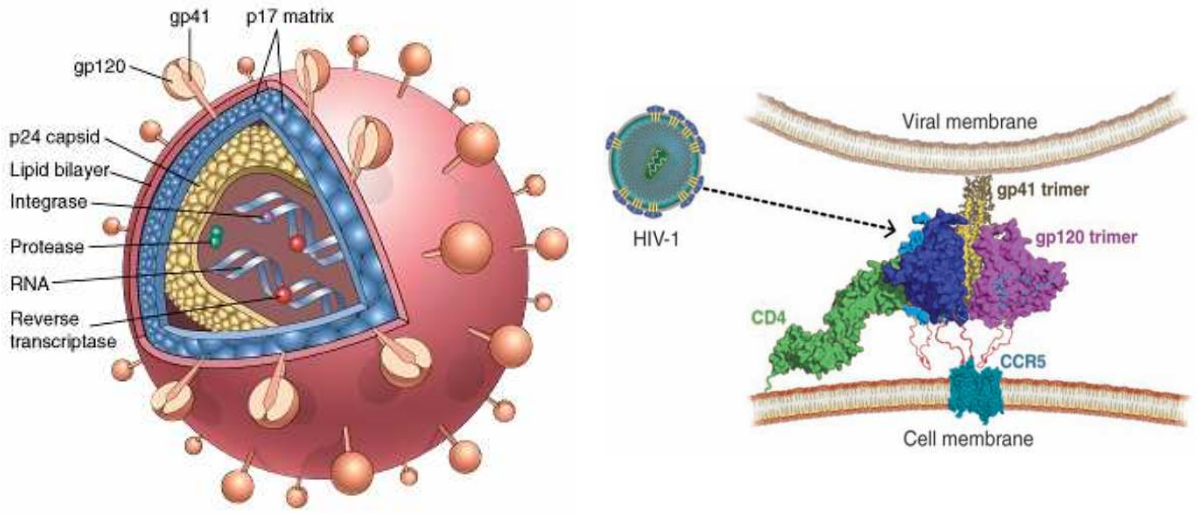

Figure 4: Schematic illustration of the HIV-1 virion [29] and the HIV-1 trimeric envelope spike [5]
Structure of the HIV virion with its proteins and genomic RNA content. Trimeric gp120 (shown in blue, pale blue and violet) interacting with CD4 receptor (green) and a co-receptor CCR5 (turquoise) on a target cell. In yellow is demonstrated the gp41 trimer.

1.1.4 Natural infection and replication cycle

The target cells for a HIV-1 infection are predominantly human $CD4^+$ T cells and cells of the macrophages and monocytes lineage [15, 30, 31]. The entry process is catalysed by the interaction of the highly glycoolysated gp120 surface protein of the trimeric Env spike with a CD4 receptor which is presented on a target cell (Figure 4) [5, 32, 33]. The binding on the CD4 molecule triggers a conformational change of the gp120 trimer molecule that exposes the co-receptor binding site. This co-receptor binding site is specific for the chemokine receptors CCR5 or CXCR4. [34]. Its presence besides the CD4 receptor is essential to allow virus entry into the target cell. The interaction between the gp120 co-receptor binding site and the co-receptor catalyses a second very complex conformational change. Thereby, the gp41 trimer undergoes a structural rearrangement, where the hydrophobic N-terminal fusion peptide intercalates into the cellular host membrane and forms an extended “prehairpin intermediate” that connects both bilayers. The intermediate stage maintained for minutes, however ultimately the C-terminal helical region (CHR) and the N- terminal helical region (NHR) of each gp41 monomer collapse to form a hairpin

structure. The CHR and NHR domains can now interact with each other and adopt a six helix bundle formation that brings both membranes in close proximity and promotes lipid mixture and the fusion between the viral and the host membrane [34-37] (Figure 5).

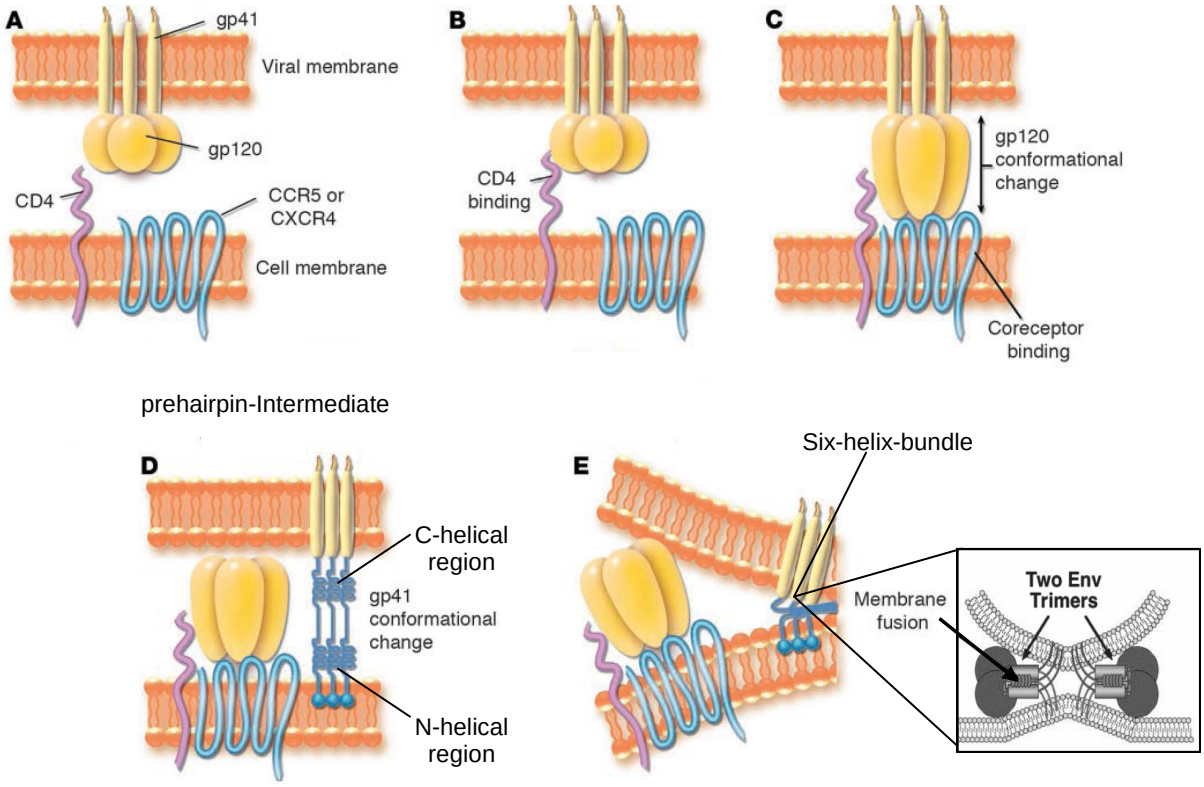

Figure 5: HIV-1 entry pathway [38]
The gp120 surface protein binds to the CD4 receptor (A and B) following by a conformational change that allows the interaction between the co-receptor binding site of gp120 and the chemokine co-receptor CCR5 or CXCR4 (C). The next step is a second rearrangement, where the N-terminal fusion peptide anchores itself into the host cellular membrane (D) and mediates the virus entry by a six helix bundle formation (E).

After fusion of both membranes, the release of the viral capsid occurs into the cytosol of the host cell through a pH-dependent fusion process [39]. Following entry the poorly stable capsid shell disrupts by a process called “uncoating” and releases the viral genome as well as the viral enzymes [40]. The viral enzyme reverse transcriptase recognises the genome and converts the positive sense RNAs in a complex process into cDNA using a cellular lysine tRNA molecule as initiating primer [20, 41]. This reverse transcriptase is highly error prone because it lacks proof-reading activity and exhibits a nucleotide misincorporation rate of 5.9×10^{-4} to 5.3×10^{-5} mutations per bp and cycle [42-45]. The nuclease activity of the reverse transcriptase degrades the RNA template and forms in a second step the complementary strand of the cDNA with the help of polypurine tracts [15,

46]. The genome of HIV-1 contains two such purine rich sections, a central polypurine (cPPT) and a 3'polypurine tract (3'PPT). The 3'PPT stops the cleavage of RNase H and allows the revealed DNA overhang to serve as primer for the second strand synthesis resulting in a completely synthesized viral DNA [47, 48]. Once generated, virus DNA is packaged in a preintegration complex composed of DNA, capsid protein, vpr, matrix and the enzyme integrase and then transported through a nuclear pore to the nucleus [22, 40, 49]. After transport, the catalytic active enzyme integrase induces the covalent integration of the viral DNA within the host cell chromatin. The integrated viral DNA forms the provirus which serves a template for RNA synthesis [20]. Human RNA polymerase transcribes the viral DNA and resulting spliced and unspliced viral mRNA are shuttled from the nucleus to the ribosomes for translation into the viral proteins [4]. The envelope protein gp160 is synthesized in the endoplasmic reticulum and subsequently cleaved into gp120 and gp41 subunits during the transport through the Golgi apparatus before arriving on the cell surface [28]. The two synthesized precursor proteins Gag and Gag-Pol enclose two single stranded copies of unspliced RNAs to form a new virion. This immature virion assembles at the cell surface where the produced envelope proteins are located and budding occurs. In this process, the non-infectious immature virion disengages itself from the host cell as an independent particle. The last step in virus replication is the reorganisation of the immature virion where the third viral enzyme, the protease, becomes active. It cleaves the Gag and Gag-Pol precursors to produce the independent enzymes protease (PR), reverse transcriptase (RT) and integrase (IN) as well as the structural proteins for matrix (MA), capsid (CA) and nucleocapsid (NC). In its mature form the HI-virus is a infectious particle and can initiate a new cycle of infection [4, 50] (Figure 6).

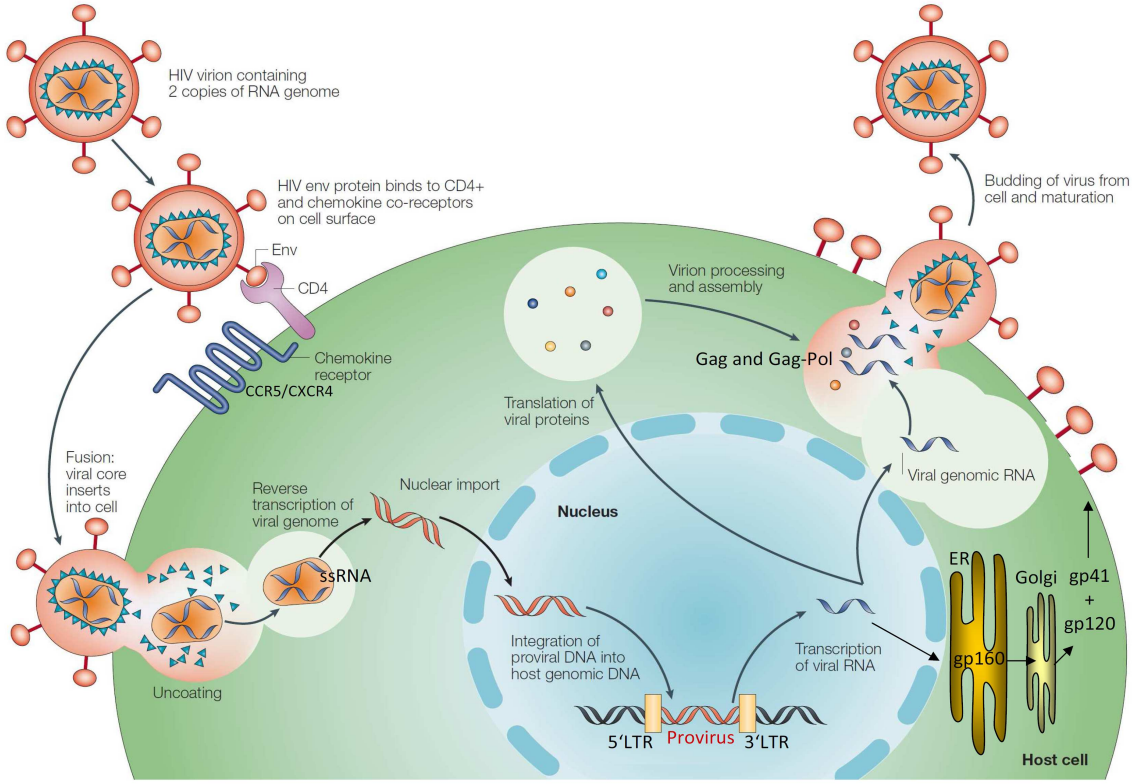

Figure 6: Replication cycle of HIV-1 [51]
Schematic presentation of the HIV life cycle. The replication starts with the attachment of gp120 to the CD4 receptor and a co-receptor. The viral genome is released into the cytosol of the host cell followed by conversion of the ssRNA into dsDNA by reverse transcriptase. The resulting dsDNA is transported into the nucleus where the integration into the chromatin of the human host cell catalysed by the enzyme integrase takes place. This integrated provirus serves as a template DNA for transcription. Viral mRNAs generated by human polymerase can now be transcribed on the ribosomes to generate new virus proteins. The env gp160 precursor is synthesized in the endoplasmatic reticulum and cleaved into gp120 and gp41 in the Golgi apparatus and transportet to the cell surface. Viral proteins and two unspliced RNAs assemble at the surface of the host cell and become independent as an immature virion by a process called budding. In a last step, the enzyme protease generates by cleavage of the precursors Gag and Gag-Pol the enzymes and structural proteins to generate a fully infectious particle.

1.2. HIV vaccines

1.2.1 Vaccines for induction of neutralising antibodies against HIV

Induction of neutralising antibodies by immunisation has been successfully applied for most pathogens and therefore is also one of the main strategies for HIV vaccine development [52]. This approach is based on the theory that the presence of such antibodies could eliminate the virus before insertion of the viral genome into the target cell and thus prevent establishment of a productive infection. During natural HIV-1 infection, neutralising antibodies develop as soon as 84 days after infection, however, since the time

frame between B cell priming and occurrence of antibodies in the blood usually takes several weeks in which HIV produces various escape variants, the clinical benefit of these antibodies is relatively low [53, 54]. More effective broadly neutralising antibodies which target epitopes that can not be altered without an immense loss of viral fitness were found in about 20-25% of the infected individuals where they arise after a prolonged time of host-virus interaction (Table 2) and predominantly in patients with high plasma viral loads [30].

Table 2: Env specific antibody response in HIV-1 infected human [30]

Antibody specificity	Time of onset after transmission (days)
gp41	23
gp120	38
Non-neutralising to CD4-binding site, MPER and CD4-inducible epotopes	40-70
Autologous-virus neutralising antibodies	Earliest ~ 84
Broad-specificity and neutralising to CD4-binding site, carbohydrate and MPER	Not usually made, but when they arise ~ 30 months after transmission in chronic infection

gp, glycoprotein; MPER, membrane proximal external region

The HIV-1 Env is thereby the main target for these molecules and several monoclonal broadly neutralising antibodies such as 2F5, 4E10, Z13, b12, 447-52D and 2G12 were isolated from infected individuals [55, 56]. These broadly neutralising antibodies recognise epitopes located in distinct Env regions: 2F5, 4E10 and Z13 target the gp41 MPER, b12 the gp120 CD4 binding site, 447-52D a quaternary V2/V3 loop epitope and 2G12 gp120 carbohydrates (Figure 7), each representing a potential target for antibody based vaccine design. Importantly, these monoclonal antibodies provide efficient protection from HIV infection when administered by passive transfer to individuals and are therefore thought to be highly advantageous for vaccine purposes. However, despite the evidence that the human immune system is capable of generating a broadly neutralising antibody response, to date the induction of such antibodies by vaccination has been achieved only with limited success [57].

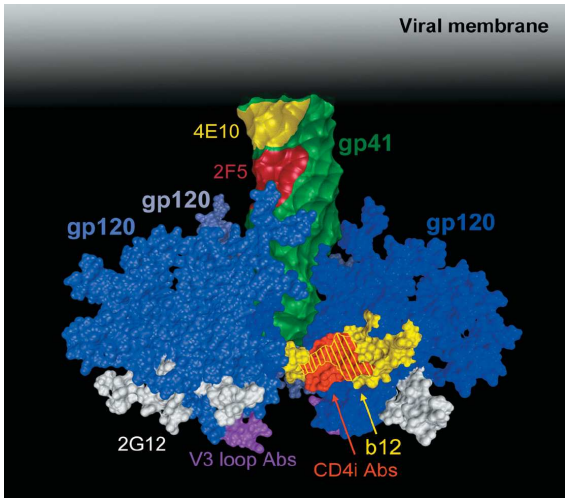

Figure 7: Model of the envelope spike of HIV-1 [55] Schematical presentation of the envelope spike containing the three subunits of gp120 (blue) and gp41 (green). This figure shows the location of epitopes that are recognized by monoclonal broadly neutralising antibodies. On the MPER region of the gp41 trimer are presented the epitopes for the antibodies 4E10 (yellow) and 2F5 (red). On the gp120 subunit monoclonal antibodies recognise conserved epitopes in the CD4 binding site (b12 in yellow/red) and a cluster of oligomannose residues (2G12 shown in grey). Last location shown in purple, are recognized by V3 loop specific antibodies like 447-52D.

One reason commonly discussed is the complex structure of the trimeric HIV-1 Env protein which has been found to be difficult to reconstitute by recombinantly produced antigens. Moreover, the trimeric spike undergoes considerable conformational changes during cell infection and to date it is not clear which of them is responsible for the induction of neutralising antibodies. The HIV Env protein also hides most of the vulnerable target epitopes through protein-protein interactions within the trimer or masks them by glycosylation [5]. Next to the problems associated with antigen design, immunological hurdles seem to be met. Almost all of the broadly neutralising antibodies that have been isolated so far are characterised by properties not usually found in the antibody population. They have a high degree of somatic mutation, carry prolonged heavy chain complementary determining regions (HCDR3s), recognise their targets through domain swap and in some cases show poly- or self reactivity [58]. There are indications that in some cases self reactivity is associated with negative B cell selection preventing expansion of such B cell populations by natural tolerance mechanisms [56, 59]. Together with the late occurrence of these antibodies during natural infection this suggests that such broadly neutralising antibodies are a result of very rare B cell activation pathways and intensive affinity maturation. As a result, alternative strategies for a sustained HIV antigen delivery might also be necessary to induce them.

1.2.2 The MPER of gp41 as target for neutralising antibodies and vaccine

As outlined above, the envelope protein of HIV-1 is the main target of the neutralising antibody response and therefore both envelope subunits, the gp120 SU protein as well as the gp41 TM protein are of particular interest for vaccine development. The TM protein exhibits more conserved sequences than its gp120 counterpart and is in contrast to the gp120 subunit not protected with an enormous glycan shield. Furthermore, it has been demonstrated that gp41 elicits a stronger immune response in infected persons with titers up to 625 fold higher than anti gp120 titers [60, 61]. The TM protein can be divided into three major regions referred to as ectodomain (extracellular domain), membrane spanning domain (MSD) and cytoplasmatic tail (intracellular, CT), (Figure 8). The ectodomain is the active part which undergoes conformational changes during virus entry and consists of the fusion peptide (FP), the fusion peptide proximal region (FPPR), the N-terminal helical region (NHR), the immunosuppressive domain (isu), the Cys-Cys-loop (C-C), the C-terminal helical region (CHR) and closely to viral membrane the membrane proximal external region (MPER). The MSD anchors the ectodomain within the lipid membrane and is followed by the relatively long CT located in the inside of the HIV-1 particle. The function of the CT is not well understood and is thought to be associated with a variety of controversial functions like involving Env incorporation into the virus or interaction with cellular proteins [60, 62, 63]. The MPER contains two highly conserved epitopes recognised by the broadly neutralising antibodies 2F5 and 4E10 (Figure 8) [31, 64].

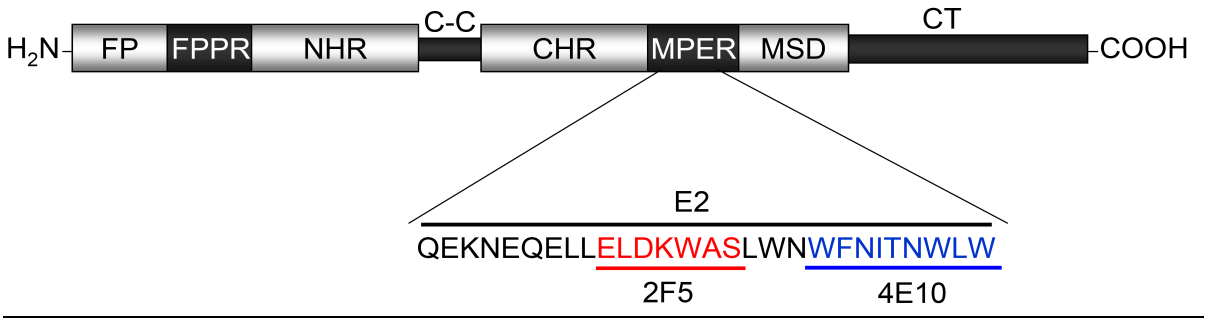

Figure 8: Schematic presentation of gp41 [4, 26]
The gp41 molecular structure of HIV-1 contains the fusion peptide (FP), the fusion peptide proximal region (FPPR), a N-terminal helical region (NHR), a Cys-Cys-Loop (C-C), a C-terminal helical region (CHR), the membrane proximal external region (MPER), the membrane spanning domain (MSD) and a cytoplasmic tail (CT). The epitopes that are recognised from the neutralising antibodies 2F5 and 4E10 are located in the MPER.

Experimental studies have shown that these antibodies are able to neutralise up to 100 % of a cross-clade panel of 90 HIV-1 strains in case of 4E10 and up to 67% in case of 2F5 (Table 3) [60, 65]. Because of their high neutralising potential, the 2F5 and 4E10 epitopes contained in the so called E2 domain (QEKNEQELLELDKWASLWN WFNITNWLW) of the MPER were on the main focus of this master thesis.

Table 3: Cross neutralising properties of 2F5 and 4E10 to HIV (a) [55]

Virus	2F5	4E10
Clade B (total = 30)	24 (80%)	30 (100%)
All clades (total = 90)	60 (67%)	90 (100%)

(a) Neutralisation was considered positive if 50% neutralisation was achieved at a mAb concentration of less than 50 µg/ml (half-maximal inhibitory dose of less than 50 µg/ml) in a relatively sensitive pseudovirus neutralisation assay.

1.2.3 Influence of the presence of the HIV-1 FPPR domain

All retroviruses infections follow a similar principle and therefore also the functional domains of the envelope proteins are highly conserved. It has been demonstrated for gammaretroviruses such as the porcine endogenous retrovirus (PERV), the feline leukaemia virus (FeLV) and the koala retrovirus (KoRV), that neutralising antibodies can be easily induced by immunisation with the TM protein of these viruses [66-69]. These antisera recognise similar to the HIV-1 neutralising monoclonal antibodies 2F5 and 4E10 an epitope in the MPER (E2, Figure 9A) and show despite the evolutionary divergence between gammaretroviruses and HIV-1, a sequence homology of three amino acids in the 4E10 epitope. Interestingly, these sera also recognise a second epitope located in the FPPR (E1, Figure 9A). This E1 domain is not recognised by the known antibodies 2F5 and 4E10, however, the presence of an HIV FPPR derived E1 peptide has been found to increase 2F5 binding to its MPER epitope (Figure 9B, [60, 70]). As a result of these experiments it was hypothesised that the interaction of the E1 and E2 domains induces a conformation that increases the binding of the 2F5 epitope and therefore antigens containing both domains might promote the induction of neutralising antibodies whereas immunogens containing the E2 domain only do not. Construction of such antigens was thus another aim of this thesis.

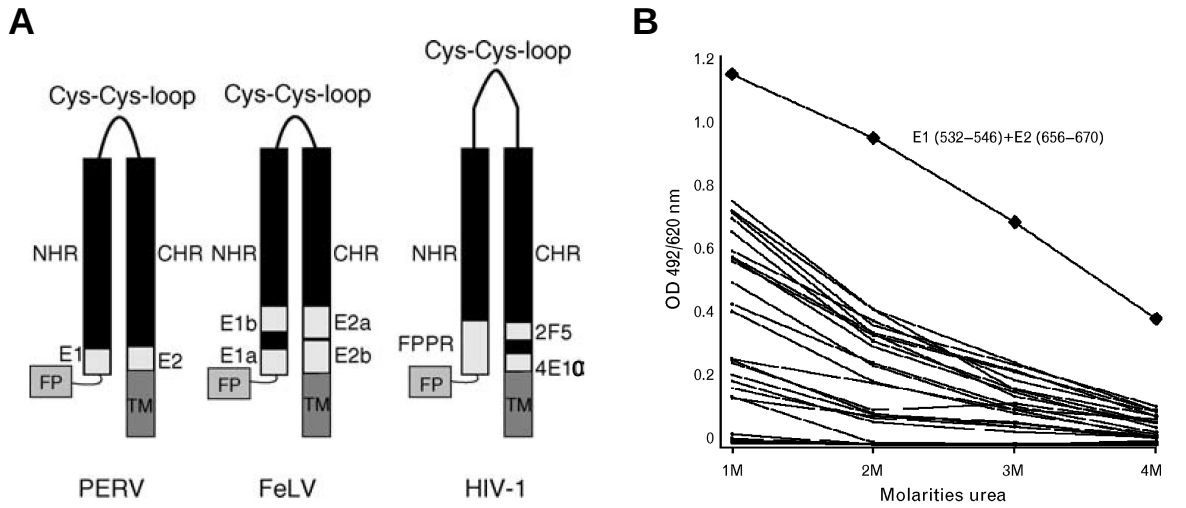

Figure 9: Immunogenic epitopes in the TM protein of gammaretroviruses or HIV-1 and reactivity of 2F5 in the presence of E2 and FPPR derived peptides [70]
(A) Localisation of E1 and E2 epitopes in the PERV and FeLV TM proteins recognised by neutralising sera and epitopes of HIV-1 broadly neutralising antibodies 2F5 and 4E10 and the FPPR domain in HIV-1 (light grey). **(B)** ELISA binding of 2F5 to the E2 peptide in the presence of various gp120/gp41 peptides. 100 ng of synthetic E2 peptide were coated with 100 ng of 211 overlapping peptides corresponding to the entire gp120/gp41 sequence of HIV-1. Strength of binding of the monoclonal antibody 2F5 was measured under different urea concentrations. Strongest binding of 2F5 was obtained when the E2 peptide was coated in presence of the E1 peptide of HIV-1.

1.3.Feline foamy virus (FFV)

1.3.1 Hybrid foamy viruses as HIV-1 vaccine carrier

Foamy Viruses (FV), are complex retroviruses and belong to the subfamily of *Spumaretrovirinae* which is a genus of the *Retroviridiae* family [71, 72]. FV are found in a wide breadth of mammalians species including primates, cats, cattle and horses [73, 74]. Humans do not belong to the natural hosts of FV (worldwide prevalence about 2%) but can be infected by primate FVs resulting in a lifelong persistent infection [71, 73, 74]. Several cases of accidental transmission of primate foamy viruses to humans have been described for persons with intensive contact to infected animals like zookeepers or bushman which were bitten or scratched by such animals. However, a transfer from human to human could not be detected even after years of follow up [75, 76]. Importantly, despite a strong virus-specific immune response and the presence of the provirus in nearly all organs, the infected host exhibits no pathogenic course of disease [71, 72, 77, 78]. This is thought to be a result of intensive evolutionary adoption of host and virus since first FV infections in non-human primates occurred over 30 million years ago [74]. FV are genetically stable and have a

proviral genome of over 13 kb [78]. Since most of the genetical information can be supplemented in *trans* they are able to harbour up to 9.2 kb of additional genetic material what makes them interesting shuttle vectors for gene therapy purposes [79]. Due to its apathogenicity, the induction of a continuous stimulation of the immune system, low prevalence in humans and a high packaging capacity, FVs are also attractive for the utilization as a safe and effective vector system for vaccine applications. The use of replication competent FVs containing HIV-1 Env immunogens is in this regard from particular interest since this approach would support B cell affinity maturation to the delivered antigens just as in natural HIV-1 infection. In this work hybrid constructs based on the feline FV (FFV) were generated with the background that after transfer of the applied strategy into infectious molecular clones, such hybrid FFV/HIV-1 vectors could be tested also *in vivo* in a small animal system. For this purpose, established FFV vectors such as pCF7 are available and will allow an effective delivery of HIV-1 antigens into the host's genome [77] (Figure 10).

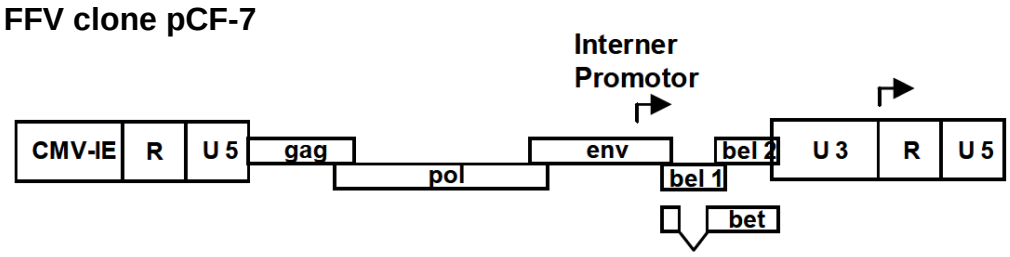

Figure 10: Schematic presentation of the genome of the infectious FFV clone pCF-7 [77]
Besides the retroviral genes Gag, Pol and Env, the FV genome encodes the three accessory proteins bet, bel 1 and bel 2 between the Env and the long terminal repeat (LTR). On the 3' end of the env gene is a second promoter that induces a basal expression of the accessory Bet and Bel 1 proteins. To assure high level transcription the original U3 region of the 5'LTR was replaced with a strong cytomegalovirus (CMV) promoter. CMV-IE = cytomegalovirus immediate early promoter, U3 = unique sequence at the 3'end of genome, U5 = unique sequence at the 5'end of genome, R= repeat sequence.

1.3.2 The FFV Bet protein as carrier for HIV-1 epitopes

In this project, the delivery of HIV-1 E1 and E2 domains should be performed by using the FFV accessory Bet as carrier protein. Bet is a 43 kDa protein and plays a regulatory function during viral replication and in counteracting cellular APOBECs [80, 81]. Bet exhibits several features making it an ideal fusion partner for vaccine purposes. It has been found to be highly expressed in infected target cells which is a requirement for quantitative presentation on the cell surface by MHC molecules. In line with this it has been

demonstrated that Bet causes a strong humoral immune response in infected cats and primates and antibodies against Bet are readily detectable by ELISA techniques (Figure 11A) [82, 83]. Furthermore, Bet is secreted from infected to neighbouring cells which allows self-distribution of the fused antigens to a wider range of cells. Since the secreted protein remains unprocessed by the proteasome, conformational epitopes fused to the Bet protein stay intact and thus can be presented to surveying B- and T-cells in a native way (Figure 11B) [84].

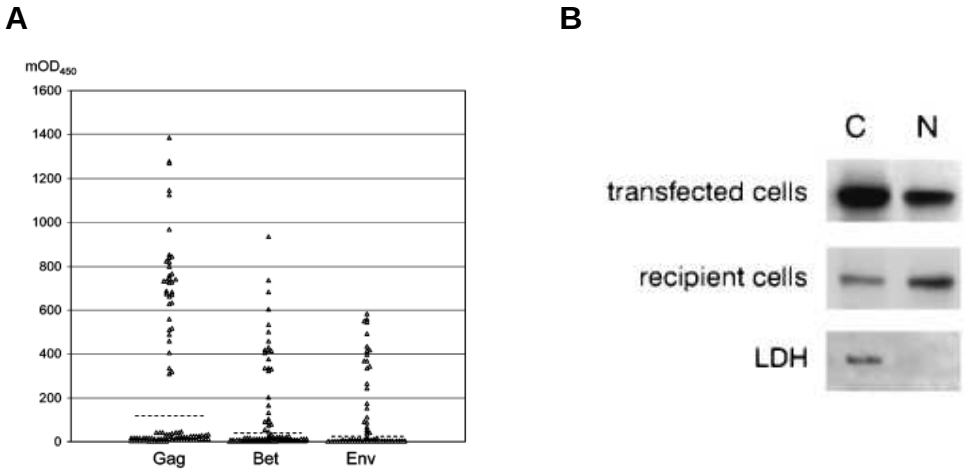

Figure 11: Illustration of ELISA reactivity [83] and secretion of the Bet protein [84]
(A) Dot-plot presentation of ELISA reactivity against foamy virus Gag, Bet and Env antigens a serological study using 99 FFV infected cat sera. **(B)** Distribution of Bet in the cytoplasm (c) and the nuclei (n) of Bet transfected and co-cultivated recipient cells. Cytosolic expressed lactate dehydrogenase (LDH) served as technical control for correct separation of cytoplasmic and nuclear fractions.

1.4.Aim of this work

In this project the strategy of using the FFV Bet protein as carrier for HIV-1 epitopes should be evaluated and the antigenicity and immunogenicity of such constructs and in particular of the introduced HIV fusion parts should be studied. Next to the classical approach of inserting the 2F5 and 4E10 epitopes only, constructs should be generated that allow the simultaneous presentation of the FPPR derived E1 domain of HIV-1 to investigate if these constructs show any benefit in regard to antigen- or immunogenicity compared to the single domain antigens. Furthermore, the obtained antisera should be analysed for any neutralising potential. Since production of milligram amounts of protein as needed for the intended immunisation studies is very elaborate in the eukaryotic system, these proteins should be expressed and purified to homogeneity by use of E. coli. Therefore it was necessary to

- generate prokaryotic expression plasmids for all constructs by molecular cloning
- establish and optimise expression and purification protocols for all proteins
- characterise the binding properties for monoclonal antibodies 2F5 and 4E10
- use the prepared proteins for immunisation studies in rats
- characterise the immune response in resulting antisera by immunological and cell culture based methods

The workflow of this project is summarised schematically in 1.5

1.5. Workflow

Generation of prokaryotic expression plasmids for FFV Bet and HIV-1 hybrid constructs

⇩

Optimisation of protein expression and purification protocols

⇩

Characterisation of generated HIV-1 chimaeric proteins

⇩

Immunisation of rats with the HIV-1 hybrid antigens

⇩

Characterisation of the antisera (titration, fusion partner specificity, neutralising activity)

2. Material and methods

2.1. Materials

2.1.1 Equipment and consumables

Äkta Explorer FLPC	GE Healthcare
Cell culture flasks	TTP
Cell culture plates	TPP
- 6 Well plates	TPP
- 96 Well plates	TPP
Cell scraper	TPP
Centrifuge 5415D	Eppendorf
Centrifuge 5805R	Eppendorf
Centrifuge tubes (15 ml, 50 ml)	TPP
CO_2-Incubator MCO-20AIC	Sanyo
Columns	
• HisTrap 1 ml, 5 ml	GE Healthcare
• Resource Q	GE Healthcare
Dialysis tubing (MWCO 12.000-14.000)	Serva
Diluting loop	GeHealthcare
ECL X-Posure Film	Thermo Scientific
ELISA-Reader	Tecan
ELISA washer	Tecan
ELISA 96 well plates	Corning
Elispot reader	AID
Developer machine (Curix 60)	AGFA
Eppendorf tubes (1,5 ml, 2 ml)	Eppendorf
Microscope	Leica
Mastercycle pro S	Eppendorf
NanoDrop ND-1000 Spectrophotometer	PQELab
Parafilm M	Pechiney
Pasteur pipettes	Volac
Pipettes (2 ml, 5 ml, 10 ml, 25 ml, 50 ml)	
- Glas pipettes	Hirschmann
- serological	TPP
Pipette, multichanel	Eppendorf
Pipette boy, Accu Jet	Neolab
pH meter	IKAMAG RET
PFVD membrane (0.2 µm)	Millipore
SDS electrophoresis chamber	CBS Scientific
Semi dry transfer cell	Biorad
Shaker device Duomax 1030	Heidolph Instruments
SterilGARD Class II TypeA/B3	Thermo Scientific
Stepper, multistep	Eppendorf
Sterile filter (Stericup-Filter 0.22 µm, 0.45 µm)	Millipore
Sonificator	Branson
Thermomixer 5436	Eppendorf

ThermoStat plus (1.5 ml tubes)	Eppendorf
Thermomixer (2.0 ml tubes)	Eppendorf
Vortexer	Heidolph
Vivaspin	Sartorius
Water bath	Memert

2.1.2 Chemicals and Kits

Acryalmide (Rotiphorese Gel 30)	Roth
Adenosin triphosphate (ATP)	Fermentas
Agarose	Roth
Ammoniumpersulfat (APS)	Roth
Ampicillin	Roth
Amylose Resin	New England Biolabs
Benzonase	Novagen
n-octyl-beta-D-glucopyranoside (Beta OG)	Qiagen
Big Dye	ABI
Brijj 35	Sigma-Aldrich
Chaps	Sigma-Aldrich
Coomassie Brilliant blue G-250	Roth
ECL (Enhanced Chemiluminescence)	Thermo Scientific
Dodedcyl-maltoside (DDM)	Roth
Dithiothreitol (DTT)	Roth
EDTA	Serva,
Ethanol	Roth
Ethidium bromide	Sigma-Aldrich
Fetal Calf Serum (FCS)	Biochrom
Glacial acetic acid	Roth
Glucose	Roth
L-Glutamin	Invitrogen
Glycerin	Roth
Glycin	Roth
HEPES	Biochrom
Guanidine hydrochloride	Roth
Imidazole	Roth
IPTG	Roth
Isopropanol	Roth
Hydrogen peroxide	Merck
Hydrochloric acid	Roth
Kanamycin sulfate	Fluka
L-arginine	Sigma-Aldrich
Lysozyme	Sigma-Aldrich
Maltose	Roth
2-mercaptoethanol	Roth
Methanol	Roth
Non-fat dry milk powder	Sucofin
MSB®Spin PCRapace (500)	Invitek
Nonidet P-40 (NP-40)	Sigma-Aldrich

Polyethylenimine (PEI)	Sigma-Aldrich
Plasmid Midi/MaxiKit	Qiagen
Nuclease Free Water	Promega
Saponin	Roth
Spin Plasmid Mini Kit Two	Invitek
Sodium dihydrogenphosphate monohydrate	Roth
Sodium chloride	Roth
Sodium hydroxid	Roth
O'Gene Ruler^{TM}, DNA Ladder Mix, Ready-to-use (0,1 µg/µl)	Fermentas
o-phenylendiamine dichloride (OPD)	Sigma
Orange G (DNA Sample Buffer)	Roth
Paraformaldehyde	Roth
Potassium acetate	Roth
Potassium hexacynaoferrate III	Roth
Potassium hexacyanoferrate II 3-hydrate	Roth
Sodium dodecyl sulfate (SDS)	Roth
Tetramethylethylenediamine (TEMED)	Roth
Terralin	Schülke
Tricin	Roth
Tris	Roth
Tris-HCL	Roth
Triton X-100	Roth
Trypsin	Invitrogen
Tween-20	Roth
Urea	Roth
X-Gal	Promega

2.1.3 Buffers

Buffers for preparation competent *E.coli*

Name	**Recipe**
TFB I	100 mM RbCl, 50 mM $MnCl_2$, 30 mM potassium acetate, 15% Glycerol, pH 5.8
TFB II	10 mM MOPS, 10 mM $RbCL_2$, 15% Glycerol, pH 6.8

Both buffers were sterile filtrated before use.

SDS-PAGE and Western Blotting

Name	**Recipe**
2 x SDS Sample Buffer	50 mM Tris-HCL, 12% Glycerol, 4% SDS, 5% 2-Mercaptoethanol, 0.01% Coomassie Brilliant Blue G-250
Gel Buffer	3 M Tris, 0.3% SDS, pH 8.4
Anode Buffer	0.2 M Tris, pH 8.9
Cathode Buffer	0.1 M Tris, 0.1 M Tricine
Transferbuffer	48 mM Tris, 39 mM Glycine, 20% Methanol, 0.03% SDS
Stripping Buffer	62.5 mM Tris-HCL pH 6.7, 2% SDS, 100 µM 2-mercapthoethanol, 92% ddH_2O

Solutions for Coomassie Blue Staining

Name	**Recipe**
Coomassie staining solution	1 g/L Coomassie Brilliant Blue G-250, 40% Methanol, 10% Glacial acetic acid
Destaining solution	10% Glacial acetic acid, 45% Methanol, 45% ddH_2O

Solutions for X-Gal staining

Name	**Recipe**
Fixation solution	2 % Paraformaldehyde, 0,2 % Glutaraldehyde
X-Gal staining solution	5 mM $K_3[Fe(CN)_6]$, 5 mM $K_4[Fe(CN)_6]$, 2 mM $MgCl_2$, 4 mg/ml X-Gal diluted in PBS

Buffers for Ni-NTA chromatography

Denaturing purification

Name	**Recipe**
GuHCL Lysis Buffer	100 mM NaH_2PO_4, 10 mM Tris-HCL; 6M GuHCL, 300 mM NaCl, pH 8.0
Wash Buffer D	100 mM NaH_2PO_4, 10 mM Tris-HCL, 8 M Urea, 300 mM NaCl, 10 mM Imidazole, pH 8.0
Elution Buffer D	100 mM NaH_2PO_4, 10 mM Tris-HCL, 8 M Urea, 300 mM NaCl, 500 mM Imidazole, pH 8.0

Non-denaturing purification

Name	**Recipe**
Wash Buffer N	50 mM NaH_2PO_4, 10 mM Imidazole, 300 mM NaCl, pH 8.0
Elution Buffer N	50 mM NaH_2PO_4, 500 mM Imidazole, 300 mM NaCl, pH 8.0

Buffers for protein purification using amylose resin

Name	**Recipe**
Column Buffer	20 mM Tris-HCL, 200 mM NaCl, 1 mM EDTA, 1 mM DTT pH 8.0
Elution Buffer	20 mM Tris-HCL, 1 mM EDTA, 1 mM DTT, 10 mM Maltose pH 8.0

Buffers for anion exchange chromatography

Name	**Recipe**
Start Buffer	20 mM Tris-HCL, pH 8.0
Elution Buffer	20 mM Tris-HCL, 1 M NaCl, pH 8.0

Buffers for protein refolding	
Name	**Recipe**
Rapid dilution - testing for best buffer conditions	50 mM NaAc, pH 5.0, 50 mM HEPES, pH 7.0, 50 mM Tris, pH 9.0
Rapid dilution (preparative)	50 mM HEPES, 100 mM L-arginine, 300 mM NaCl, pH 8.0
Dialysis Buffer	50 mM NaH_2PO_4, 300 mM NaCl, 10 mM Imidozole, pH 8.0

Buffers for agarose gelelectrophoresis	
Name	**Recipe**
Tris-acetate-EDTA (TAE, 50x)	2 M Tris, 50 mM EDTA, 1 M acetate

2.1.4 Media for bacteria culture

All media were autoclaved prior use.

Media	**Recipe**
LB	10 g/L Tryptone, 5 g/L Yeast extract, 100 mM NaCl, pH 7,0
2YT	16 g/L Tryptone, 10 g/L Yeast extract, 100 mM NaCl, pH 7,0
TB	12 g/L Tryptone, 24 g/L Yeast extract, 4 ml/L Glycerol, add 100mL 170 mM Potassium dihydrogene phosphate after autoclaving
SOC	20 g/L Tryptone, 5 g/L Yeast extract, 8.6 mM NaCl, 2.5 mM KCl, 20 mM $MgSO_4$, 20 mM Glucose, pH 7,0

2.1.5 Media for cell culture

Dulbecco's Modefied Eagle Medium (DMEM)

DMEM was used for cultivating HEK 293-T and TZM-bl cell lines. Prior use media were supplemented with 10% [v/v] FCS, 2 mM L-Glutamine, 10 mM HEPES and 5 mM Penicillin/Streptomycin.

2.1.6 Bacteria strains

Strain	**Genotype**	**Vendor**
E.coli Top10	F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 deoR recA1 endA1 araΔ139 Δ(ara,leu)7697 galU galKλ- rpsL(StrR) nupG	Invitrogen
E.coli DH5α	F- φ80*lac*ZΔM15 Δ(□*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ^-	Stratagene
E.coli SCS1	*recA1 endA1 gyrA96 thi-1 hsdR17 (r*κ- *m*κ+*) supE44 relA1*	Stratagene [85]
E.coli T7-Shuffle	F´ *lac, pro, lacI^Q / Δ(ara-leu)7697 araD139 fhuA2 lacZ::T7 gene1 Δ(phoA)PvuII phoR ahpC* galE (or U) galK λatt::pNEB3-r1-cDsbC* (Spec^R, *lacI^q*) *ΔtrxB rpsL150(Str^R) Δgor Δ(malF)3*	NEB
E.coli C43 (DE3)	F – *ompT hsdSB (rB- mB-) gal dcm* (DE3)	Lucigen
E.coli Rosetta 2	F$^-$ *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* pRARE2 (Cam^R)	Novagen
E.coli K12	e14- (McrA-), recA1, endA1, gyrA96, thi-1, hsdR17 (rK- ,mK+), supE44, relA1, D(lac-proAB), [F' , traD36, proAB, lacIq ZDM15]	NEB

2.1.7 Cell lines

HEK 293T cells

The adherent cell line descended from human embryonic kidney cells and exhibits an epithelial morphology. 293T is an easily transfectable derivate of 293 cells and stably expresses the large T-antigen of SV40. The cell line was purchased from the ATCC (Catalog Number: CRL-11268).

TZM-bl cells

The parental HeLa cell line was generated from JC53-bl clone 13 and stably expresses large amounts of CD4 receptor and CCR5 co-receptor that makes the cell line highly sensitive to infection with diverse isolates of HIV-1. Luciferase and ß-galactosidase were integrated as reporter genes under the control of HIV-1 LTR promoter and therefore allow simple and quantitative analysis of virus infection by measurement of luciferase or ß-galactosidase activity. The cell line was obtained from the NIH AIDS Research & Reference Reagent Program (Catalog Number: 8129).

2.1.8 Animals

Immunisations experiments were performed in twelve weeks old female Wistar rats with 220-250 g body weight which were purchased from Charles River. Each group contained 4 animals.

2.1.9 Antibiotics

Antibiotic	**Working concentration**	**Vendor**
Ampicillin	100 µg/ml	Roth
Cloramphenicol	34 µg/ml	Roth
Penicillin/Streptomycin	100 U/ml, 100 µg/ml	PAA
Kanamycin	25 µg/ml	Fluka

2.1.10 Plasmids

Plasmid	Description	Vendor
pQE-Xa-30	Prokaryotic expression vector featuring IPTG controlled T5 promoter expression, a Factor Xa protease cleavage site, a N-terminal His tag sequence, a Col E1 origin of replication, a ampicillin resistance gene (Ampicillin)	Qiagen
pCL1	Cloning vector containing HIV-1 E1 and E2 sequences modified by Dr. J. Kreuzberger (RKI, Berlin)	GeneArt
pBC12-Bet	CMV driven, eukaryotic expression vector encoding the full length FFV Bet protein.	Prof. Martin Löchelt (DKFZ Heidelberg)
pMAT	Proprietary GeneArt cloning vector containing HIV-E1-loop-E2 sequence. Carries the ampicillin resistance gene and a Col E1 origin of replication.	GeneArt
pMal p5x	Prokaryotic expression vector encoding the maltose binding protein gene (malE) under control of the tac promotor (Ptac) for expression of maltose binding protein fusion proteins in the periplasm of E. coli. Further encodes the lac repressor gene ($laqI^q$), a Factor Xa protease cleavage site and the ampicillin resistance gene.	NEB
pNL4-3	Full length molecular infectious clone of HIV-1 pNL4-3.	NIH

2.1.11 Antibodies

Primary Antibodies

Antibody	Application	Source	Vendor
Penta His	Western Blot	Mouse monoclonal	Qiagen
2F5	Western Blot,	Human monoclonal	Polymun scientific
4E10	Western Blot	Human monoclonal	Polymun scientific
anti E1 serum	Western Blot	Rat	J. Kreutzberger, RKI

Secondary Antibodies

All secondary antibodies were purchased from Dako GmbH.

Antibody	Application	Source
Anti-mouse HRP-linked	Western Blot	Rabbit Polyclonal
Anti-human HRP-linked	Western Blot	Rabbit Polyclonal
Anti-Rat HRP-linked	ELISA	Rabbit Polyclonal

2.1.12 Restriction enzymes

Restriction enzymes for molecular cloning were purchased from Fermentas Life Science as "Fast Digest" enzymes.

Restriction enzyme	Recognition sequence 5'→3'	Incubation temperature
*Hind*III	5'...A^A G C T T...3'	37°C
*Nae*I	5'...G C C^G G C...3'	37°C
*Mly*I	5'...G A G T C $(N)_5$^...3 '	37°C
*Pdm*I = *Xmn*I	5'...G A A N N^N N T T C...3'	37°C
*Bgl*II	5'...A^G A T C T….3'	37°C
*Bam*HI	5'...G^G A T C C…3'	37°C
*Stu*I	5'...A G G^C C T…3'	37°C
*Pvu*I	*5'...C G A T^C G...3'*	*37°C*

2.1.13 Other enzymes

Enzyme	Vendor
Lysozyme (from chicken egg white, 20.000 U/mg)	Sigma
Benzonase (25 U/µl)	Novagen

Shrimp alkaline phosphatase (SAP) (1 U/µl)	Fermentas
T4 DNA-Ligase (5 U/µl)	Fermentas
Polymerases • Hot Start DNA-Polymerase (1 U/µl) • Platinum *Pfx* DNA Polymerase (1 U/µl) • Firefly DNA Polymerase (1 U/µl)	 Sengenetic Invitrogen Steinbrenner GmbH

2.1.14 Primer

All primers have been synthesized from eurofins mwg operon and resuspended to yield a concentration of 100 µM. All primers were diluted to a working concentration of 10 µM.

Primer	**sequence 5'→3'**	**GC**	**Tm**	**AT**
FFV Bet_fwd	atggcttcaaaatacccggaagaaggacca	46.7%	63.0°C	62°C
FFV Bet_rev	tcatctgactctgaagctggtgcagccggctag	57.6%	67.7°C	62°C
FFV Bet E1_fwd	tcaggttcagctggtgctggcgcaggtggaggcgcagcaggtagcaccatg	64.7%	75.8°C	56°C
FFV Bet E1_rev	tataagctttagctcagcagctgacgtgc	44.8%	60.4°C	56°C
FFV Bet E2_fwd	tcaggttcagctggtgctggcgcaggtggaggccaggaaaaaaacgaacaggaactgctggaactggat	55.1%	74.3°C	56°C
FFV Bet E2_rev	tataagctttaccacagccagttggtaatatt	34.4%	57.9°C	56°C
1.2_fwd	caagaaaagaatgaacaagaattattgga	27.0%	55.0°C	5°C
1.2_rev	ttccattgcatccagctc	50.0%	56.2°C	54°C
2.1_fwd	gaattacaggccctggaggc	60.0%	61.4°C	53°C
2.1_rev	agacaataattgtctggcctgtac	41.7%	59.3°C	53°C
FFV BetSeq1_fwd	cgccgctacacttactaaaac	47.6%	53.7°C	52°C
FFV BetSeq1_rev	cccaaaggggatcatgttcag	52.4%	55.8°C	52°C
FFV BetSeq2_fwd	gactctgaagctggtgca	55.6%	54.8°C	52°C
005_fwd	aaaatttatttgctttgtgagcg	30.4%	51.2°C	50°C
006_rev	ccagatggagttctgaggtca	52.4%	56.0°C	50-52°C

2.1.15 Software and online tools

Name	Function
DNASTAR Lasergene	Generation of plasmid maps, analysis of sequencing runs, restriction site analysis
GelDoc 2000	Biorad gel documentation system
Oligo Analyzer	Primer design (http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/)
Expasy translate tool	Translation of nucleotide into protein sequences (http://web.expasy.org/translate/)
SDSC Biological Workbench	Nucleotide, protein translation and alignment tool (http://workbench.sdsc.edu/)

2.2. Molecular biological methods

2.2.1 Isolation of plasmid DNA

Isolation of plasmid DNA was performed with the Invisorb Spin Plasmid Mini Kit Two (Invitek, Germany), the Plasmid Midi Kit (Qiagen, Germany) or with the Endo Free Plasmid Maxi Kit (Qiagen, Germany) dependent on the amount of DNA needed. The principle of these kits is based on the alkaline lysis of transformed bacteria and subsequent binding of plasmid DNA to anion exchange columns. Briefly, 2-250 ml of *E.coli* overnight cultures grown in LB medium with antibiotics were centrifuged and the pellets were resuspended in lysis buffer. After removal of cell debris by centrifugation, plasmid containing supernatants were loaded under low salt conditions onto the column either by centrifugation (mini-prep) or by gravity flow (midi and maxi-prep). After washing and removal of residual ethanol, purified plasmid DNA was directly eluted using 50 µl ddH_2O in case of the spin column protocol. DNA from midi and maxi preparations was first eluted under high salt conditions and then precipitated by addition of 0.7 volumes of isopropanol and centrifugation. Resulting DNA pellets were resuspended in approximately 70-150 µl ddH_2O dependent on pellet size and intended concentration.

2.2.2 Estimation of concentration and purity of plasmid DNA

DNA concentration as well as purity was measured spectrometrically using a NanoDrop device. Therefore, the photometer was calibrated with ddH_2O and then 1.5 µl of sample applied. For estimation of plasmid DNA concentration the assumption that an absorption of 1 at 260 nm corresponds to 50 µg/ml dsDNA was used [86]. Potential protein contaminations were detected by evaluation of the absorption quotient at 260 and 280 nm. Samples having values between 1.8 and 2 were considered to be highly purified plasmid DNA. When samples were to dilute or contained substances interfering with spectrometric detection, DNA concentration was estimated by agarose gel electrophoresis.

2.2.3 Agarose gel electrophoresis

DNA samples were separated according to their length by agarose gel electrophoresis. For standard separations, 1% agarose gels were used. For this purpose agarose was mixed with

1 x TAE buffer and boiled until the agarose was completely dissolved. Ethidium bromide at a concentration of 0,001% (v/v) was added to the agarose gel solution to visualize DNA fragments subsequently. The liquid agarose solution was poured in an electrophoresis chamber and incubated until polymerisation. After placing the gel in an electrophoresis chamber (Biorad, USA) with TAE buffer, DNA samples were mixed with sample buffer, loaded and separated at a voltage of 120 V. Gels were documented using a UV transilluminator system and software (Biorad, USA). To analyse the size of DNA fragments, a commercial DNA Ladder (O'GeneRuler, DNA Ladder Mix ready-to-use, Fermentas) was run in parallel.

2.2.4 DNA fragment purification from agarose gels

For isolation of DNA fragments from agarose gels, DNA bands with the intended size were excised from the gel with a scalpel under UV exposure. Since long wave UV light exposure can damage DNA and introduce mutations, this step was performed as quickly as possible. Afterwards DNA fragments were purified with the Invisorb Spin DNA gel extraction kit (Invitek, Germany) according to the manufacture's instructions. Gel purified fragments were eluted at least in 20 µl of ddH_2O [87].

2.2.5 Restriction digestion

Prokaryotic restriction endonucleases cleave double-stranded DNA sequence-specifically, with a palindromic sequence as recognition site, resulting in 5'- and 3'-overhangs (sticky ends) or blunt ends that can be used for ligating such fragments to compatible foreign DNA [88]. For analytical digestions, 1 µg of DNA was incubated with the restriction enzyme in a 20 µl restriction mixture for 0.5 h at 37°C. For cloning purposes, DNA templates were digested in preparative restriction mixtures of 100 µl and incubated longer (2 h at 37°C) to assure complete digestion. In all cases mixtures were provided with sufficient amounts of restriction enzyme (5 U/µg DNA) and filled up with ddH_2O to obtain a DNA concentration of 0.05 µg/µl (Table 4).

Table 4: Set-up of analytical and preparative restriction digestions

	analytical	preparative
Plasmid-DNA	1 µg	5 µg
10x *Fast Digest Green Buffer*	2 µl	10 µl
Fast Digest Enzyme (10 U/µl)	0.5 µl	2.5 µl
ddH_2O	ad 20 µl	ad 100 µl

2.2.6 Dephosphorylation and phosphorylation of DNA fragments

Religation of linearised vector DNA is a common problem during blunt-ended or non-directional cloning and reduces the efficiency of ligation with DNA inserts [88]. To avoid this, preparative DNA digestions were treated with shrimp alkaline phosphatase (SAP, Fermentas, Germany) to remove 5' phosphate residues at the ends of the linearised vector. For that, after enzymatic digestion the mixture was supplied with 2 µl of SAP (1 U/µl) for additional 30 min at 37 °C. To increase the efficiency of insert integration of blunt-ended PCR inserts, 5 µg insert DNA was phosphorylated using T4 Polynukleotid Kinase (PNK, Fermentas, Germany) in a 20 µl reaction mixture containing 2 µl 10 x T4 PNK buffer A, 1 mM ATP and ddH_2O. After 1 h incubation at 37°C, phosphorylation was stopped by heating the samples for 10 min at 75 °C. To remove buffer components and enzyme, the mixture was purified using the MSB Spin PCRapace kit (Invitek, Germany) as recommended by the manufacturer.

2.2.7 Primer

Sense- and antisense primers for PCR were designed with the computer programme OligoAnalyzer (IDT Technology, Germany) assuring a length of 15 to 30 base pairs, a moderate GC content of 40-50% and a calculated annealing temperature between 50 and 65°C. Additionally, if possible, both primers were designed to have a melting temperature difference (ΔT_M) of not more than 2°C to ensure a similar hybridisation kinetic in their annealing phase [89]. In several cases mutagenic primers were used that introduced new

restriction sites to allow subcloning, introduce new amino acids or also mutations. These primers are described separately at the respective position in the results part.

2.2.8 PCR Cloning

The expression plasmids were generated by PCR cloning. Therefore amplification of FFV Bet as well as HIV E1, E2 and E1-loop-E2 sequences was necessary. As polymerase for this approach the Platinum Pfx DNA polymerase (Pfx) from Invitrogen was used. The Platinum Pfx polymerase is a hot start polymerase which possesses 3'-5' proof reading activity and fast chain extension capability [90]. A standard PCR reaction for amplification of FFV Bet and HIV E1 and E2 sequences was set up (Table 5, Table 6). For successful amplification of HIV E1 and E2 sequences, DMSO containing enhancer solution included in the Pfx Kit had to be added to increase primer specificity, avoid primer dimer formation and break up secondary structures of the GC rich template. Obtained PCR products were then separated by agarose gel electrophoresis and isolated for further experiments as described (2.2.7).

Table 5: Master mix for amplification of FFV Bet

Total	50 µl	final concentration
10 x buffer	5 µl	1 x
$MgSO_4$ (50mM)	1 µl	1 mM
dNTP's (10mM)	1.5 µl	0.3 mM
3'-Primer (10µM)	1.5 µl	0.3 µM
5'-Primer (10µM)	1.5µl	0.3 µM
DNA (0.62 µg/µl)	1 µl	12.4 ng l
Pfx polymerase (2,5U/µl)	0.4 µl	0.016 U/µl
ddH_2O	38.1 µl	

Cycler conditions

94°C, 10 min

94°C, 40 s → Denaturing
62°C, 20s → Annealing
68°C, 30 s → Elongation
x 25

68°C, 2 min

04°C, ∞

Table 6: Master mix for amplification of HIV E1 and E2

Total	60 µl	final concentration
10 x buffer	6 µl	1 x
$MgSO_4$ (50mM)	0.8 µl	0.6 mM
dNTP's (10mM)	1.8 µl	0.3 mM
3'-Primer (10µM)	1.8 µl	0.3 µM
5'-Primer (10µM)	1.8µl	0.3 µM
DNA (0,43 µg/µl)	1.2 µl	8.6 ng
Pfx polymerase (2,5U/µl)	0.48 µl	0.02 U/µl
10 x Enhancer	6 µl	1 x
ddH_2O	34.12 µl	

Cycler conditions

94°C, 10 min

94°C, 40 s → Denaturing
56°C, 20s → Annealing
68°C, 30 s → Elongation
x 35

68°C, 2 min

04°C, ∞

2.2.9 Mutagenesis PCR

To obtain truncated variants of E1-loop-E2 constructs, the GeneArt synthesized plasmid pMAT was mutated by full-plasmid mutagenesis PCR. To allow direct ligation of the amplified products, primers were phosphorylated prior to use (Table 7). Samples were incubated for 1 h at 37°C and inactivated by heating at 75°C for 10 min. Phosphorylated primers were then directly used for PCR amplification without a further purification step. Table 8 describes the setup of a standard PCR mix with phosphorylated primers for amplification of HIV-1 E1-loop-E2 constructs.

Table 7: Mixture for primer phosphorylation

Primer (100 pmol/µl)	0,9 µl
10 x T4 PNK buffer A	0,9 µl
10 mM ATP	0,9 µl
T4 PNK	0,8 µl
ddH_2O	5,5 µl

Table 8: Master mix for mutation of pMA-T vector

Total	60 µl	final concentration
10 x buffer	6 µl	1 x
$MgSO_4$ (50mM)	2.64 µl	2.2 mM
dNTP's (10mM)	1.98 µl	0,33 mM
3'-Primer-P (10µM)	1.98 µl	0.33 µM
5'-Primer-P (10µM)	1.98µl	0.33 µM
DNA (0.056 µg/µl)	1.5 µl	1.4 ng
Pfx polymerase (2,5U/µl)	0.53 µl	0,2 U/µl
ddH_2O	43.39 µl	

Cycler conditions

94°C, 10 min

94°C, 40 s → Denaturing
53°C, 20s → Annealing
68°C, 30 s → Elongation
} x 35

68°C, 2 min

04°C, ∞

2.2.10 Ligation

Insertion of foreign DNA fragments into an expression vector or self circularization of a linear PCR insert is called ligation. During ligation, the enzyme ligase catalyses the formation of a phosphodiester bond between the 5'-phosphates and the 3'-hydroxy group of two DNA strands in the presence of ATP [91]. In case of vector self circulation as used for mutated E1-loop-E2 constructs, the 5'-phosphate bonds with the 3'-hydroxy group of the same DNA strand and forms a closed circle (plasmid). The setup of a classical ligation reaction mixture and one used for vector relegation are given below. All mixtures were incubated 1 h at room temperature prior to transformation in competent bacteria.

Ligation of PCR insert with vector DNA

Vector DNA	50-100 ng
PCR insert	10-100 ng
10 x ligase buffer	1 µl
T4 ligase (5U/µl)	1 µl
ddH_2O	5,5 µl
Total	10 µl

Self circularization of linear DNA

DNA	10-20 ng
10 x ligase buffer	2 µl
T4 ligase (5U/µl)	1 µl
ddH_20	12 µl
Total	20 µl

2.2.11 Transformation of competent bacteria

The delivery of plasmids for DNA amplification or expression in *E. coli* was performed by use of chemical competent bacteria by heat shock. In detail, 1-10 ng plasmid DNA was incubated with 50 µl of competent cells for 30 min on ice. After incubation, cells were heat pulsed for 40 sec at 42°C to permeate cell membranes and allow incorporation of the expression plasmids into bacteria. Afterwards samples were placed on ice for two minutes so that the membrane pores are able to close. Then 300 µl SOC- or LB-media was added to the bacteria solution and the mixture incubated at 37°C and 350 rpm. Time of incubation depended on the antibiotic resistance gene encoded on the backbone of the plasmid and took 15 min for ampicillin and up to 1 h for chloramphenicol or kanamycin resistant bacteria. For selection of positive transformed bacteria cells were plated on agar plates containing the appropriate antibiotic and incubated at 37°C until the next day.

2.2.12 Preparation of chemical competent cells

For preparation of chemical competent cells, bacteria were spread on LB-agar plates without antibiotics and incubated overnight at 37°C. One single bacterial colony was then transferred to 5 ml LB medium and cells grown again overnight at 37°C and 220 rpm. 100 ml pre-warmed LB medium were inoculated with 1 ml overnight culture and incubated at 37°C under shaking until the bacterial suspension reached an OD_{600} value of 0.5. Then bacterial suspension was chilled on ice for 5 min and centrifuged at low speed (5 min, 4000 x g, 4°C). The supernatant was carefully discarded while keeping the collected cells constantly on ice. In the next step, cells were resuspended in 30 ml ice cold TFB I buffer. Cell suspension was kept on ice for further 90 min and afterwards centrifuged again for 5 min at 4000 x g and 4°C. After discarding the supernatant carefully, the cell pellet was resuspended in 4 ml ice cold TFB II buffer. 50 µl of resuspended competent cells were aliquoted in sterile microcentrifuge tubes and snap-frozen in liquid nitrogen. Chemical competent *E.coli* cells were stored at -80°C until use.

2.2.13 Colony PCR

To identify positive clones in transformed bacteria, colony PCR was used. Therefore single colonies were directly taken from agar plates as template material and mixed with the Master Mix for colony PCR containing appropriate primers (Table 9).

Table 9: Master mix for Colony PCR

Total	15 µl	final concentration
Firefly buffer (10 x)	1.5 µl	1 x
MgCl (25 mM)	2 µl	3.33 mM
dNTP's (10 mM)	0.63 µl	0.42 mM
3'-Primer (10 µM)	0.3 µl	0.2 mM
5'-Primer (10 µM)	0.3µl	0.2 mM
DNA	1 colony	1 colony
Firefly polymerase (2,5U/µl)	0.2 µl	0.033 U/µl
ddH_2O	10.07 µl	

Cycler conditions

95°C, 10 min

95°C, 30 s → Denaturing
see 2.1.14 → Annealing
72°C, 30 s → Elongation
x 25

72°C, 2 min

04°C, ∞

2.2.14 Preparation of glycerine stocks for long term storage of bacteria

Bacteria clones containing the desired plasmid were stored long term in a glycerol solution. 600 µl of 50% sterile filtrated glycerol solution were mixed with 900 µl of bacteria overnight culture. After snap freezing the bacteria samples in liquid nitrogen clones were subsequently stored in a 1.5 ml tube at - 80°C.

2.2.15 Sequencing of constructed plasmids

Plasmid DNA of constructs yielding a positive result in the colony PCR screening were isolated (see 2.2.1) and sequenced by a fluorescence based cycling sequencing using Big Dye 3.1 to ensure integrity of cloned plasmids. For this purpose following protocol was used (Table 10).

Table 10: Master mix for sequencing

Total	10 µl	final concentration
ABI buffer (5 x)	2 µl	1x
BigDye	0,4 µl	
Primer (10 µM)	0,6 µl	0,6 µM
Plasmid DNA	2,0 µl	10-20 ng
ddH_2O	10 µl	

Cycler conditions

96°C, 1,5 min

96°C, 10 s → Denaturing

see 2.1.14 → Annealing

60°C, 4 min → Elongation

x 35

04°C, ∞

2.3. Proteinchemical methods

2.3.1 SDS-PAGE

Extraced proteins can be separated electrophoretically on a denaturing acrylamide gel according to their molecular weight. SDS-PAGE based on the protocol of Schägger and Jagow [92] was performed using 10% separation gels and 4% stacking gels (Table 11). Protein samples were mixed with 2 x sample buffer containing ß-mercaptoethanol and than loaded into the gel. Protein separation was performed at 100 - 120 V for 45 min along with a protein ladder (PageRulerTM, Fermentas) as marker for determination of protein size. Proteins were visualized by staining either by Coomassie Brilliant Blue or by antibodies after transfer to PVDF membranes.

Table 11: Mixture fort wo SDS-Gels

	4% stacking gel	10% separation gel
SDS-Gelbuffer	2 ml	5 ml
Aqua dest.	3.2 ml	5 ml
Rotiphorese (30)	0,8 ml	5 ml
APS	100 µl	100 µl
Temed	10 µl	10 µl

2.3.1.1. Coomassie staining

For Coomassie staining SDS-gels were incubated for 15 min in a 1% Coomassie brilliant Blue solution under permanent shaking followed by dye removal using destaining solution until the bands were clearly visible. For removal of last traces of background colour, gels were placed in distilled water.

2.3.2 Expression screening and solubility testing

To find suitable expression conditions for the intended proteins expression screening in 96 deep well plates was performed. For this, the respective expression plasmid was transformed into the four *E.coli* expression strains SCS-1, T7-Shuffle, C43 and Rosetta 2 that are protease deficient and avoids the degradation of the produced target protein. A single transformant of these bacteria was picked to inoculate overnight cultures. 100 µl of these overnight cultures were then transferred into 1 ml of LB, 2YT, TB or SOC medium prepared in 96 deep well plates and grown for 3 h at 37°C to reach the exponential growing phase. Protein production was induced by adding 0.1 mM or 0.5 mM IPTG along with a non induced control and then samples grown for additional 3 h at two different growing temperatures of 30°C and 20°C. Subsequently, cells were pelleted by centrifugation at 3700 rpm for 10 min. Medium was discarded and cell pellets were resuspended with 200 µl of lysis buffer (PBS + 0,1 mg/ml lysozyme), incubated on ice for 10 min and then further disrupted by sonification using 5 pulses of ultrasound at a duty cycle of 40%. About 60 µl from total lysates were saved for later analysis of total protein expression by SDS-PAGE. The remaining material was subjected to centrifugation (16.000 g, 20 min, 4°C) to analyse expression in the soluble fraction by SDS-PAGE and Western blot. A schematical figure of the plate layout used is given in Figure 12.

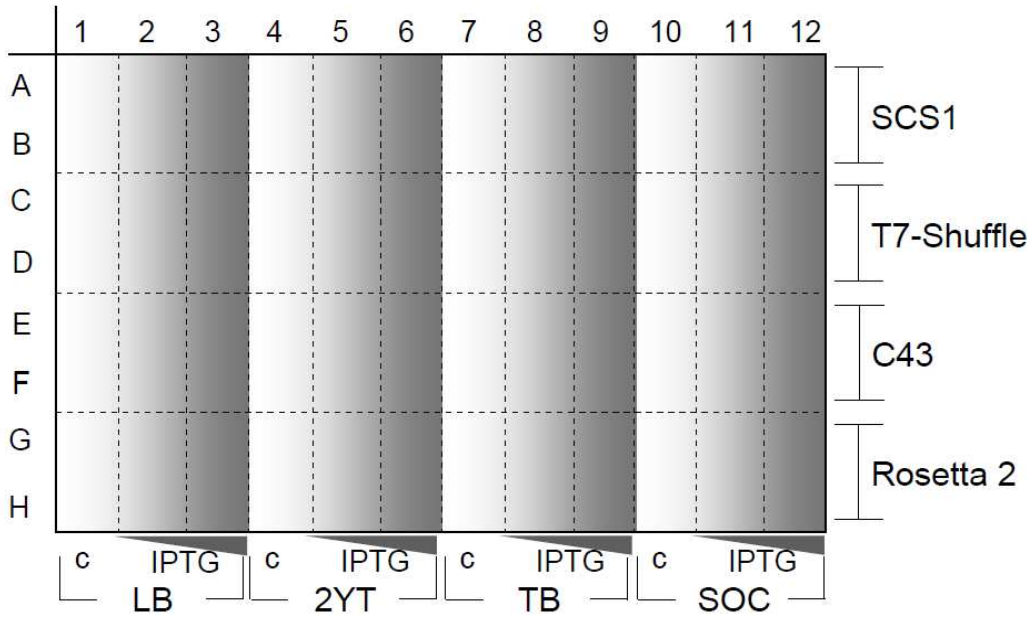

Figure 12: Layout of the 96 deep well plate used for small scale expression optimisation.
Four bacteria strains SCS1, T7-Shuffle, C43 and Rosetta 2 containing the respective expression plasmid were inoculated in LB, 2YT, TB or SOC medium and grown for 3h at 37°C. Protein expression was induced with 0.1 or 0.5 mM IPTG, along with non-induced cells as controls (c) and incubated at two different growing temperatures 20 and 30°C before pelleting and lysis.

2.3.3 Time course expression analysis

Bacteria growth and protein expression changes remarkably during prolonged culture and directly influences the level and quality of the produced recombinant protein. To determine protein toxicity and the best time point for harvest of soluble protein, time course expression analysis for FFV Bet was performed. An overnight culture of pQE-Bet transformed into *E. coli* strains SCS-1 and T7-Shuffle were inoculated in 200 ml TB medium for SCS-1 and 2YT medium for growing of T7-Shuffle. Media were additionally supplied with 0.4% glucose to inhibit basal protein expression. After 2 h, OD_{600} was measured and non-induced samples (t_o) taken as control. Protein production was induced with 0.1 mM and 0.5 mM IPTG and cells further incubated at 20°C. Additional samples were taken in a range of 1 h till 24 h after induction in time intervals as indicated and OD_{600} was measured. After 24 h, all samples were lysed in PBS + 0.1 mg/ml lysozyme and soluble and insoluble fractions separated by centrifugation (16.000 g, 4°C, 20 min). The presence of FFV Bet was analysed in all fraction by SDS-PAGE and Coomassie blue staining as well as Western blot using anti-His antibodies.

2.3.4 Solubilisation test with non-ionic detergents

To increase the amount of proteins available for non-denaturing purification, various mild non-ionic detergents were tested for their ability to solubilise the target protein. For this purpose, the protocol to extract the soluble protein fraction was used, but additionally, several non-ionic detergents (Table 12) were added to the lysis buffer (PBS, 0.1 mg/ml lysozyme) in a concentration of 1 x or 5 x of their critical micelle concentration (CMC). After separation of soluble and insoluble material by centrifugation (16.000 g, 20 min, 4°C) the soluble protein fractions were analysed by Coomassie staining and Western blot.

Table 12: Used non-ionic detergents, CMC = critical micelle concentration

Detergent	CMC % [w/v]	Detergent	CMC % [w/v]
NP40	0.0179	DDM	0.0090
Triton-X 100	0.0155	Chaps	0.4900
Triton-X 114	0.0133	Brij 35	0.1130
Tween 20	0.0700	Saponin	0.1000
beta OG	0.7000		

2.3.5 Large scale protein expression

For preparative protein purification a large scale expression was performed using the conditions found in previous small scale expression screenings (Table 13). In general, two litres pre-warmed medium as indicated was inoculated with 75 ml of a bacterial overnight culture grown in LB medium to obtain an OD_{600} of 0.1. The suspension was then incubated at 37°C on a shaker for 2-3 h until the bacterial suspension reached an OD_{600} of 0.8-1.0. Protein expression was induced by addition of IPTG followed by additional growth at either 30°C or at 20°C as indicated (Table 13). Finally, cells were pelleted in four fractions of 500 ml pellets by centrifugation at 10.000 rpm for 10 min. Supernatants were discarded and cell pellets stored at -20°C until protein purification.

Table 13: Optimised expression conditions for generated constructs

Construct	Expression strain	IPTG conc.	Growing temperature	Growing time	Growing media	Purification
pQE-Bet	SCS-1	0.5 mM	20°C	2	TB	non-denaturing
pQE-Bet	SCS-1	0.5 mM	37°C	3 h	2YT	denaturing
pQE-Bet-E1 **PQE-Bet-E2** **pQE-Bet-E1-Loop-E2**	SCS-1	0.5 mM	37°C	3 h	2YT	denaturing
pMal	Rosetta 2	0.5 mM	20°C	2-3 h	LB	non-denaturing
pMal-E1-Loop-E2	Rosetta 2	0.5 mM	20°C	2-3 h	LB	non-denaturing

2.3.6 Purification of His-tagged proteins

For purification of His tagged proteins under non-denaturing conditions, cell pellets were resuspended as 10x concentrate with lysis buffer N (PBS and 0.1 mg/ml lysozyme) and incubated for 10 min on ice. After sonification (3 x 20 cycles at 60% duty cycle) cells were centrifuged at 10.000 rpm for 20 min and 4 °C to separate soluble and insoluble proteins. Expressed recombinant proteins possess a His-Tag on their N-terminus forming chelates with bivalent Ni^{2+} cations that are immobilised on Ni-NTA columns [93]. This allows

protein purification by Ni-NTA chromatography by one of the procedures described below.

2.3.6.1. Purification under non-denaturing conditions

Supernatants were taken to analyse the soluble protein fraction by affinity chromatography on 1 or 5 ml Ni-NTA columns (HisTrap FF Crude Crude, GE Healthcare) installed in an Äkta Explorer 10 s FPLC System. Before applying samples, the column was equilibrated with wash buffer N (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazol, pH 8.0). Lysate was loaded onto the column with the help of a sample pump at flow rates of 1-2 ml/min and then washed with wash buffer N until the OD_{280} decreased below 0.01 absorption units. Bound material was recovered by applying elution buffer N (50 mM NaH_2PO_4, 300 mM NaCl, 500 mM imidazol, pH 8.0) as a linear gradient of 20 column volumes and fractions collected automatically in 96 deep well plates.

2.3.6.2. Purification under denaturing conditions

For purification under denaturing conditions, the remaining insoluble fraction was lysed in buffer D (100 mM NaH_2PO_4, 10 mM TrisCl, 6 M GuHCL, 300 mM NaCl, 10 mM imidazol, pH 8.0) and extracted for 2 h under slow continual rotation at 4°C. Non-solubilised material was separated from solubilised denatured protein by centrifugation (10.000 rpm, 20 min, 4°C) and the resulting supernatant taken for protein purification by Ni-NTA columns equilibrated with wash buffer D (100 mM NaH_2PO_4, 10 mM TrisCl, 8 M urea, 300 mM NaCl, 10 mM imidazol, pH 8.0). After loading, unbound material was removed with wash buffer D and proteins released with a linear gradient of 20 column volumes elution buffer D (100 mM NaH_2PO_4, 10 mM TrisCl, 8 M urea, 300 mM NaCl, 500 mM imidazol, pH 8.0). As mentioned above elution fractions were collected in 96 deep well plates in 1.5 ml fractions.

2.3.7 Purification of MBP-tagged proteins

The expressed proteins of the pMal-p5x vector contain a MBP fusion protein on their N-terminus with a tight affinity to amylose matrix for affinity purification (NEB, Germany). For purification of soluble expressed MBP E1-loop-E2, 500 ml pellets were resuspended in

20 ml column buffer (20 mM TrisHCl, 200 mM NaCl, 1 mM EDTA, 1 mM DTT) followed by three rounds of sonification on ice (20 cycles each, duty cycle 40%). Homogenised cell lysate was centrifuged (10.000 g, 20 min, 4°C) and the resulting supernatants diluted in column buffer to a final volume of 50 ml. For protein purification, 100 ml of crude extract were loaded on a column buffer equilibrated 4 ml amylose column with the Äkta Explorer FPLC System. The loaded column was washed intensively with 20 column volumes and the target protein recovered with elution buffer (20 mM TrisCL, 200 mM NaCl, 1 mM EDTA, 1 mM DTT, 10 mM maltose) by using a 50 % gradient over 10 column volumes. Finally, the column was washed with 100% elution buffer with a 5 fold column volume to remove any residual material. Elution fractions were collected in a 96 deep well plate in a volume of 1.5 ml for each fraction.

2.3.8 Ion exchange chromatography

During purification of MBP E1-loop-E2, MBP and the intended MBP fusion protein were eluted as a mixture from the amylose resin. To separate both species, elution fractions from affinity chromatography were pooled and diluted 1:2 to decrease salt concentration in the sample to 100 mM NaCl. The solution was then subjected to ion exchange chromatography using a 1 ml Resource Q column (GE Healthcare, Germany) which is a strong anion exchanger based on quaternary ammonium ligands (DEAE). The Resource Q column was equilibrated with start buffer (20 mM TrisCl, pH 8.0) and diluted samples loaded with a pump. Unbound material was removed by washing with five column volumes of start buffer and samples subsequently eluted in 1 ml fractions using a 50% linear gradient of elution buffer (20 mM Tris, 1 M NaCl, pH 8.0) over 20 column volumes.

2.3.9 Estimation of protein concentration by OD_{280} measurement

The yield of protein in the elution fractions were identified applying computer evaluation software of the Äkta Explorer system after purification. Therefore, amount of expressed target protein were calculated by peak area and the extinction coefficient of the respective protein. The molecular chemical properties of generated and purified target proteins are summarised in Table 14.

Table 14: Molecular chemical properties of purified proteins

	Molecular weight	pI	Ext. coefficient
FFV Bet	45.6	5.33	1.61
FFV Bet-E1	49.0	5.86	1.48
FFV Bet-E2	49.8	5.57	1.97
FFV Bet-E1-Loop-E2	54.4	5.34	1.80
MBP	47.2	5.24	1.40
MBP-E1-Loop-E2	53.8	4.97	1.74

2.3.10 Screening for suitable refolding conditions

To establish a suitable refolding protocol for denatured proteins a screening protocol described by Vincentelly et al., 2004, [94] was adopted. This method is based on the fact that the optical density of precipitated proteins increases at wavelengths of 340-400 nm where soluble proteins usually do not absorb (Figure 13). Comparison of the ratio of the protein absorbance at 370 nm of a soluble (or solubilised) protein compared to the absorbance of the same protein in a different buffer allows thus to estimate its impact on protein stability and aggregation. Vincentelly et al. [94] used this observation to find suitable conditions for refolding of denatured proteins by screening for buffers that allow removal of detergents without increasing precipitation. Denatured protein obtained from affinity chromatography was therefore concentrated to 2 mg/ml and reduced by incubation with 20 mM beta-mercaptoethanol for at least three hours. Subsequently protein was mixed by 1:20 dilution in various refolding buffers (Table 15). Refolding mixtures were prepared in triplicates in a 96 microtiter plate and incubated for 24 h at 4°C in the respective buffers. Absorbance of the diluted and the reference sample (solubilised protein in 8 M urea) were analysed at 370 nm. Additionally a control plate without protein was run in parallel to examine potential self absorbance of the individual buffers. Results were expressed as percentage difference to the absorbance of the soluble, denatured FFV Bet protein in 8 M urea.

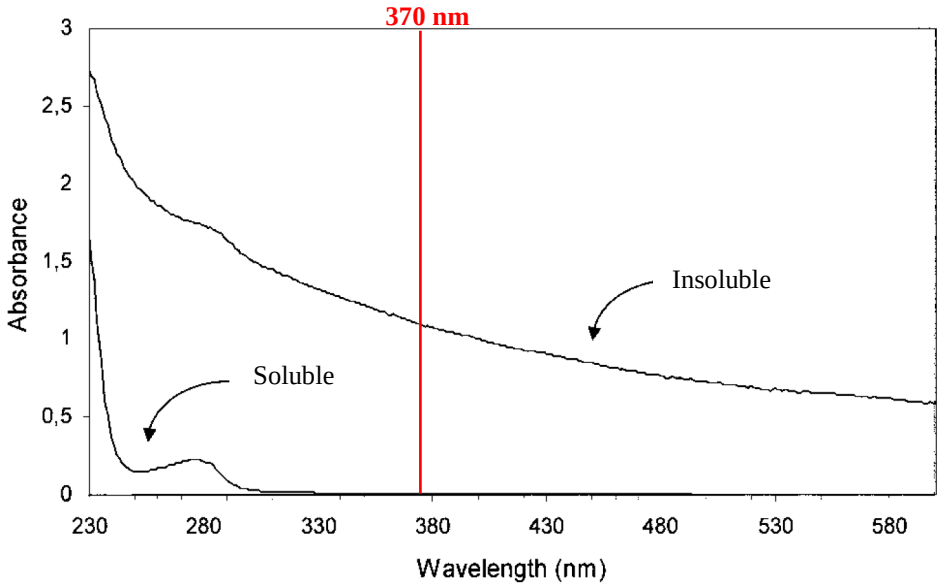

Figure 13: Absorbance spectra of precipitated and soluble forms of a protein [94]

Table 15: Buffer conditions for refolding optimisation

Buffer conditions	
Buffer / pH	50 mM NaAc, pH5; 50 mM HEPES, pH 7, 50 mM Tris, pH 9
Ionic strength	100 mM NaCl, 300 mM NaCl
Thiol reagent	10 mM ß-mercaptoethanol
Additives	L-Arginine, Glycerin, PEG 4000, Sucrose and combinations

2.3.11 Preparative refolding of denatured proteins

Purified denatured proteins were refolded by rapid dilution in a suitable refolding buffer identified in 2.3.10. As in small scale experiments proteins were reduced with 20 mM ß–mercaptoethanol for three hours and then concentrated to 2 mg/ml. Denaturant was removed by rapidly pipetting the protein solution into stirring refolding buffer (50 mM HEPES, 100 mM L-arginine and 300 mM NaCl, pH 9.0). Solutions were then incubated overnight at RT. To remove the refolding buffer, proteins were dialysed against a 100-fold excess of phosphate buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0) overnight at 4°C

and then concentrated by Ni-NTA chromatography under non-denaturing conditions as described in 2.3.6.1

2.4.Immunological methods

2.4.1.1. Western blot analysis

For detection of proteins by antibodies, samples separated by SDS-PAGE were transferred onto a PVDF membrane (Millipore) by semi-dry electroblotting. For this purpose PVDF membranes were first activated in methanol and subsequently equilibrated in Transfer Buffer for a few seconds. To build the blotting sandwich, one thick filter paper was pre-soaked with Transfer Buffer and placed onto the bottom of the blot chamber (anode). Then, the pre-wetted PVDF membrane, the SDS-Gel and an additional layer of filter paper which was also incubated in Transfer Buffer was added. The top cover of the blotting chamber functioned as cathode in this setting. Transfer was performed at a voltage of 20 V for 30 min. After transfer, the membrane was recovered from the sandwich and unspecific binding sites blocked by incubation with 5% [w/v] milk powder diluted in PBS-Tween (PBS / 0.05% [v/v] Tween 20) for 1 h. PVDF membranes were then incubated for 1 h at room temperature or at 4°C overnight with the first antibody diluted in 1% milk powder in PBS-T. To remove unbound antibodies, membranes were washed three times with PBS-T (PBS / 0.05% [v/v] Tween 20) for 5 min. Subsequently, an appropriate secondary, horseradish peroxidase (HRP) conjugated antibody was applied for 30 min at room temperature in a dilution as indicated followed by 3 x 5 min washing steps in PBS-T. For protein detection Enhanced Chemiluminescence (ECL) solution and light sensitive films were used.

2.4.2 Membrane stripping

Membrane stripping was performed to remove antibodies from an already developed PVDF membrane to allow analysis by further primary and secondary antibodies. Therefore, membranes were activated with methanol again and then incubated in stripping buffer for 1 h at 60 °C. Membranes were washed 3 x 5 min with PBS-T and finally blocked

with 5% milk powder in PBS-T followed by incubation with antibodies as described in **Fehler! Verweisquelle konnte nicht gefunden werden.**.

2.4.3 Immunisation

Wistar rats in groups of four were immunised three times at intervals of three weeks with 250 µg of antigen per rat and immunisation. Antigens emulsified in Freund's adjuvant were injected either subcutaneously (1st injection) or subcutaneously and intramuscularly (2nd and 3rd injection) at several sites of the animal. Blood was taken from these animals before experimentation (pre-immune sera, PI), two days before boost injections and three weeks after the last boost (serum 1, 2 and 3, respectively) (Figure 14). The immunisation studies for Bet-E1-loop-E2 and MBP-E1-loop-E2 proteins were performed identically but with a two weeks immunisation interval (**Figure** 15). The immunisation protocols are provided in the appendix for detailed information.

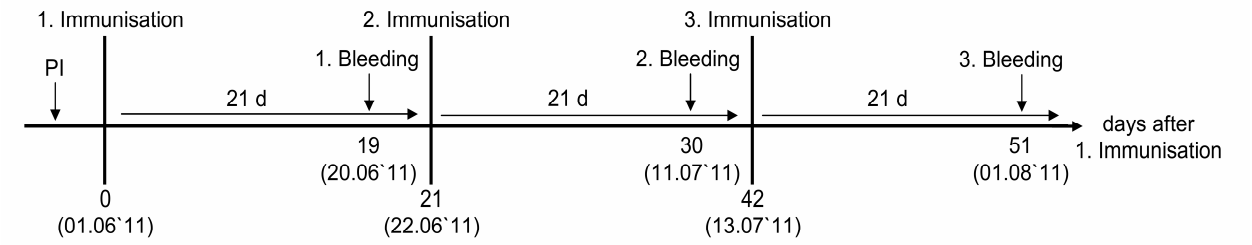

Figure 14: Immunisation schedule for Bet-E1, Bet-E2 and Bet-E1/Bet-E2 experiments

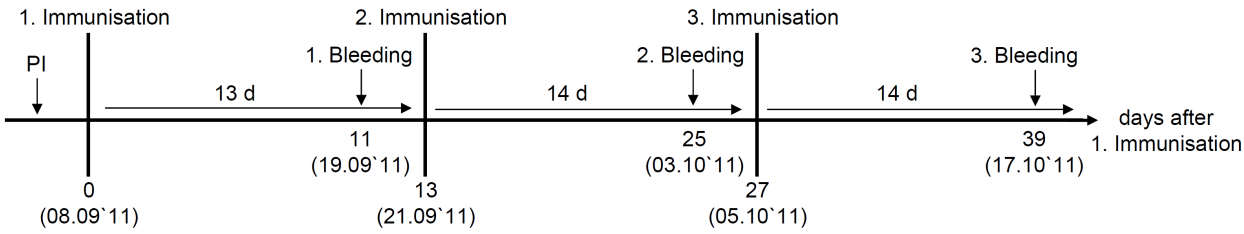

Figure 15: Immunisation schedule for Bet-E1-loop-E2 and MBP-E1-loop-E2 experiments

2.4.4 Serum preparation

Serum from immunised animals was obtained by incubating blood samples after bleeding for 2 h at room temperature and inducing coagulation by gently stirring with a glass bar before further storage overnight at 4°C. The interaction between thrombocytes and the negatively charged surface of the glass bar activates factor XII and XI which induces the coagulation cascade [91]. Clotted blood was centrifuged for 10 min at 2000 rpm and 4°C. Resulting supernatant was transferred in new tubes followed by a second centrifugation step (10000 rpm, 4 min, 4°C). Supernatants were then again transferred in new tubes and heated to 56°C for 30 min in a water bath. After decomplementation, sera were centrifuged a third time and supernatant stored in fresh tubes at -80°C until use.

2.4.5 Enzyme linked immunosorbent assay (ELISA)

ELISA was used to analyse immune sera for the presence of antibodies against the administered antigens as well as HIV peptides. ELISA plates were coated by diluting the antigen in water to a final concentration of 4 ng/µl and then applying 50 µl (200 ng/µl) of this solution to each well. Plates were dried overnight at 37°C. To prevent unspecific binding of serum antibodies the plates were blocked with 5% [w/v] BSA diluted in PBS-Tween (PBS / 0.05% [v/v] Tween 20) for 1 h at 37°C. For each serum a tenfold dilution series in 1% BSA with PBS-T was prepared in the range of 10^2 to 10^8. Plates were washed three times with PBS-T to remove the blocking solution and subsequently incubated with the diluted serum samples for 1 h at 37°C. Plates were washed again three times followed by incubation with a secondary anti-rat IgG - HRP antibody diluted 1:3000 in 1% BSA with PBS-T for 30 min at 37°C. Excessive antibodies were removed by final six washing steps with PBS-T. To develop the ELISA a colorimetric proceeding was used. Therefore, an OPD-tablet (o-phenylendiamine dichloride, 30 mg) was dissolved in 30 ml PBS with 200 µl H_2O_2 and 40 µl of this solution was applied to each well. After an incubation time of 10 min, the reaction was stopped by adding of 40 µl 5N H_2SO_4 and the absorbance of each well measured in an ELISA reader at an OD of 492 nm.

2.4.5.1. Calculation of antibody endpoint titer

For calculation of antibody endpoint titers an ELISA cut off was defined for each animal using the mean absorbance of the pre-immune serum plus three standard deviations. After calculation of this threshold the absorbance measured for immune sera at every dilution step in a tenfold dilution series was plotted against the dilution step and a logarithmic regression curve fitted to that data with the help of Microsoft Excel (Figure 10, black line). The obtained regression function (Figure 16, red circle) was solved by insertion of the calculated cut off as x-value (Figure 16, inset). The solution of this equation (Figure 10, inset and orange circle) represents the highest serum dilution that gives a signal about noise and was defined as endpoint titer.

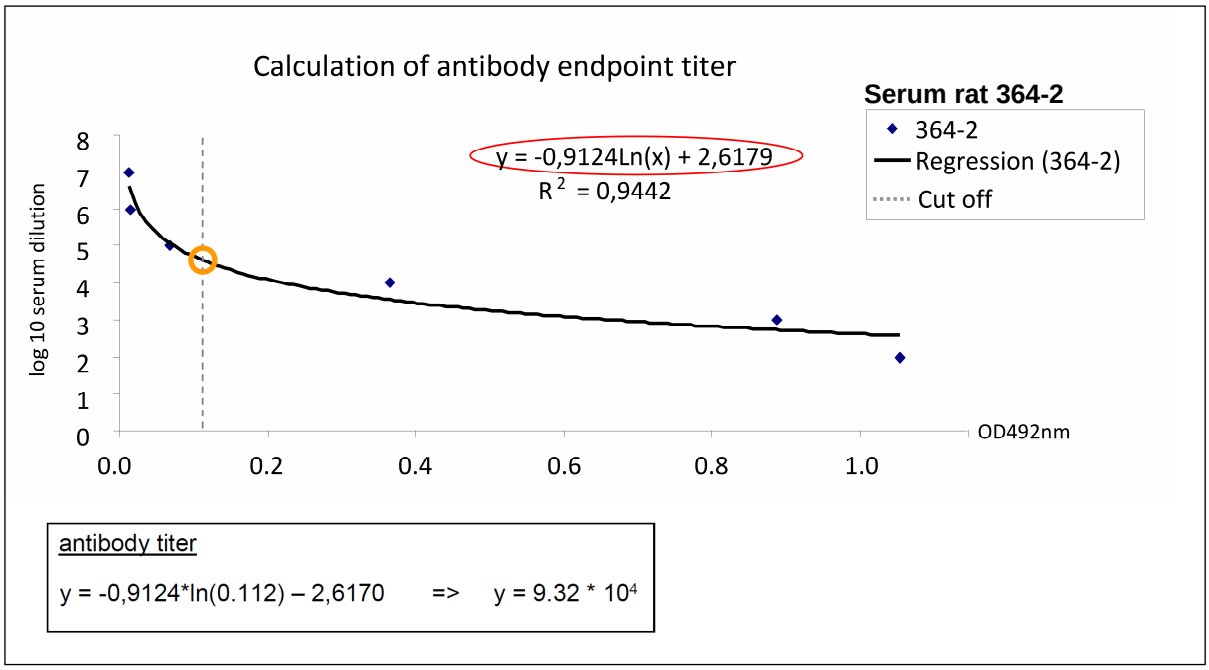

Figure 16: Exemplary diagram for endpoint titer determination.

2.5. Cell culture methods

2.5.1 General cell culture conditions

All cell lines were cultivated in DMEM medium in a 37°C incubator supplying 5% CO_2 atmosphere. Prior use media was supplemented with 10% [v/v] FCS, 2mM L-Glutamine, 10mM HEPES and 5 mM Penicillin/Streptomycin.

2.5.2 Freezing and thawing of cells

Cells were stored for long term in liquid nitrogen using freezing medium which contains 90% FCS and 10% DMSO. To avoid the toxic side effect of DMSO thawing of cells was performed as fast as possible. Therefore, the cryotube was placed in a 37°C water bath until a little piece of ice was still visible. Cell suspension was transferred to and gently mixed in pre-warmed cell culture media to dilute DMSO to a non toxic concentration and placed into cell culture flasks. After cells were adhered to the bottom of the culture vessel, culture media was replaced to remove residual DMSO.

2.5.3 Passaging of adherent cell lines

To split cells, confluence was analysed microscopically and DMEM of 80-90% confluent cells was removed and cells were washed once in 15 ml PBS. After aspirating PBS, 1 ml of a Trypsin/EDTA solution was added to the cells and incubated at 37°C until most of the cells were completely detached. Trypsin digestion was stopped with fresh DMEM and after resuspending, cells were split in a ratio of 1:10 into new media.

2.5.4 Transfection of cells

For delivery of plasmids into HEK-293T cells for new virus preparation, cells were transfected with polyethylenimine (PEI). This method based on a condensation of negatively charged plasmid DNA into positively charged particles of PEI that form spontaneously under physiological conditions. These particles are able to interact with the surface of cells followed by endocytosis of the particles [95]. For transfection 1 x 10^5 cells were seeded in 3 ml into each well of a 6 well cell culture plate and incubated overnight at 37°C. For every well 3 µg plamid DNA and 150 mM NaCl were mixed with 300 µl fresh medium (1:10 of cell culture volume). PEI solution was pre-warmed at 60°C until the cloudy solution became clear and then 3 µl (1 µl per µg plasmid DNA) added to the DNA/NaCl mixture. After incubation for 15 min at room temperature, 300 µl of the mixture were pipetted to cells in the 6 well plate.

2.5.5 Preparation of viral supernatants

To produce viral particles HEK293T cells were transfected with an infectious molecular clone of HIV-1 pNL4.3 as described above. Two days after transfection virus containing medium was collected and centrifuged for 10 min at 1500 rpm to pellet cells. Resulting supernatants were then additionally filtered through a 0.22 µm filter to remove any residual cell debris. Virus containing supernatant was aliquoted and snap-frozen in liquid nitrogen prior to storage at -80°C.

2.5.6 Determination of virus titer with TZM-bl cells

The TZM-bl cells are engineered indicator cells that permit easy determination of virus infection by X-Gal staining. They contain an integrated reporter gene for ß-galactosidase under the control of the HIV-1 LTR. Infection of cells by HIV-1 results in the expression of the tat transactivator protein which in turns activates reporter gene expression (ATCC, Catalog Number: CRL-11286). To determine virus titers, $1x10^4$ of TZM-bl cells were resuspended with DMEM and seeded into a 96 well cell culture plate one day before starting the assay. Virus was titrated by serial dilution of supernatants in cell culture medium and transfer of 50 µl of each dilution on 80-90% confluent TZM-bl cells. After incubation for two days at 37°C cells were analysed by X-Gal staining for the presence of integrated virus. Therefore supernatants were aspirated and cells were washed gently with 150 µl PBS to avoid detaching of the cells. Fixation was performed with 45 µl/well fixation solution and incubation for 10 minutes at room temperature. Cells were washed again with 200 µl PBS and subsequently stained with 45 µl/well of an X-Gal containing staining solution for 1-2 hours at 37°C. Positive cells which show distinct blue nuclear staining when infected were counted with an ELISPOT device and viral titers calculated with the equation given below.

$$\text{Viral titer [IU]/ml} = \frac{\text{infected cells} \times \text{dilution}}{\text{volume used for infection}} \times 1000$$

2.5.7 Neutralisation-Assay (NT-Assay)

TZM-bl indicator cells were seeded at a density of 1 x 10^4 cells per well in a 96 well cell culture plate. On the next day rat sera (2.4.4) were diluted with culture media 1:20 in a separate 96 well plate and 50 µl of the serum dilution pipetted into the wells of the dilution plate to yield triplicates. Then 200 infectious units of titrated virus supernatant in a volume of 50 µl were added to each serum dilution. Sera were incubated with the virus for 30 min at 37°C and then the whole serum-virus mixture was added to the cells. As controls, cells that were incubated with virus and the monoclonal antibody 2F5 in dilutions as indicated, non-infected cells as well as an infection control which was incubated only with virus was run in parallel. Whether cells were infected or sera neutralised was analysed 48 h after serum-virus treatment by washing, fixing and staining the cells as described above (2.5.6). Identically, spots were counted with help of the ELISPOT reader. Neutralisation was defined as the reduction of viral infection by more than 50% when sera from immunised animals and their respective pre-immune serum were compared.

3. Results

3.1. Expression and purification of FFV Bet

3.1.1 Generation of the FFV-Bet expression plasmid

In order to produce recombinant FFV Bet/HIV-1 hybrid proteins and to examine their immunogenicity and in particular to characterise the antibody response generated against the introduced HIV-1 fusion parts, the prokaryotic expression plasmid pQE-Bet was constructed that built the molecular basis for the subsequent cloning of Bet/HIV-1 fusion constructs connected by a 15 aa spacer but also for the establishment for suitable expression and purification protocols. The parental prokaryotic expression vector pQE-30-Xa (Figure 17) was lineraised with the restriction enzymes *Stu*I and *Bam*HI. The full length FFV-Bet sequence was amplified from the eukaryotic expression plasmid pBC12-Bet by proof-reading PCR with primer FFV Bet_fwd containing a 5' blunt end and primer FFV Bet_rev introducing a four amino acid spacer followed of the restriction site *Bgl*II which is compatible with *Bam*HI overhangs. Both digestion mixtures were separated by agarose gel electrophoresis for gel extraction and subsequent cloning. The purifed vector DNA had a size of 3493 bp and the FFV-Bet insert 1179 bp after restriction digestion (Figure 17B).

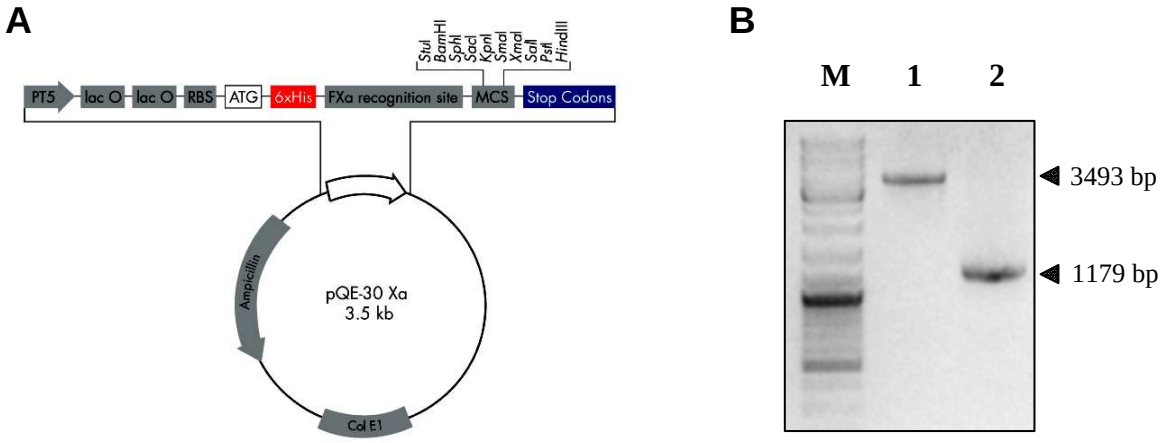

Figure 17: Parental expression vector pQE-30-Xa and purification of vector and inserts for cloning.
(A) The parental expression vector pQE-30-Xa encoded main features. PT5 - T5 promotor, lac O – lac operon, RBS – ribosome binding site, ATG – start codon, 6xHis, N-terminal His-tag, FXa, factor Xa protease cleavage site, MCS – multiple cloning site with unique restriction sites. **(B)** Purification of *Stu*I/*Bam*HI digested pQE-30 Xa vector (lane 1) and *Bgl*II digested FFV Bet PCR insert (lane 2), M-marker. The sizes of both fragments are indicated.

Both fragments were ligated using T4 ligase and the ligation mixture transformed into chemical competent *E.coli*. Grown colonies were picked and analysed by colony PCR with the primers 005_fwd and 006_rev that recognized two sequences flanking the pQE vector multiple cloning site and therefore can be used to screen for the successful insertion of the FFV-Bet sequence. About 92% of the picked clones contained the FFV Bet insert and thus resulted in the amplification of a 1149 bp insert (Figure 18).

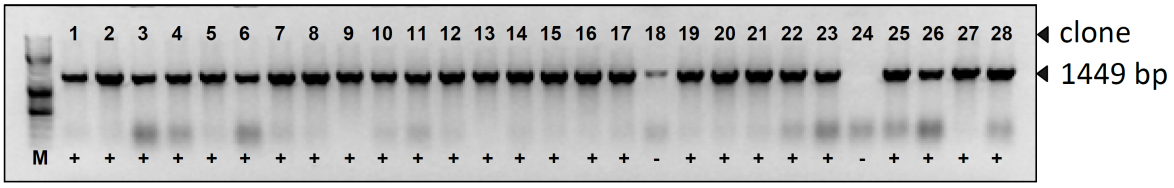

Figure 18: Colony PCR for screening of positive clones
15 µl of Colony PCR mixtures were analysed by agarose gel electrophoresis. Clones that contained the full length FFV-Bet insert gave a signal at 1449 bp as indicated.

Plasmid DNA of those clones that gave a positive signal in colony PCR were isolated and sequenced with pQE and FFV-Bet specific primers (005_fwd, 006_rev, FFV BetSeq1_fwd) to ensure insert integrity. By aligment of obtained sequencing results with the published reference sequence (Uniprot Acc. Nr O93036) clone pQE-Bet 10 was found to be completely identical and in frame with the encoded N-terminal 6xHis-tag and the factor Xa cleavage site (not shown). The plasmid map of the generated pQE-Bet expression plasmid shows the genes and restriction sites, a schematic presentation of the resulting FFV-Bet protein (45.6 kDa) containing the His tag and the factor Xa cleavage site (Figure 19).

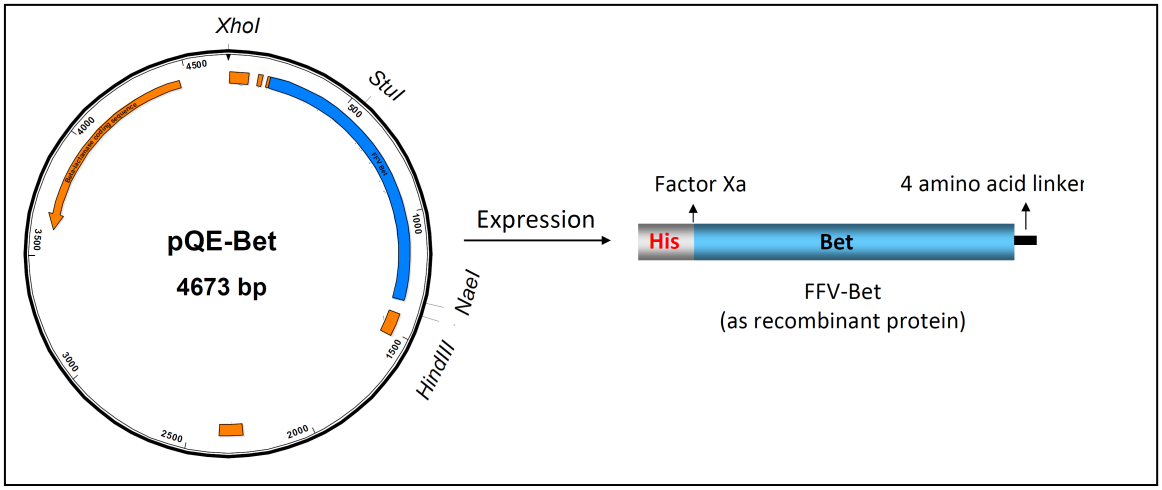

Figure 19: Plasmid map of pQE-Bet vector and resulting recombinant FFV-Bet.
The pQE-Bet vector and recombinant FFV-Bet protein after expression which is tagged with His residues on its N-terminus and a four amino acid linker on the C-terminus for subsequent connection with HIV-1 sequences.

3.1.2 Screening for best expression conditions for pQE-Bet

Protein expression and solubility is strongly influenced by the genetic background of the bacteria used and the culture environment under which protein production takes place. To quickly establish parameters for high level overexpression, the expression plasmid pQE-Bet was transformed into the four specialised *E.coli* expression strains SCS-1, T7-Shuffle, C43 and Rosetta 2 to examine which bacteria strain expresses the recombinant Bet protein best. Furthermore four culture media, different temperatures, and inducer concentrations were tested in parallel to determine their influence on bacteria behaviour (Table 16).

Table 16: Tested conditions to determine best protein expression parameters

Bacteria strains	Temperature	IPTG concentration	Media
SCS-1, T7-Shuffle,	20°C	0.1 mM	LB, 2YT,
C43, Rosetta 2	30°C	0.5 mM	TB, SOC

Induced cells grown under the various conditions were lysed with lysis buffer (PBS, 0.1 mg/ml lysozyme, benzonase) and further disrupted by sonification. The soluble fraction was separated from total lysates by centrifugation and both fractions were analysed by SDS-PAGE and Western blot analysis (Figure 20).

Best protein expression was achieved in the bacteria strain SCS-1 followed by a low-level expression in T7-Shuffle with highest amounts of protein produced in enriched media like 2YT, TB and SOC (black circles in Figure 20). In *E.coli* strains C43 and Rosetta 2 there was no obvious expression detectable. Neither IPTG concentration nor growing temperature seemed to influence amounts of produced Bet protein in total lysates remarkably (Figure 20).

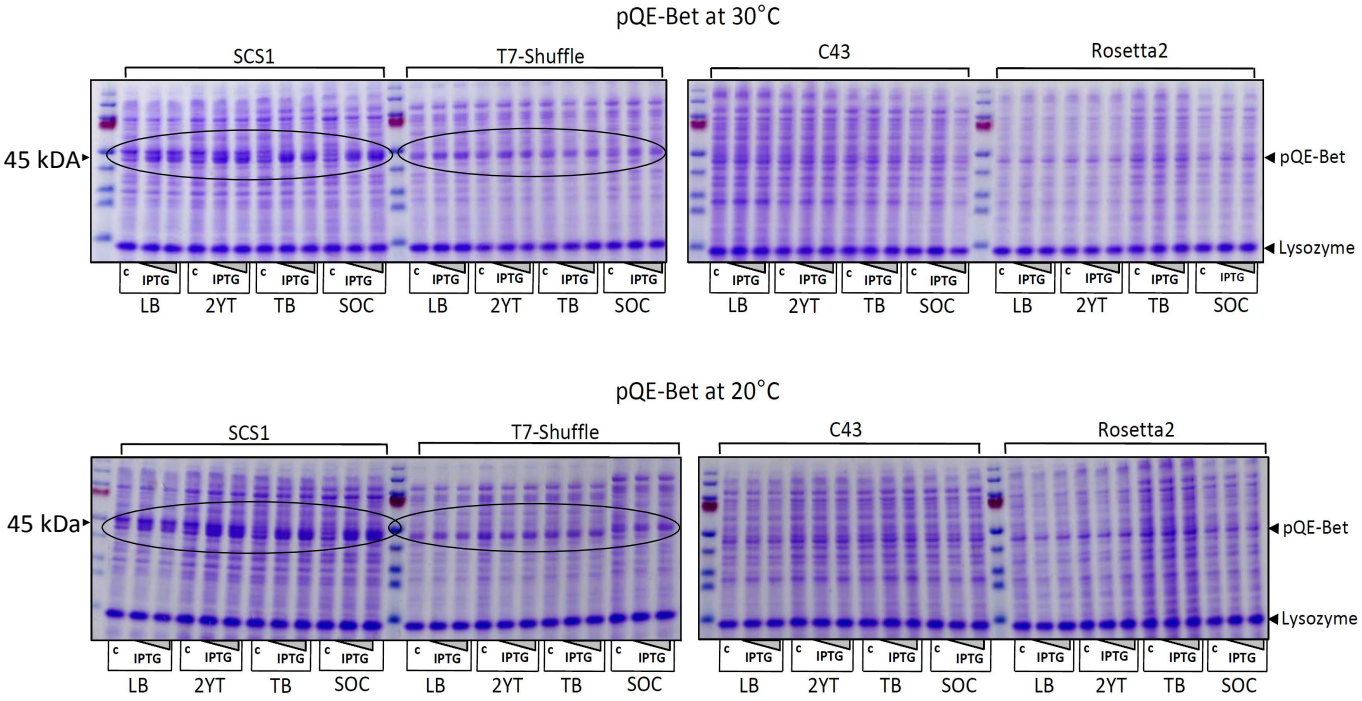

Figure 20: Bet protein expression screening in total lysates
100 µl of an overnight culture of SCS1, ShuffleT7, C43 and Rosetta 2 containing the expression plasmid pQE-Bet were added to 1 ml LB, 2YT, TB or SOC medium and grown for 3h at 37°C. Protein expression was induced by addition of no (c, control) 0.1 or 0.5 mM IPTG (grey bars) and further growing for another 3h at two different temperatures (20°C and 30°C). Afterwards cells were collected by centrifugation and the medium was discarded. Pellets were resuspended in 200 µl lysis buffer (PBS, 0.1 mg/ml lysozyme, benzonase) and further disrupted by sonification. 20 µl of total lysates were analysed by SDS-PAGE and Coomassie staining.

For the functionality of proteins it is necessary to produce them in a soluble and possibly native form. Therefore, the soluble protein fractions of the performed expression screen were analysed for the presence of FFV-Bet. In comparison to the results with total lysates, it was not possible to detect the FFV-Bet protein in the soluble fraction by Coomassie blue staining (Figure 21). However, expression was detected analysing the soluble Bet fractions by Western blot Figure 21). Best expression was achieved in the SCS-1 and the T7-Shuffle host at a growth temperature of 20°C in comparison to C43 which exhibited a rather low level of Bet expression at the same temperature (Figure 21). In Rosetta 2, no apparent expression at 20°C as well as for 30°C was measurable underlining the importance for selection of a suitable bacterial host for successful expression. Notably, in all four bacteria strains an increased expression of soluble FFV-Bet at lower temperature in contrast to the higher growth temperature was detectable.

Media that aided soluble Bet expression depended mostly on the individual bacteria strain. SCS-1 produced increased amounts of soluble Bet in TB and SOC media while for T7-Shuffle 2YT was the most efficient medium. Identically to total lysates, the use of different IPTG concentrations had no influence on soluble protein production. In contrast, rather a quite high level of basal promoter activity ('leakyness') was found that could only be eliminated in presence of glucose (see lanes of SOC medium in Figure 21). As a result subsequent expression experiments were performed with glucose supplied media.

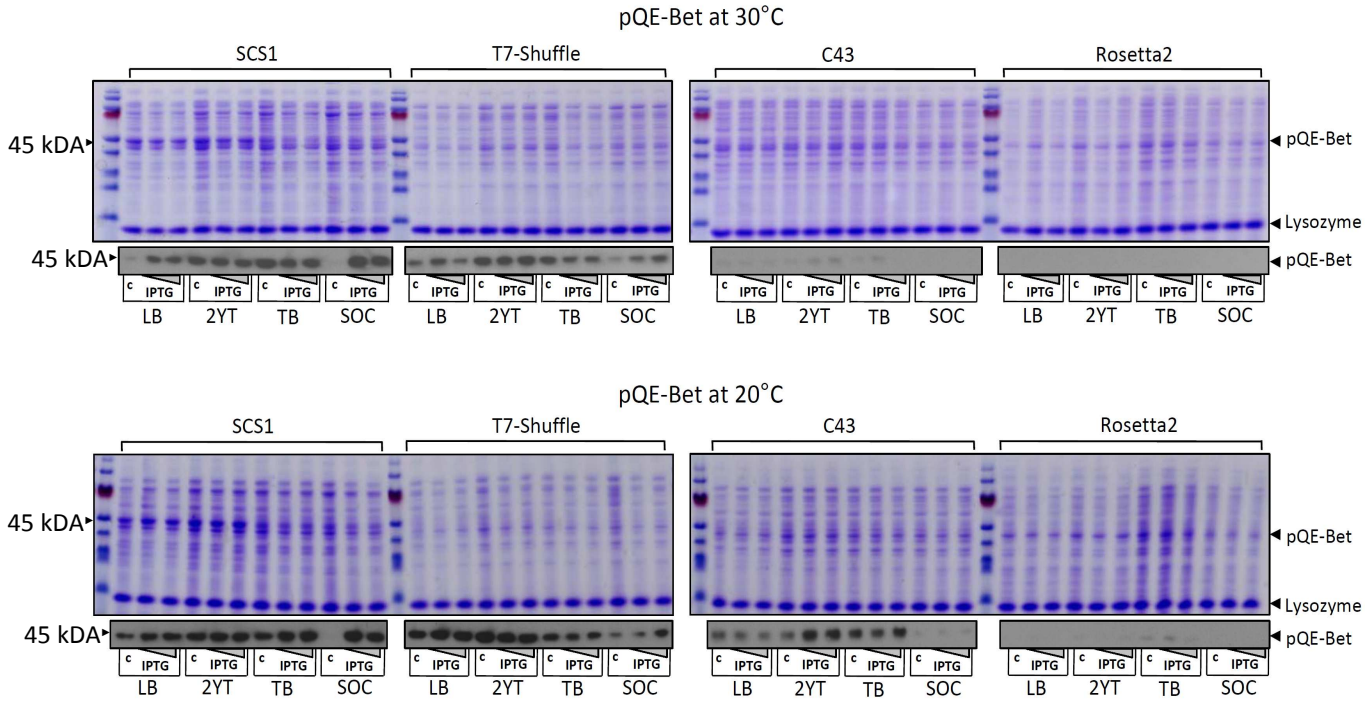

Figure 21: Analysis of expressed FFV Bet in the soluble fraction
After protein expression as described in Figure 20, remaining total lysate were centrifuged (16.000 g, 4°C, 20 min) and 20 µl of supernatants were analysed by Coomassie blue staining and 6 µl were examined by Western blot using Penta-His as primary antibody (1:5000) and anti-mouse as secondary antibody (1:5000).

Taken together, this expression screening revealed suitable expression conditions for high level expression of FFV Bet but that the majority of this protein was expressed in an insoluble form and only minor amounts of soluble Bet protein were produced. Since the presence of soluble protein was only detectable by Western blot yields from a non-denaturing purification were expected to be very low. To increase the amount of soluble Bet protein, further optimisation was performed with the best Bet expression strains SCS-1 and T7-Shuffle under conditions found as a result of this expression screen.

3.1.3 Time course analysis of Bet expression

The overall expression of a recombinant protein in a prokaryotic host is a balance between soluble and insoluble forms and strongly depends on the protein that is expressed. Factors like temperature, time point of induction and the degree of protein induction regulated by IPTG strongly influences this balance. Some proteins are only expressed in its soluble form until a certain time point and afterwards stored as inclusion bodies. Higher IPTG concentrations and temperatures result in fast production of the target protein but correlate with inclusion body formation [93]. Also, it is possible that an expressed protein has a toxic effect on bacteria leading to its fast proteasomal degradation or even death of cells. Systematic analysis of these parameters is thus important to obtain recombinant proteins with higher yields and quality. To investigate if the FFV Bet protein is toxic and to find an optimal time point for harvesting maximal amounts of soluble FFV Bet, a time course experiment was performed. Therefore, transformed Shuffle T7 in 2YT medium and SCS1 bacteria in TB medium were induced with 0.1 and 0.5 mM IPTG at a mild temperature of 20°C and optical density as well as FFV Bet protein expression in the soluble and insoluble fraction monitored by SDS-PAGE and Western blot. Growth curves of both expression strains showed that the expressed Bet protein did not influence SCS-1 and T7-Shuffle health (Figure 22). SCS-1 exhibited a better growth rate in comparison to T7-Shuffle, however, both strains were growing with similar rates and increased steadily.

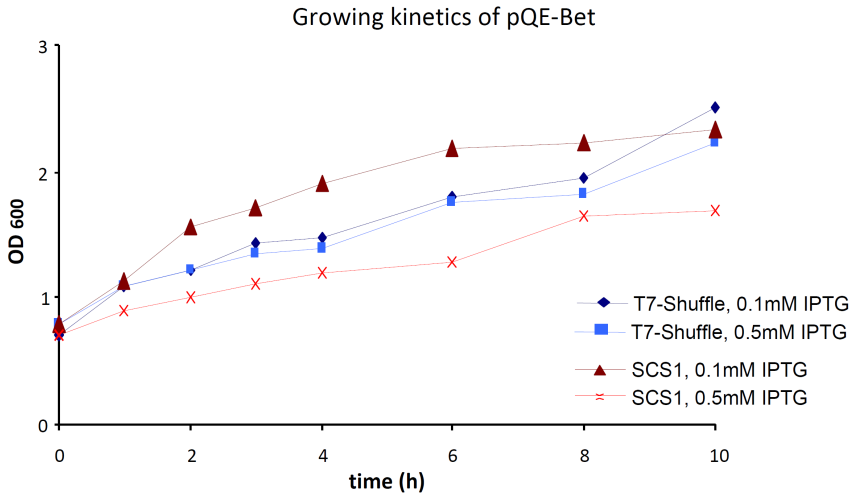

Figure 22 Growth kinetics of pQE-Bet transformed in SCS-1 and T7-Shuffle
SCS-1 and T7-Shuffle bacteria cultures were induced with low and high IPTG concentrations as indicated and OD_{600} monitored for the given intervals.

Protein induction with lower IPTG concentrations (0.1 mM IPTG) had a positive influence on bacteria growth and this effect was much more pronounced in the case of SCS-1 compared to T7-Shuffle (Figure 22). However since bacteria growth does not automatically correlate with the expression of soluble proteins, samples from every time point along with non-induced control were lysed and soluble and insoluble fractions analysed. As in the previous experiment, Bet protein was easily detected in the insoluble fractions by Coomassie staining as early as 1 hour after induction but not in the soluble compartment (Figure 23). Western blot analysis revealed that a weak expression of soluble Bet peaked at the first two hours after induction with 0.1 mM as well as 0.5 mM IPTG but at later time points Bet was stored in inclusion bodies. As a result of this experiment, subsequent large scale expressions for purification under non-denaturing conditions were performed in the SCS-1 bacterial strain induced with 0.5 mM IPTG and grown for 2 h at 20°C.

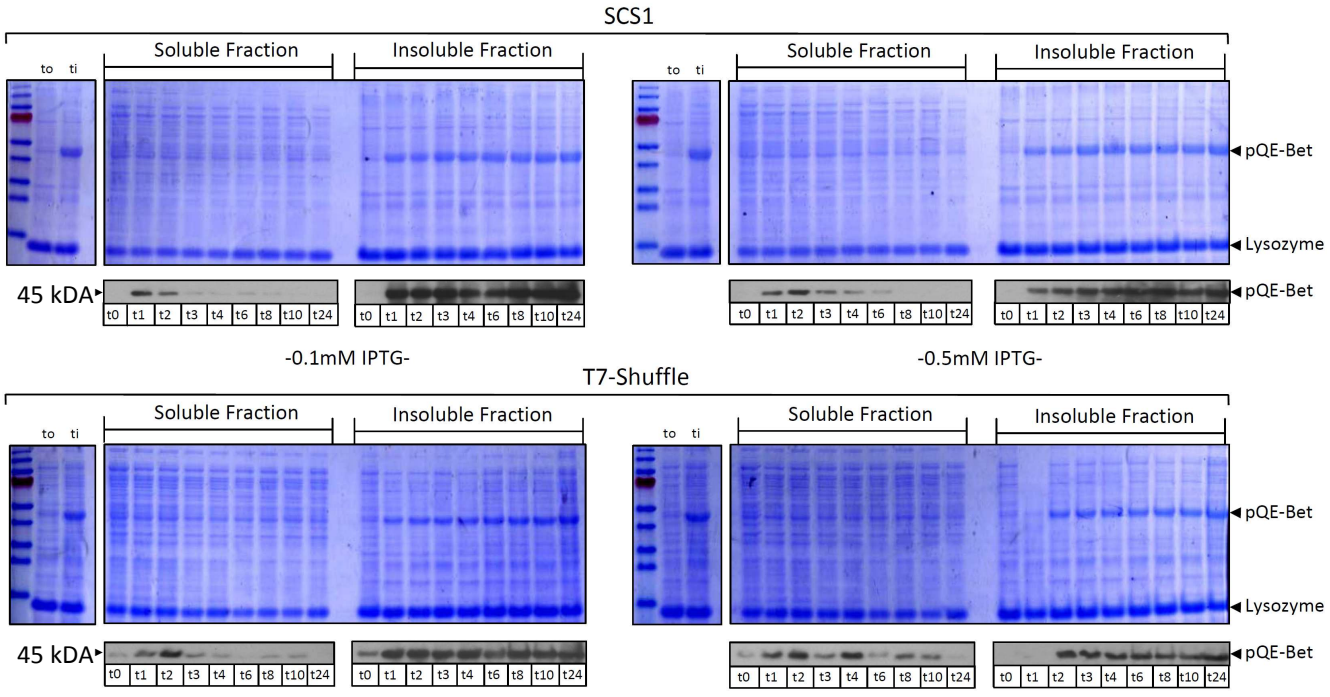

Figure 23: Soluble and insoluble Bet expression in SCS-1 and T7-Shuffle over 24 h
Overnight cultures of SCS-1 and T7-Shuffle were inoculated into 200 ml fresh media TB or 2YT medium to yield an OD_{600} of ~0.1 and grown at 37°C until OD_{600} increased to 0.6. Non-induced samples were taken (t_0) and protein expression was induced with 0.1 mM and 0.5 mM IPTG. Bacteria were than incubated for further 24 h at 20°C and samples collected at the indicated time points. After 24 h samples were lysed and soluble fractions separated from insoluble material. Every protein fraction was analysed by Coomassie blue staining (15 µl of sample) and Western blot (6 µl of sample) with Penta-His primary antibody (1:5000) and anti-mouse secondary antibody (1:5000).

3.1.4 Testing solubility of pQE-Bet with non-ionic detergents

Due to their amphipathic nature, non-ionic detergents are able to solubilise proteins aggregates which are a result of hydrophobic interactions and therefore precipitate into the insoluble fraction. Importantly, non-ionic detergents are thereby mild solubiliser and thus allow maintaining the soluble structure of the protein [96]. In the following experiment, the ability to solubilise insoluble expressed Bet protein by several non-ionic detergents was tested. Therefore, induced cells were lysed under non-denaturing conditions and two concentrations of the indicated detergent at 1x and 5x of the detergent specific critical micelle concentration (CMC) were added to the mixture. After mixing and short incubation soluble and insoluble fractions were separated by centrifugation and analysed by Coomassie blue staining and Western blot (Figure 24). In all cases an intensive band of Bet protein was detected in the insoluble fraction but not in the soluble supernatant by Coomassie blue staining (Figure 24). To determine if weak amounts of soluble Bet were contained in the soluble protein fraction a Western blot was performed. However, Western blot analysis confirmed that none of the selected non-ionic detergents could efficiently solubilise the Bet protein into the supernatant.

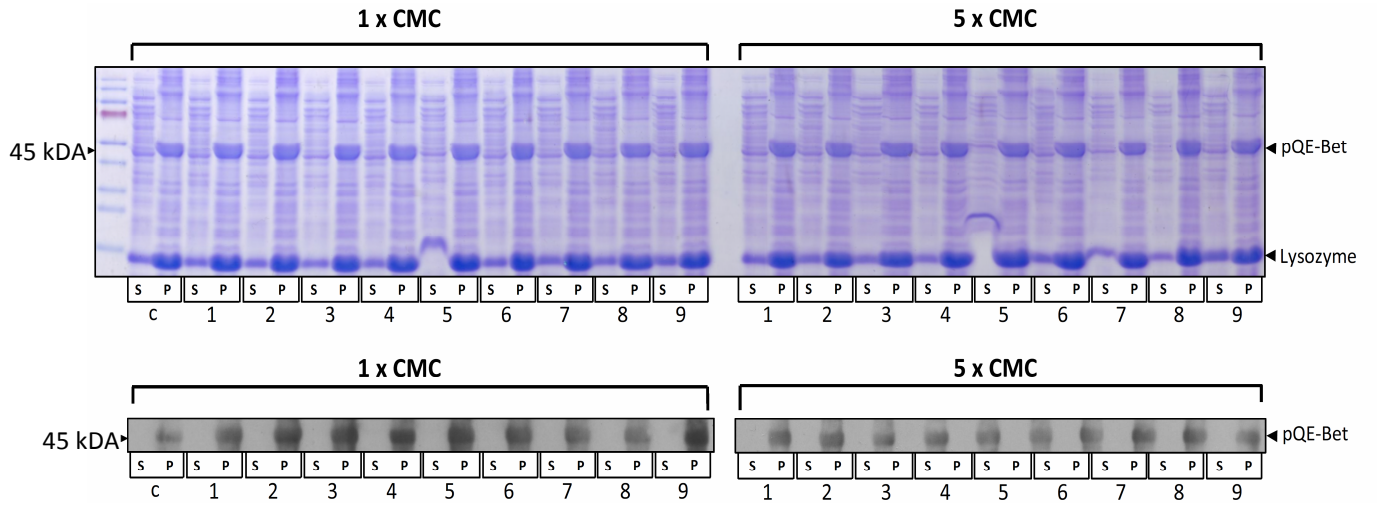

Figure 24: Detergent screen with non-ionic detergents to solubilise FFV-Bet
Several non-ionic detergents were added in a concentration of 1 x CMC and 5 x CMC to lysis buffer during bacterial lysis to determine their solubilisation potential. Supernatant (S) and pellet (P) were separated and 18 µl of each sample were analysed by Coomassie blue staining and 6 µl by Western blot using Penta-His as primary antibody (1:5000) and anti-mouse as secondary antibody (1:5000). c=control without detergents, 1=NP40, 2=Triton X 100, 3=Tween 20, 4=beta OG, 5=DM, 6=CHAPS, 7=Brij 35, 8=Saponin, 9= Tween 80.

3.1.5 Subcloning of FFV-Bet into pMal vector for an increased solubility

The cytoplasmic expression of insoluble proteins can often be increased when the insoluble target protein is fused to a highly soluble fusion partner. Maltose binding protein, NusA, Glutathione-S-transferase, the Calmodulin binding protein, thioredoxin and others are used for that purpose. From all of these commonly used tags, the MBP protein has been found to be particularly effective in pulling fusion proteins into solution by simultaneously allowing affinity purification [97]. To test this approach for FFV Bet, an expression construct was generated that fuses the Bet sequence to a signal peptide containing MBP protein that promotes expression in the periplasm of *E. coli* and therefore also formation of disulfide bridges. Additionally, a His-tag was fused to the Bet protein by PCR to allow purification of the protein after cleavage from the large MPB fusion partner. The FFV Bet sequence was amplified from the pBC12-Bet vector and inserted into the *Xmn*I/*Bam*HI digested pMal-p5x vector multiple cloning site (Figure 25A and B). Positive clones were identified by colony PCR resulting a PCR insert of 787 bp (Figure 25C) and after sequencing this construct was subjected to an expression optimisation screening as described for the pQE-Bet construct (see 3.2). Total lysates and supernatants were analysed by Coomassie blue staining and Western blot.

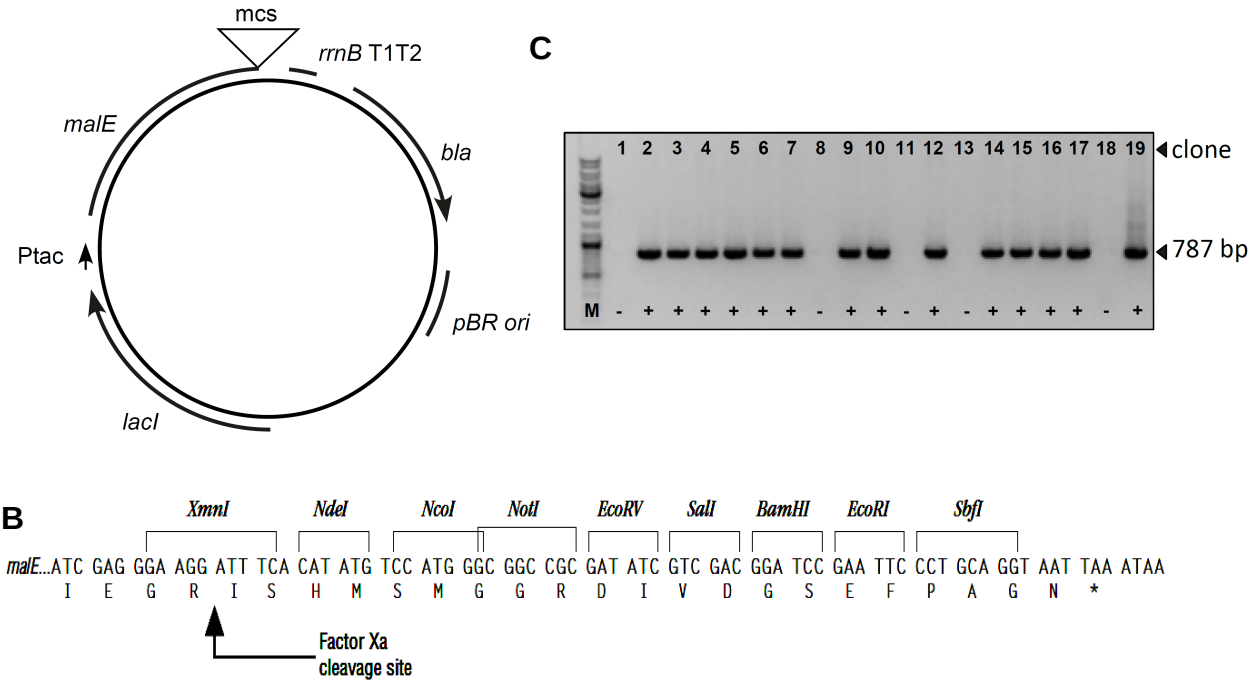

Figure 25: Cloning of the MBP-Bet expression plasmid
(A) Plasmid map and main features of the parental expression vector pMal-5px and its multiple cloning site **(B)**. The amplified His-Bet sequence was introduced into the *Xmn*I/*Bam*HI sites of the vector. **(C)** Colony PCR of transformants to identify positive clones.

Results showed that the best conditions for expression of the 90 kDa MBP Bet protein in total lysates was achieved in the *E.coli* strain Rosetta 2 growing in TB medium and to some extend also in LB, 2YT and TB media with SCS-1 (Figure 26A). Bacteria strains C43 and T7-Shuffle exhibited no obvious MBP-Bet overexpression at any condition (not shown). Analysing the soluble fraction it was not possible to detect any MBP-Bet expression at the expected size of 90 kDa neither at 20°C nor 30°C by Coomassie staining (Figure 26, B). Western blots performed with a Penta-His primary antibody detected the His tagged Bet protein as a double band migrating at 43 kDa, however the full length protein with 90 kDa was not recognised. These results suggested that although quite well expressed, fusing Bet to the potent MBP protein had no benefit for protein solubility and that the translated fusion product is obviously highly instable.

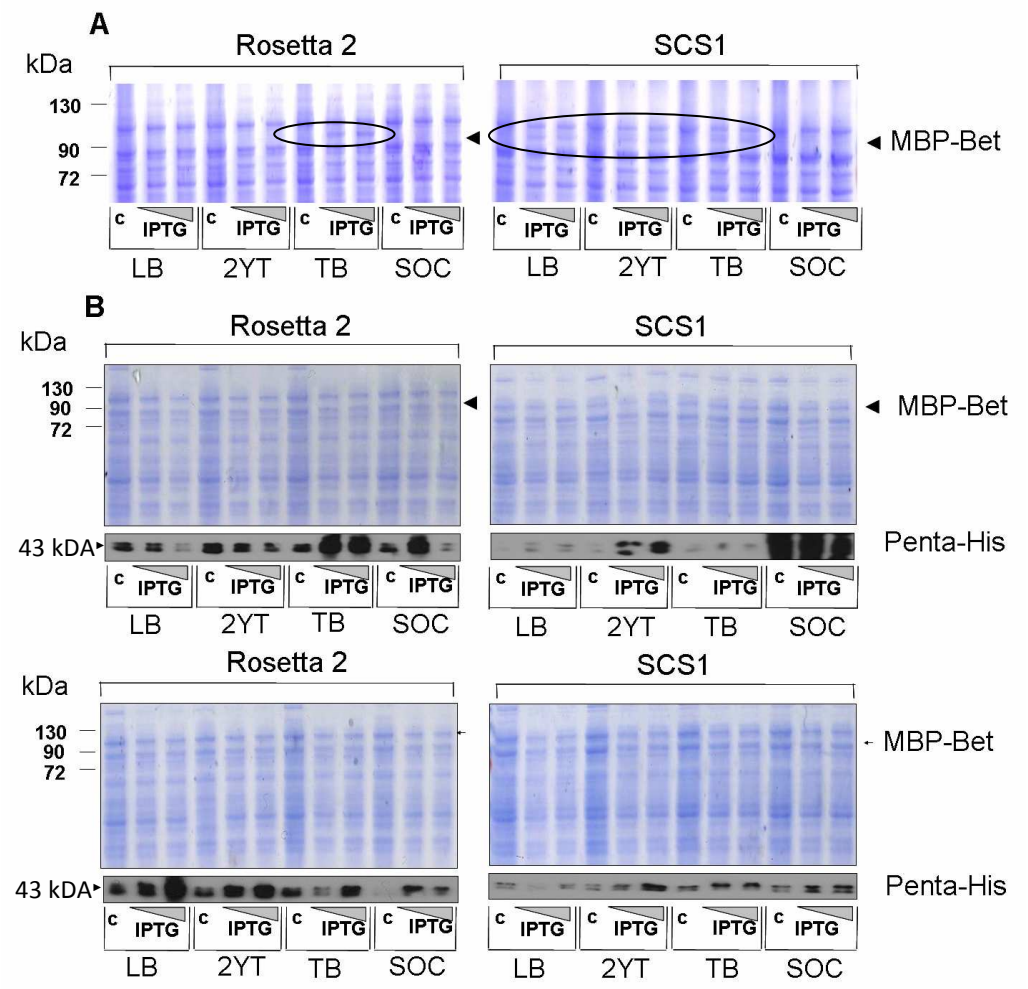

Figure 26: Screening of MBP-Bet expression
(A) Overall expression of the 90 kDa MBP-Bet at 20°C in total lysates of producing bacteria strains Rosetta 2 and SCS-1. **(B)** Expression of MBP-Bet in the soluble fraction of the same bacteria as in A, either at 20°C or 30°C (upper and lower panel). Western blot using Penta-His antibody (1:5000) detected expression as a double band at 43 kDa but not at the expected size of the full length protein at 90 kDa (not shown).

3.1.6 Purification of FFV Bet under non-denaturing conditions

Since attempts to increase the yield of soluble FFV-Bet protein by any of the above mentioned methods were not successful, it was sought to estimate the amount of soluble protein that can be recovered when optimised expression parameters for soluble expression (see 3.1.3) are applied. Therefore, a 500 ml pellet of induced cells was lysed under non-denaturing conditions and supernatants were taken for FPLC-assisted purification by Ni-NTA affinity chromatography. After loading the soluble cell lysate onto the column unbound material was washed away and Bet protein eluted with an imidazol gradient. Elution fractions were collected in a 96 deep well plate and concentrations were calculated by peak integration with the help of the Äkta Explorer Unicorn Software. With this approach approximately 2 mg protein were eluted from the column (Figure 27A). Elution fractions were analysed by Coomassie blue staining and Western blot (Figure 27B). Both methods showed that only minimal amounts of Bet protein were eluted under these conditions and that several unrelated proteins with a higher molecular weight than for Bet were co-purified with this approach (Figure 27B). Additionally, Western blot analysis demonstrated a highly degraded Bet protein (Figure 27B) in each elution fraction. In consequence, attempts to purify Bet under non-denaturing conditions were stopped here and further experiments focused on the establishment of a denaturing purification strategy with subsequent refolding.

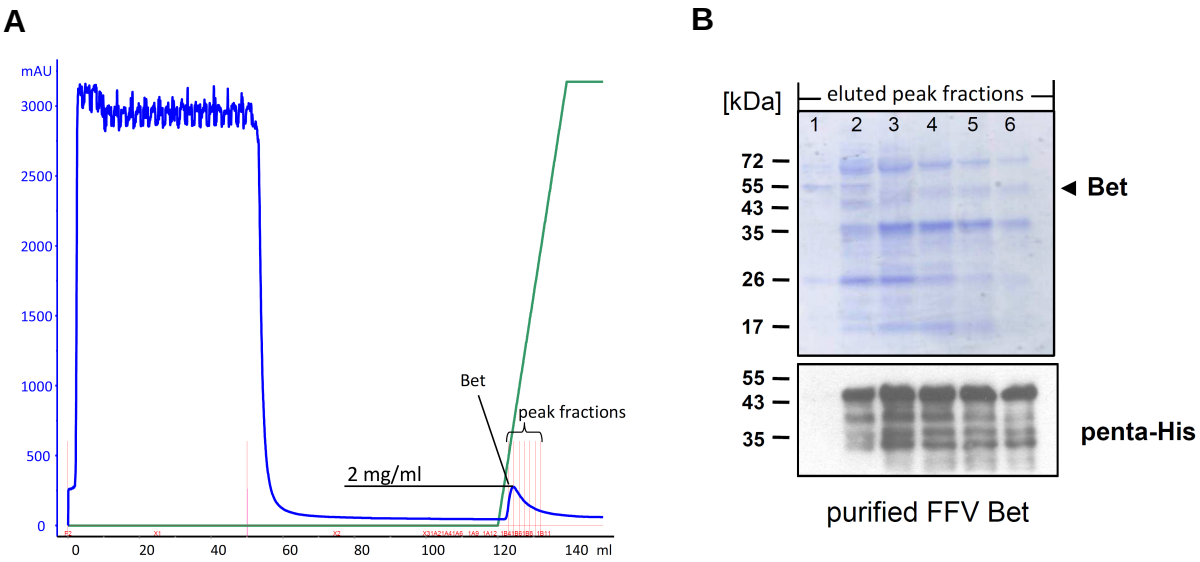

Figure 27: Purification of FFV-Bet protein under non-denaruring conditions.
(A) A 500 ml pellet containing expressed FFV-Bet was resuspended in lysis buffer N (PBS, 0.1 mg/ml lysozyme) and supernatant purified under non-denaturing conditions using a 5 ml Ni-NTA column installed in an Äkta Explorer system. After intensive washing, protein was eluted using an imidazol gradient (10-500 mM). Yields were calculated by peak area (2 mg). **(B)** Coomassie blue staining and Penta-His Western blot analysis (1:5000) of elution fractions. The expected size of FFV Bet and molecular weights are indicated.

3.1.7 Purification of FFV-Bet under denaturing conditions

To assess the large amounts of FFV-Bet which were stored as inclusion bodies and to avoid the degradation side effect, purification of FFV-Bet was performed under denaturing conditions using the insoluble fraction. A 500 ml pellet of induced cells grown under conditions for maximal protein expression (see 3.1.2) were lysed under non-denaturing conditions to remove cytosolic proteins and the pelleted fraction extracted with GuHCl buffer. This suspension was centrifuged again to remove non-solubilised material and the supernatant used for purification on the Äkta FPLC System. After washing, proteins were eluted with an imidazol gradient as before but in contrast to purification under non-denaturing conditions all buffers were supplied with 8 M urea as solubilising agent. Amounts of protein were calculated with the Äkta software and collected elution samples of the peak fractions were analysed by Coomassie blue staining and Western blot (Figure 28). The Äkta diagram showed that about 7-fold higher yields of FFV-Bet can be obtained by this means in comparison to the non-denaturing purification procedure (Figure 28A). Moreover the protein was already sufficiently pure and did not degrade as demonstrated by Coomassie gel and Western blot analysis (Figure 28B).

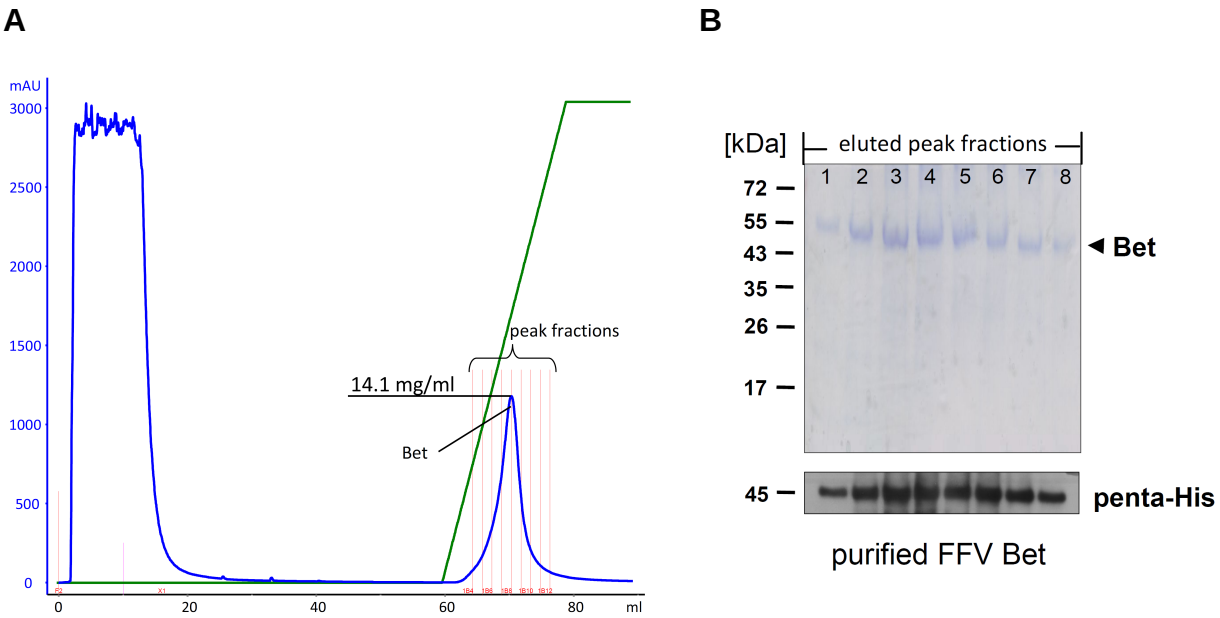

Figure 28: Purification of FFV-Bet under denaturing conditions
(A) The insoluble fraction of a 500 ml induced FFV-Bet pellet was extracted with 6 M GuHCL for 2 h. The lysate was centrifuged and supernatant which contains solubilised denatured FFV-Bet was loaded on the Äkta using a 5 ml Ni-NTA column. After washing with 8 M urea containing buffer, protein was eluted with 8 M urea buffer and an imidazol gradient from 10 mM to 500 mM. Yield of FFV-Bet was calculated by peak integration. (B) Eluted fractions were analysed by Coomassie blue staining and Western blot analysis using a primary antibody against the His tag (dilution 1:3000). Molecular weights and the expected size of FFV Bet are indicated.

3.1.8 Establishment of a refolding protocol for FFV Bet

In a next step, denaturants from the previous purification protocol had to be removed to physiological buffers to allow renaturation of the tertiary structure of Bet. Since the protein was precipitating when simply dialysed against PBS, are more comprehensive protocol was necessary. To test conditions that allow high level renaturation a 96 well refolding protocol adopted from the screening method described by Vincentelly et al., 2004, [94] was used. In this denatured FFV-Bet protein was reduced and concentrated to 2 mg/ml and then diluted 1:20 in various refolding buffers. After 24 hours, precipitation was assessed by measuring the optical density of the samples at 370 nm subtracted by the blank absorbance obtained with the individual buffer only. Results were expressed as percentage difference to the absorbance of the solublised denatured FFV Bet protein in 8 M urea. The analysis of this refolding screening experiment showed a strong precipitation of FFV-Bet at an acidic pH of 5.0 but not at higher pH values (Figure 29). From all additives tested sucrose and in particular L-arginine had the strongest influence on preventing protein aggregation and were therefore obviously suitable additives for supporting protein refolding. Salt concentrations of NaCl as well as reducing agents exhibited no significant influence on protein aggregation in this assay (Figure 29B).

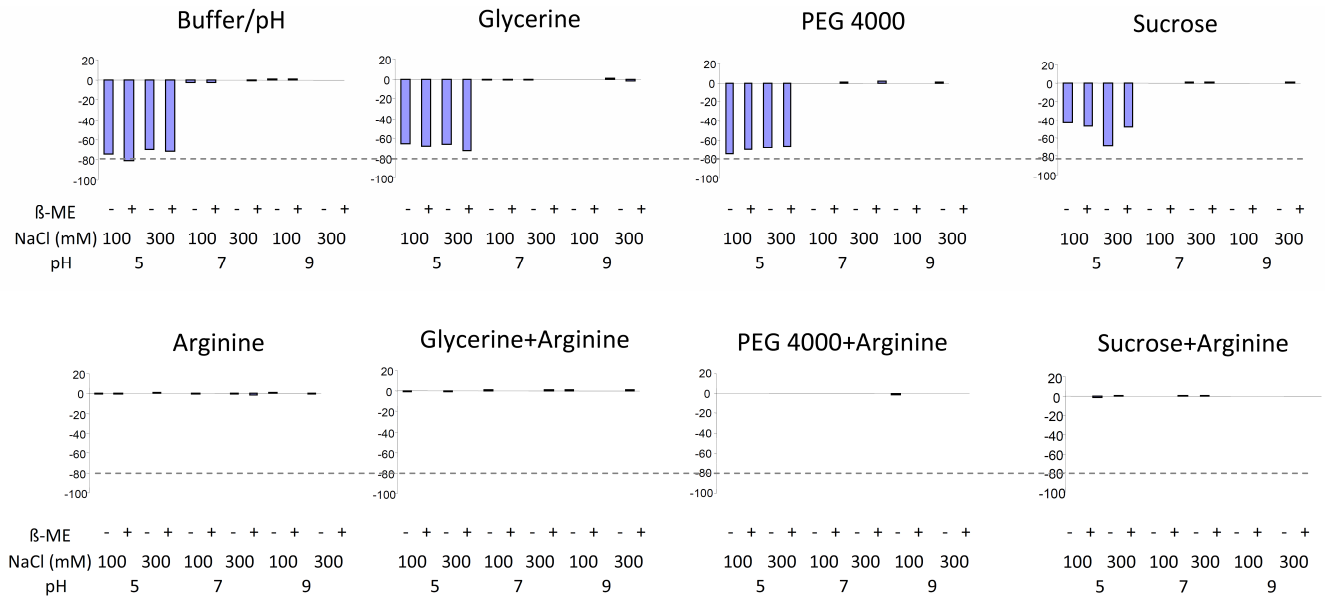

Figure 29: Microtiter plate screening to find suitable refolding conditions for FFV Bet.
Denatured FFV-Bet protein concentrated to 2 mg/ml was diluted 1:20 in different refolding buffers to examine the most efficient refolding conditions regarding pH, salt concentration, reducing agent and additives like sucrose, L-arginine, glycerine, PEG4000, and combination of those. After 24 h protein aggregation was measured by reading OD_{370} values of the samples. Results are presented as percentage differences to the absorbance of solubilised Bet protein in 8 M urea. ß-Me = 2-mercaptoethanol.

As result of this screening, a refolding buffer containing 50 mM HEPES, 300 mM NaCl, 100 mM L-arginine, pH 9.0 was selected for preparative refolding in the subsequent experiments.

3.1.9 Large scale refolding with denatured FFV Bet

For preparative refolding of FFV Bet the conditions established in microtiter plates were scaled up accordingly. For this purpose, several elution fractions of denatured Bet (~5 mg protein) were pooled, reduced and concentrated to 2 mg/ml protein followed by rapid 1:20 dilution in stirring refolding buffer and further incubation for 24 h at room temperature. In comparison to the small scale conditions, some material was precipitating during this dilution step, probably due to differences in the mixing speed and thus detergent removal kinetics in that larger volume. Due to the resulting high dilution of Bet, the protein should be concentrated again by affinity chromatography using native buffer conditions. L-arginine, which is incompatible with Ni-NTA chromatography, was therefore removed by dialysis against a 100-fold excess of phosphate buffer for 24 hours at room temperature. After removal of any precipitated material by centrifugation and filtration, the solution was loaded onto Ni-NTA columns using the dialysis buffer supplied with imidazol. Bound renatured proteins were eluted with an imidazol gradient as described for the non-denaturing purification. Elution fractions were analysed by Coomassie blue staining which confirmed the presence of highly pure renatured Bet at the expected size of 45 kDa (Figure 30).

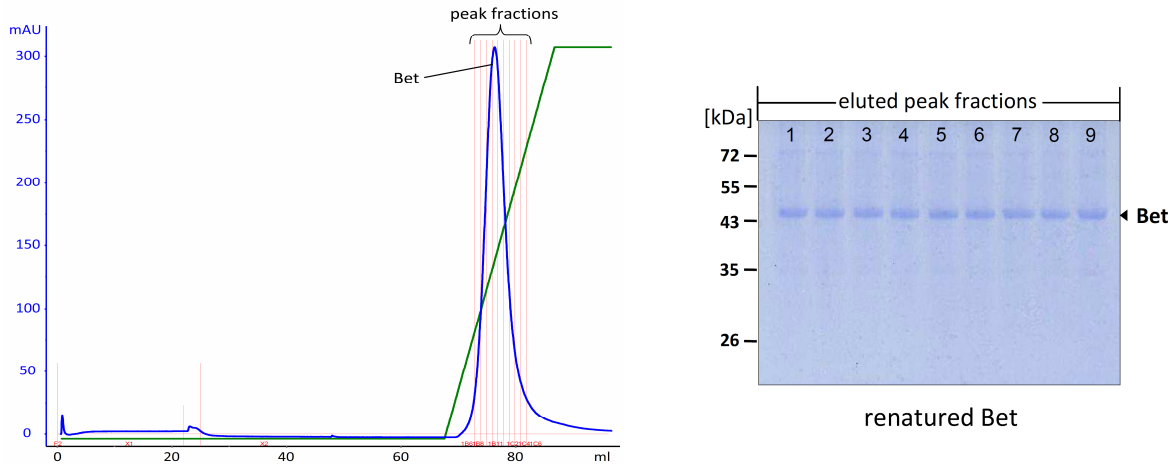

Figure 30: Concentration of the renatured FFV-Bet protein
After refolding and dialysis of the renatured FFV Bet protein, it was subsequently concentrated on the Äkta using a 1 ml HisTrap column and native buffer conditions. Fractions were anlysed by Coomassie blue staining.

3.2. Expression and purification of Bet/HIV-1 hybrid proteins

3.2.1 Construction of FFV Bet-E1 and Bet-E2 expression plasmids

To generate constructs containing HIV-1 domains, after the establishment of suitable expression, purification and refolding protocols for the FFV Bet protein, the HIV-1 E1 and E2 sequences were inserted into pQE Bet expression vector. This way recombinant Bet-E1 and Bet-E2 hybrid proteins were obtained (Figure 31). HIV E1 and E2 sequences including an 11 amino acid linker sequence were amplified from plasmid pCL1 with primer pairs FFV Bet-E1_fwd and FFV Bet-E1_rev or FFV Bet-E2_fwd and FFV Bet_rev, respectively (Figure 32A). Primers were modified so that the PCR products contained a 5' blunt and 3' *Hind*III restriction site to introduce them into the *Nae*I and *Hind*III linearised pQE-Bet vector (Figure 32B). Positive clones were identified by colony PCR with FFV Bet and E1 or E2 specific primers which revealed the expected PCR products of 892 bp (E1) and 913 bp (E2) in almost all colonies (Figure 32C). Plasmid DNA of positive clones was isolated and the integrity of the inserts was confirmed by sequencing. Plasmids were then transformed into the SCS-1 bacterial strain for expression.

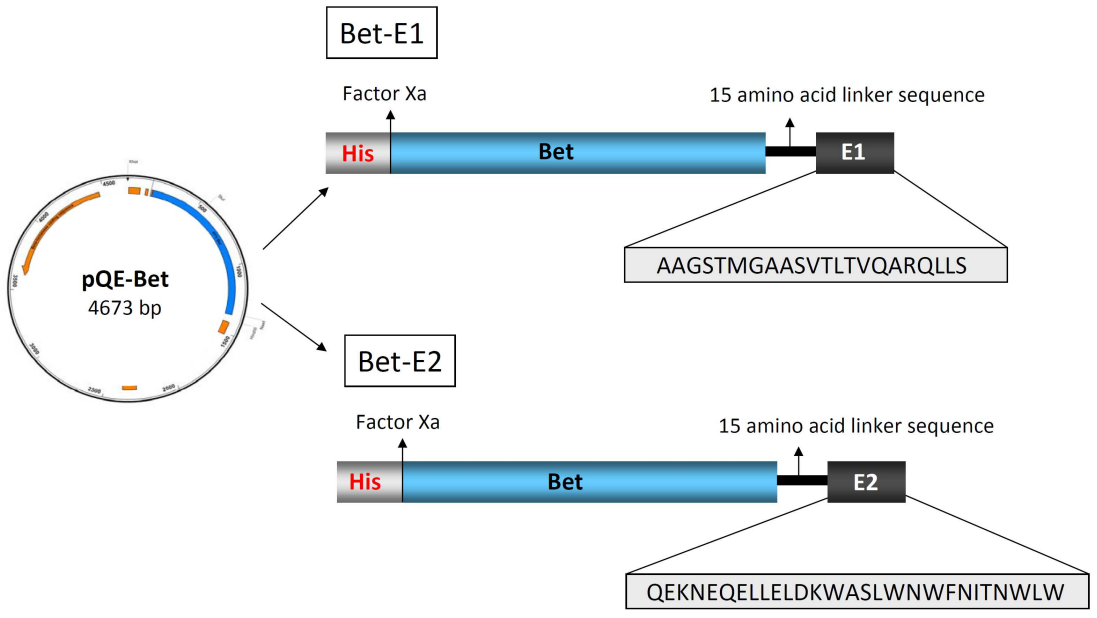

Figure 31: Schematical illustration of the pQE-Bet plasmid map and of Bet-E1 and Bet-E2
The pQE-Bet vector was used as basis for the insertion of the FFV Bet/HIV-1 expression plasmids. The E1 or the E2 sequence is connected by a 15 aa linker to the FFV Bet protein.

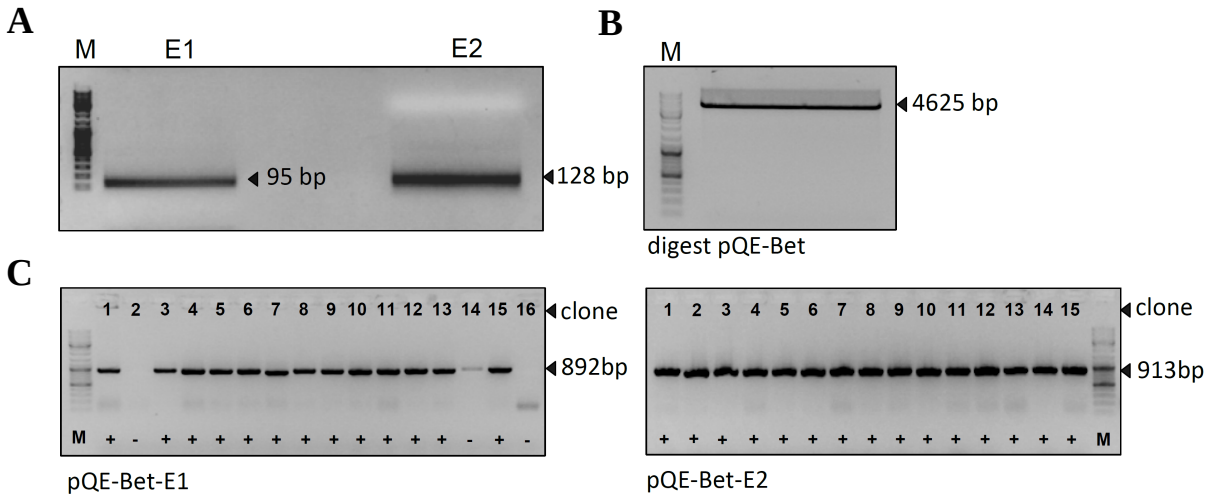

Figure 32: (A) Molecular cloning of FFV Bet E1 and Bet E2 expression plasmids. (A) Separation of PCR products of E1 and E2 with the expected insert size of 95 bp and of 128 bp for purification. **(B)** Purification of *Nae*I and *Hind*III digested expression vector pQE-Bet for molecular cloning with E1 and E2 sequences. **(C)** Colony PCR on colonies obtained after transformation using the primers FFV-Bet-Seq_fwd and FFV-Bet-E1_rev for E1 and FFV Bet-Seq_fwd and FFV Bet-E2_rev for E2. Positive and negative clones (+/-) are indicated, M, marker.

3.2.2 Generation of FFV Bet and MBP E1-loop-E2 hybrid proteins

Since it was not clear whether the introduced HIV-1 parts of FFV Bet-E1 and Bet-E2 expressed on two independent proteins can really find each other when administered together or the interaction of the relatively small inserts might be prevented by the 45 kDa large FFV Bet fusion partner, an additional hybrid construct was prepared that stabilises the E1 and E2 interaction. For this second approach, the plasmid pMA-T was synthesized by GeneArt that contains the E1 and E2 sequences connected by a extended loop (Figure 33, [98]).

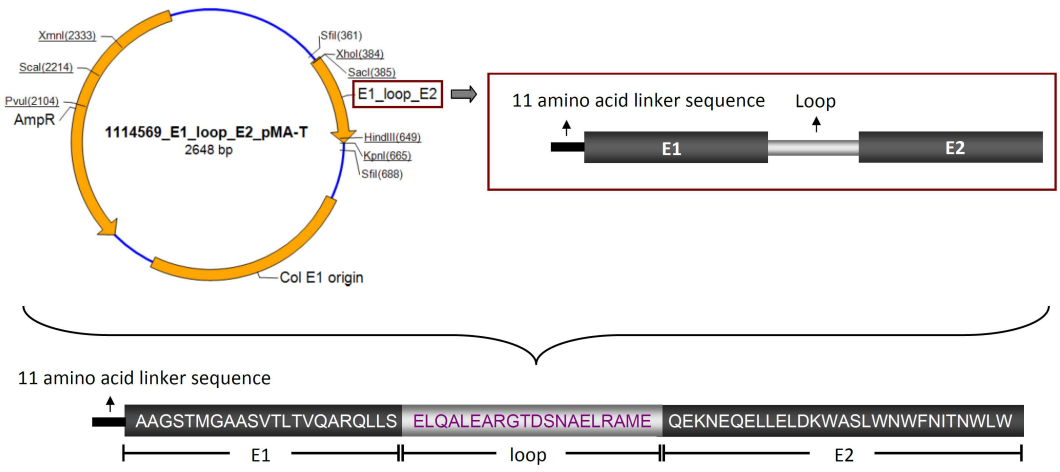

Figure 33: Plasmid map of the pMA-T vector and the encoded E1-loop-E2 construct.
The pMA-T vector contains the E1-loop-E2 sequences which are connected by a flexible loop. The pMA-T vector was used for digestion of the E1-loop-E2 sequence for following cloning steps.

The length of the loop sequence in this construct is crucial for the presentation of the E1 and E2 domains in a way that allows increased 2F5 binding as seen for the free peptides [60, 70]. Therefore, primers for the pMA-T vector were designed that allow truncation of the E1-loop-E2 sequence by single amino acids for the generation of various sub-variants. Nine truncated pMAT expression clones were generated during this work and are currently investigated in a separate project. In this thesis, the focus was concentrated on one loop construct that was deleted by one amino acid on the N-terminal and the C-terminal part of the loop enclosed sequences. This construct was shown in an alpha helical wheel projection to present both HIV domains as demonstrated in the gp41 crystal structure [99] and thus was one of the favoured constructs (Mühle et al. unpublished data). For generation of this loop construct, two rounds of mutagenesis PCR had to be performed (Figure 34). In the first round an E1 reverse primer (2.1_rev) and a mutated forward primer (2.1_fwd) with a single truncated amino acid that recognised the N-terminal domain of the enclosed loop sequence was used to eliminate the first amino acid on the N-terminus. All primers were phosphorylated prior use, so that the resulting amplicon could directly be religated to a full plasmid after PCR. This new truncated plasmid was taken for a second PCR amplification step using an E2 forward primer (1.2_fwd) and a truncated reverse primer (1.2_rev) that annealed on the C-terminal region of the loop sequence and reduced it by one amino acid on the C-terminus.

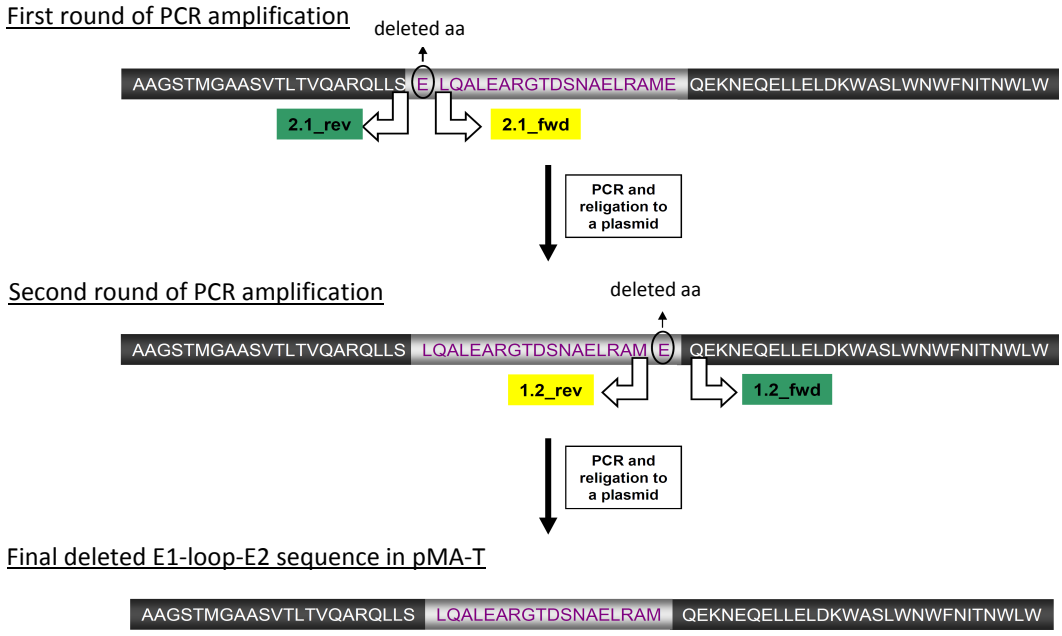

Figure 34: Mutagenesis PCR of pMA-T to truncate the loop enclosed sequence for one single aa on each terminus. Illustration shows the PCR products that were obtained after amplification with mutated primers. In the first round of amplification the first aa on the 5' end of the loop sequence was deleted using the primers 2.1_rev and 2.1_fwd following of a religation of the vector and a second PCR. Thereby, another aa on the 3' end of the loop was deleted with the primers 1.2_rev and 1.2_fwd.

The truncated E1-loop-E2 insert was then isolated by cleavage of the modified pMA-T vector with the restriction enzymes *Hind*III and *Mly*I (blunt end) for subsequent cloning (Figure 35A). As in 3.2.1 the insert was ligated into the *Nae*I/*Hind*III linearised pQE-Bet vector to obtain the pQE-Bet-E1-loop-E2 expression construct. Positive clones were identified as described for the two single domain Bet/HIV-1 hybrid constructs (see 3.2.1 and Figure 35B) and verified by sequencing. To rule out the possibility that the denaturation process during Bet purification might irreversibly destroy the conformation formed by the HIV-1 E1-loop-E2 fusion part or this conformation will not be completely restored after refolding, the same insert was also fused to the MBP fusion protein in the pMal vector to obtain a soluble expressed protein which can be purified under physiological conditions. Therefore the pMal vector was digested with *Xmn*I *and Hind*III and the loop insert was introduced. Positive pMal-E1-loop-E2 constructs were identified by enzymatic digestion with *Hind*III and *Bgl*II because no compatible primers for colony PCR were available (Figure 35C). Plasmid maps of the expression vectors as well as the resulting recombinant proteins are given in Figure 35.

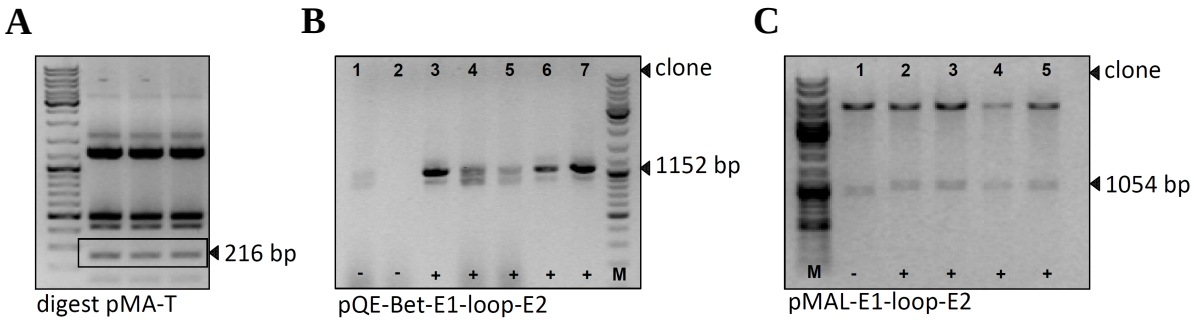

Figure 35: Molecular cloning of E1-loop E2 constructs.
(A) *Mly*I and *Hind*III digestion of the pMA-T vector containing the truncated E1-Loop-E2 sequence with an expected size of 216 bp which was used for subsequent cloning into pQE-Bet and pMal vectors. **(B)** Colony PCR of transformants using the primer FFV Bet-Seq_fwd and FFV Bet-E2_rev. Positive clones result an PCR product of 1152 bp. The smaller PCR product can be a result of unspecific primer binding. **(C)** Enzymatic control digestion of pMal-E1-loop-E2 clones with the restriction enzymes *Hind*III and *Bgl*II resulting in a cleavage product of 1054 bp for positive clones. Negative clones exhibited a cleavage size of 870 bp. +/- positive and negative clones, M-marker.

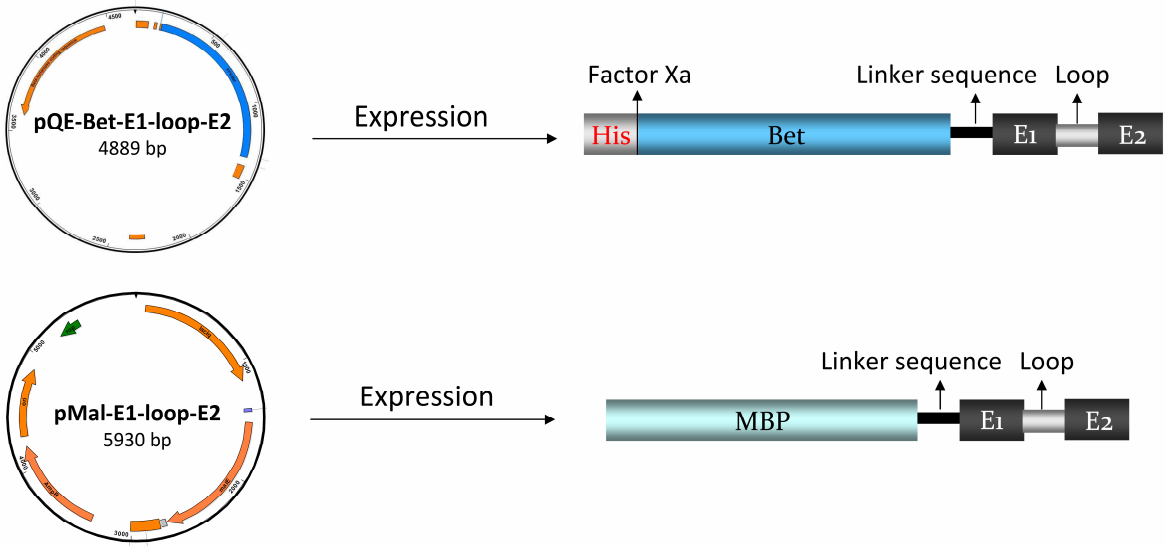

Figure 36: Schematically presentation of pQE-Bet-E1-loop-E2 and pMal-E1-loop-E2 vectors and the resulting recombinant proteins after expression. The prokaryotic expression vectors allow the production of the FFV Bet-E1-loop-E2 protein that contains additionally a His tag and a Factor Xa cleavage site on its N-terminus and the MBP-E1-loop-E2 protein.

3.2.3 Purification of Bet/HIV-1 hybrid proteins

The established FFV-Bet protocols were applied for production of the Bet/HIV-1 fusion proteins. First, the insoluble fractions of FFV Bet-E1, Bet-E2 and Bet-E1-loop-E2 induced cells were taken for purification under denaturing conditions as described (see 3.1.7). Eluted peak fractions of all three hybrid constructs showed a sufficiently pure protein for all generated antigens with yields in a range of 11.3 to 14.4 mg protein (Figure 37A-C). Purity of the eluted peak fractions of all three Bet/HIV-1 fusion proteins were analysed by Coomassie staining (Figure 37A-C).

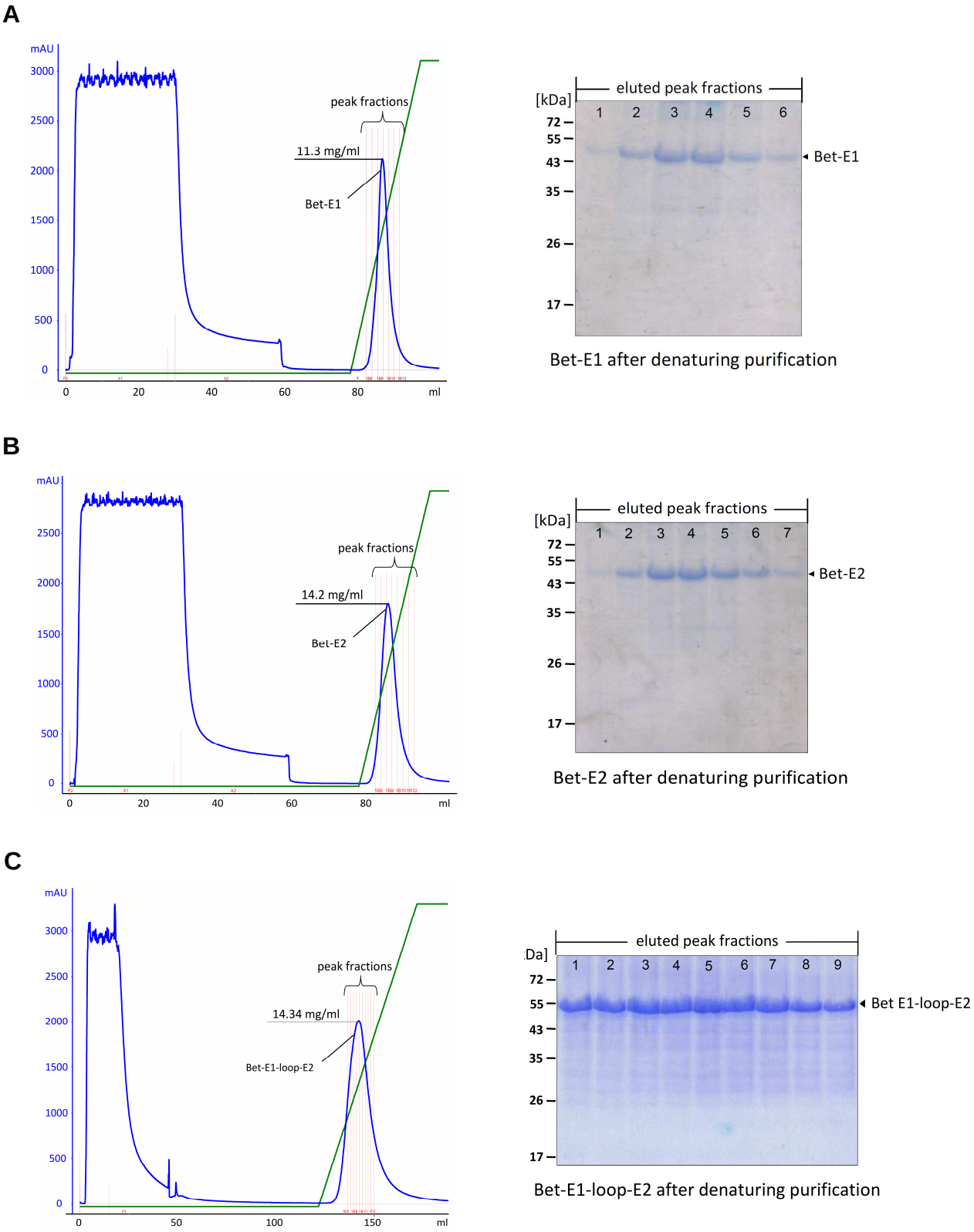

Figure 37: Purification of Bet/HIV-1 hybrid proteins under denaturing conditions
The insoluble fractions of 500 ml pellet of each induced **(A)** FFV-Bet-E1, **(B)** Bet-E2 and **(C)** Bet-E1-loop-E2 cells were extracted with 6 M GuHCl for 2 h. The lysates were centrifuged and supernatants which contained solubilised proteins were used for purification on the Äkta FLPC system. After washing of unbound material, proteins were eluted with an imidazol gradient of 10 mM to 500 mM. Yields of FFV-Bet/HIV-1 hybrid proteins were calculated by the Äkta software and eluted peak fractions analysed by Coomassie staining (20 µl).

3.2.4 Renaturation of the FFV Bet/HIV-1 hybrid proteins

As for the FFV Bet protein, the three denatured FFV Bet/HIV-1 hybrid proteins were renatured by rapid dilution in refolding buffer and afterwards subjected to a dialysis step as decribed in chapter 3.1.9. After dialysis, Bet-E1, Bet-E2 and Bet-E2-loop-E1 were concentrated by loading onto NiNTA-columns using physiological buffer conditions (also see 3.1.9). Eluted peak fractions of the renatured FFV Bet/HIV-1 hybrid proteins were analysed by Coomassie staining to determine the integrity and purity of the target proteins. Results revealed proteins with a purity of more than 90% with expected molecular weights of 49 kDa, 49.8 kDa and 54 kDa for Bet-E1, Bet-E2 and Bet-E2-loop-E1, respectively (Figure 38A-C). For immunisation all three antigens were concentrated to 0.5 mg/ml, aliquoted and then stored at -20°C. For short term storage proteins were kept at 4°C until needed.

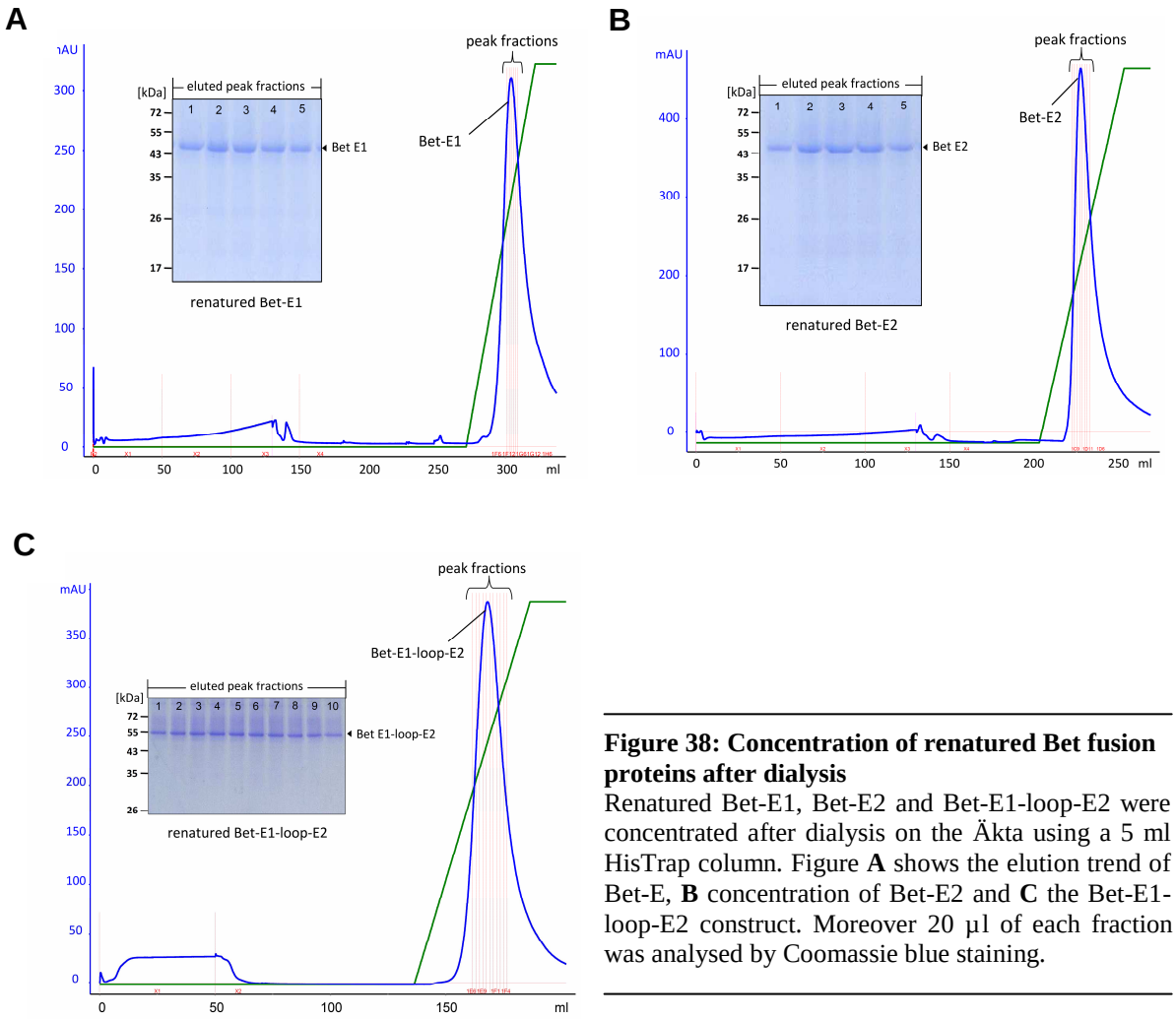

Figure 38: Concentration of renatured Bet fusion proteins after dialysis
Renatured Bet-E1, Bet-E2 and Bet-E1-loop-E2 were concentrated after dialysis on the Äkta using a 5 ml HisTrap column. Figure **A** shows the elution trend of Bet-E, **B** concentration of Bet-E2 and **C** the Bet-E1-loop-E2 construct. Moreover 20 µl of each fraction was analysed by Coomassie blue staining.

3.2.5 Expression and purification of the MBP-E1-loop-E2 protein

In contrast to expression conditions found suitable for Bet containing proteins, the MBP fusion protein was not expressed under these conditions. To maximise production, an expression screen for the pMal loop construct was performed and the soluble protein fraction was analysed in analogy to 3.1.5. This expression screen revealed that best protein production for soluble MBP-E1-loop-E2 protein could be achieved in the Rosetta 2 strain growing at 20 °C for 3 h after 0.5 mM IPTG induction (data not shown). After expression under these optimised conditions pellets of induced pMal-E1-loop-E2 cells were lysed under non-denaturing conditions as described above. After centrifugation to remove the insoluble material, the crude extract was loaded on an amylose column to isolate the MBP fusion protein from cytosolic *E. coli* proteins. Bound proteins were washed and eluted with a 10 mM maltose gradient resulting in yields of 5 mg protein per litre expression culture (Figure 39A). Similar to the pMal-Bet construct (Figure 263.1.5), the produced MBP-E1-loop-E2 protein was instable and dissociated to about 30% of full length protein and 70% pure MBP (Figure 39B). However, next to this degradation problem a soluble protein with extremely high purity was obtained (Figure 39B).

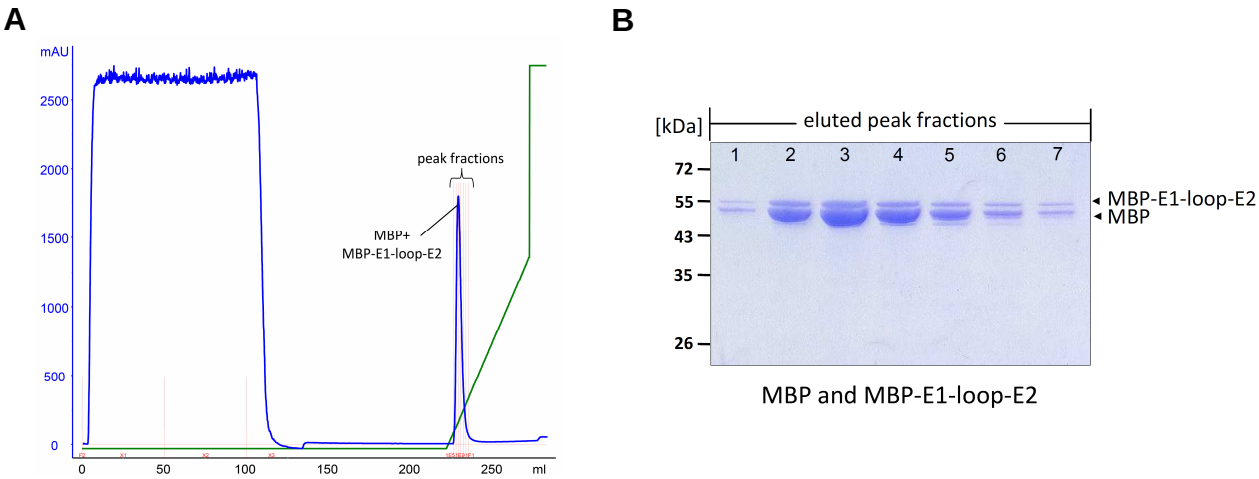

Figure 39: Native purification of MBP and MBP-E1-loop-E2
(A) Crude extract containing the target MBP fusion proteins were purified using 4 ml amylose resin. MBP and MBP fusion proteins were eluted at the same time by use of a 10 mM maltose gradient. **(B)** 25 µl of each eluted peak fraction was analysed by SDS-PAGE and Coomassie blue staining.

To separate the dissociated MBP species from the full length MBP hybrid protein, the amylose elution peak fractions were pooled, 1:2 diluted to decrease salt concentration and loaded on a Resource Q column (see 2.3.8). Whereas the MBP protein was eluted at a salt concentration of 100 mM NaCl, the MBP-E1-loop-E2 was eluted at 220 mM NaCl

resulting in two clearly separated peaks in the chromatogram (Figure 40A). SDS-PAGE analysis of the eluted peak fractions confirmed the successful separation of these two protein species resulting in a highly pure MBP/HIV-1 hybrid protein (Figure 40B).

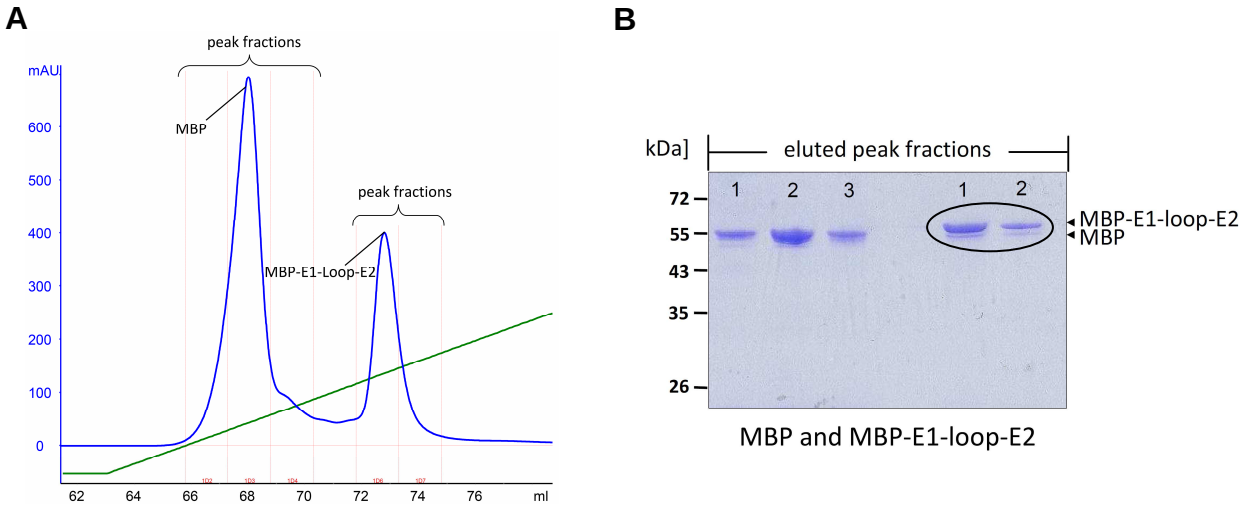

Figure 40: Separation of MBP and MBP-E1-loop-E2 by ion exchange chromatography
(A) After purification by amylose column, eluted peak fractions were pooled and MBP were separated from the MBP full length fusion protein using an Resource Q anion exchange column. **(B)** 35 µl of the eluted peak fractions of the Resource Q run were examined by Coomassie blue staining.

3.3. Characterisation of antigenicity of produced proteins

3.3.1. Antigenicity of recombinant proteins in Western blot

To assure protein integrity and to examine the reactivity of the produced antigens with HIV broadly neutralising antibodies, equal amounts of refolded proteins were separated by SDS-PAGE or transferred to PVDF membranes (Figure 41A and B). Western blots were probed with 2F5 and 4E10 to check for the presence of introduced HIV MPER domains as well as with a FPPR specific rat antiserum produced in previous immunisation studies (rat 334-1). This rat serum recognises the core epitop HXBII gp41 aa 541 TLTVQARQL 570 what was analysed by epitop mapping (J. Kreuzberger, RKI, unpublished data). As expected, the monoclonal antibodies 2F5 and with a weaker extent also 4E10 reacted with all antigens containing the MPER epitope but not with the unmodified Bet or the Bet E1 protein (Figure 41B). Interestingly, the refolded Bet loop construct seemed to react with these antibodies with lower intensity and this effect was even more pronounced for the soluble expressed MBP loop construct resulting in a decreased Western blot signal (Figure 41B). In case of the FPPR specific antiserum, only constructs containing the E1 epitope

were recognised, but not the Bet or the Bet E2 protein (Figure 41). Similar to the findings above, reactivity to the constructs containing both HIV epitopes connected by a loop was decreased compared to the protein containing the E1 epitope alone.

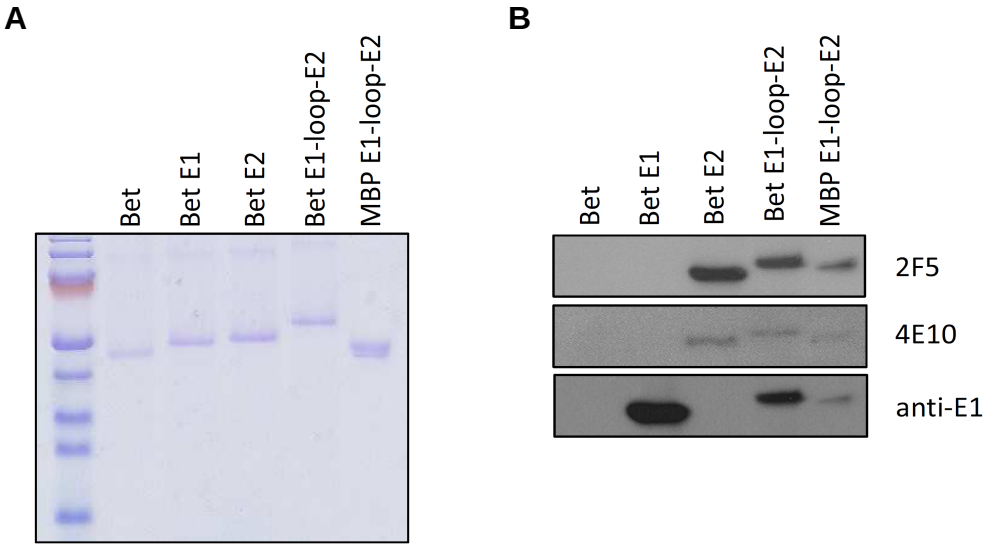

Figure 41: Recognition of produced proteins by monoclonal antibodies 2F5 and 4E10 and a FPPR specific antiserum. (A) 2 µg of produced recombinant proteins were loaded for SDS-PAGE and analysed by Coomassie staining. **(B)** 150 ng of the same proteins were transferred onto a PVDF membrane and tested with the primary antibodies 2F5 (1:30000), 4E10 (1:10000) and an anti-E1 rat serum (1:800) to confirm the presence of the introduced HIV-1 domains. As secondary antibody an anti-human IgG antibody in case of 2F5 and 4E10 and an anti-rat IgG antibody for the anti-E1 serum (1:3000) was used.

3.3.2. Antigenicity of recombinant proteins in ELISA

The antigenicity of the HIV MPER domain has been found to be sensitive to the conformational status in which it is presented to antibodies. It has been demonstrated that denatured gp41 is generally better recognised from the antibody 2F5 whereas 4E10 reactivity decreases [64]. In the experiment in 3.3.1 the binding of monoclonal antibodies to the antigens reduced and denatured by SDS-PAGE was investigated. To analyse the situation when antigens were presented in a non-denatured form, all antigens were coated with equivalent amounts on ELISA plates and binding of monoclonal antibodies 2F5 and 4E10 was investigated. To allow an estimation of the strength of the reactivity, antibodies were serially diluted. Results showed that in case of 2F5 only minor differences in binding were detected between the individual constructs. Bet-E2 as well as the combination of Bet-E2 with Bet-E1 was recognised to a similar extent, with a minimal stronger signal for the combination of both antigens at lower antibody dilutions (Figure 42). Next, the MPB loop construct and Bet loop construct were detected by 2F5 with an only slightly decreased

intensity compared to Bet-E2 and the combination of Bet-E2 with Bet-E1. This situation looked quite different when 4E10 binding was analysed. Here, the Bet E1-loop-E2 antigen reacted with about threefold higher intensity with 4E10 than the single domain Bet-E2 construct. Then with decreasing reactivity the combination of the Bet-E1 and Bet-E2 and the MBP E1-loop E2 antigen followed (Figure 42). In summary, these results showed that all produced antigens were full length proteins containing the intended HIV-1 fusion part, although the constructs were recognised with different intensity by the broadly neutralising antibodies. Importantly, the presence of the E1 domain seemed to be highly beneficial for the presentation of the 4E10 epitope and increased binding about threefold.

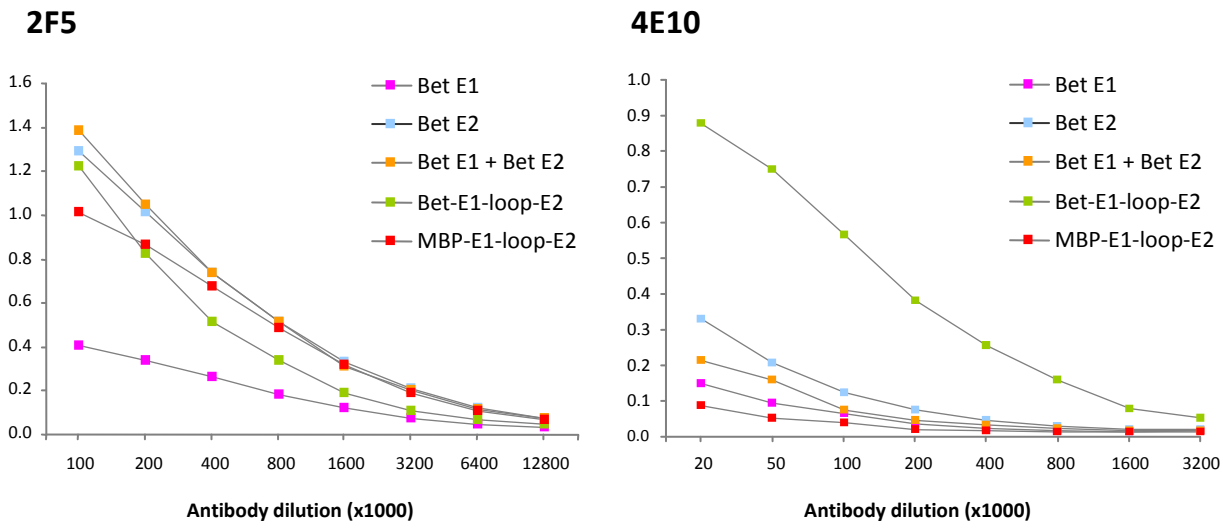

Figure 42: Equal amounts of antigen (100 ng/well, filled up to 200 ng/well with recombinant Bet protein except for Bet E1 and Bet E2 antigens) were coated on ELISA plates and incubated with serially diluted monoclonal antibodies 2F5 (11,3 µg/µl) or 4E10 (11,9 µg/µl) as indicated. Bound antibodies were detected with an anti human IgG-HRP antibody diluted 1:3000 and OD492 measured in a microplate reader.

3.4. Immunisation with the Bet and MBP HIV-1 hybrid proteins

The hybrid proteins Bet-E1, Bet-E2, Ber-E1-loop-E2 and MBP-E1-loop-E2 were used for immunisation studies of rats to investigate their immunogenicity. For that, two independent immunisation studies were performed. The first immunisation was done with the FFV Bet-E1 and FFV Bet-E2 antigens in three different rat groups with the number 364, 365 and 366. Rat group 364 was immunised with the antigen Bet-E1. The group 365 was immunised with FFV Bet-E2 and the group 366 with both single domain antigens. Rat

groups 365 and 366 that were vaccinated with either the Bet-E2 domain or a mixture of the Bet-E2 domain and Bet-E1. In the second immunisation study, two rat groups (374 and 375) were used. These rats were immunised with the Bet and the MBP HIV-1 hybrid protein containing the E1 and E2 domain and the loop (Table 17). For every injection 500 µl of antigen with a concentration of 0.5 mg/ml were mixed with an equal volume of Freund's adjuvant and 1 ml of this mixture was used per rat and immunisation in case of all FFV Bet/HIV-1 hybrid constructs. The amount of administered MBP-E1 loop-E2 antigen was lower because of technical difficulties in producing this protein due to the instability already mentioned.

Table 17: Overview of performed immunisations with the Bet- and MBP-HIV hybrid antigens

Rat group	Number of animals	Immunised antigen	Amount of antigen per injection	Remarks
364	4	 FFV Bet-E1	250 µg Bet-E1	Rat 1 died after the first boost
365	4	 FFV Bet-E2	250 µg Bet-E2	-
366	4	 FFV Bet-E1 and Bet-E2	250 µg Bet-E1, 250 µg Bet-E2	-
374	4	 MBP-E1-loop-E2	~150 µg MBP-E1-loop-E2	Rat 1 died before immunisation, Rat 2 died after the first boost
375	4	 FFV Bet-E1-loop-E2	250 µg Bet-E1-loop-E2	Rat 2 died after the second boost

3.5. Characterisation of immune sera

3.5.1 Immune response against administered antigens

To investigate to which level immunised animals produced antibodies specific for the immunogen they were vaccinated with, sera of all five rat groups were first titrated against the administered antigen by ELISA. For that, coated ELISA plates were probed with sera serially diluted from $1:10^2$-10^8 and antibody endpoint titers calculated with measured OD values. Results showed that all antigens induced a strong immune response and antibody titers rised qickly to 10^4-10^6 already after the first immunisation (Figure 43). In almost all animals the immune response was strongest after the second immunisation and then remained at this level or in some cases was slightly decreasing (Figure 43A-F). The antibody titers of group 364 which were immunised with Bet-E1 peaked after the second immunisation at a level of 10^6 (Figure 43A). A similar result was seen for sera from rat group 365 when these were tested against the Bet-E2 antigen (Figure 43B). Here, the endpoint titers increased to values of 10^6 (Figure 43B). In case of sera from rat group 366 immunised with both single domain antigens, anti Bet-E1 titres also reached levels of 10^5 and 10^6 for the FFV Bet-E2 antigen (Figure 43C and D). For unknown reasons, the immune response of rat number three in this group was quite weak against Bet-E1 as well as Bet-E2 (Figure 43C and D). The sera of the HIV-1 loop antigens MBP-E1-loop-E2 and FFV Bet-E1-loop-E2 also exhibited a strong signal to their immunogen with the highest titers ranging from 10^5 to 10^6 after the first injection and increasing up to 10^7 in case of rat 375-1 immunised with the FFV Bet-E1-loop-E2 (Figure 43E and F). Notably, the immune response in group 375 was quite variable and antibody titers differed by three magnitudes between the individual rats. Taken together, all HIV-1 hybrid antigens were highly immunogenic and induced high levels of antibody titers even after the first injection.

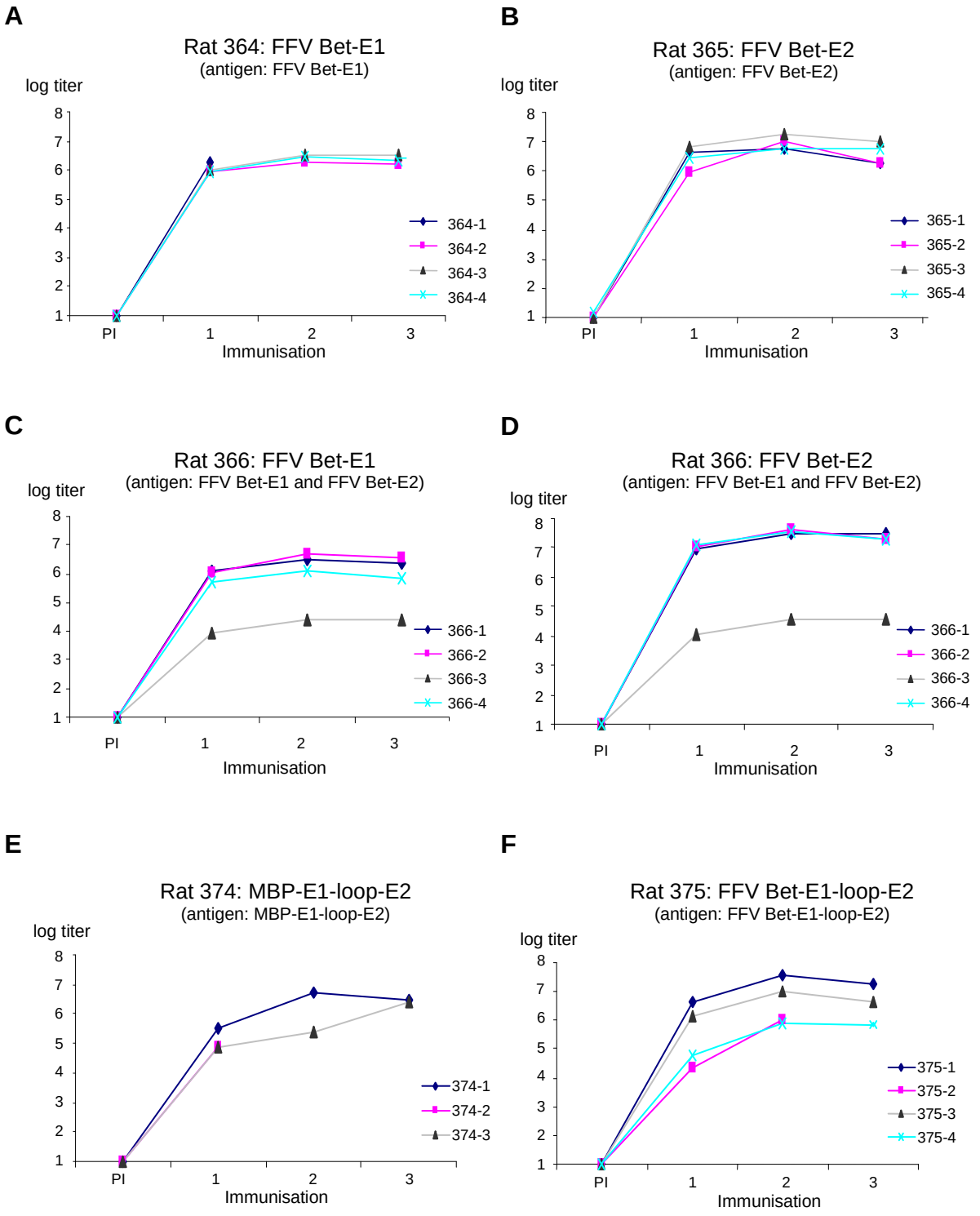

Figure 43: Antibody endpoint titers of the antisera titrated against their specific immunogen.
Sera of immunised rats were diluted in PBS containing 1% BSA and 0.05% Tween 20 in a range of 10^2 to 10^8 and applied on ELISA plates coated with 200 ng/well of the HIV-1 hybrid antigen indicated in brackets. After washing, a HRP conjugated anti-rat antibody (1:3000) and OPD was used for development of the plates and absorbance of individual wells measured in an ELISA reader at 492 nm. Diagrams show the antibody enpoint titers of rat sera to the given immunogen **(A)** group 364 against FFV Bet-E1 **(B)** group 365 against FFV Bet-E2 **(C-D)** group 366 against both independent FFV Bet/HIV-1 hybrid antigens **(E)** group 374 against MBP-E1-loop-E2 and **(F)** group 375 against FFV Bet-E1-loop-E2. PI = pre-immune, 1 = first immunisation, 2 = second immunisation, 3 = third immunisation

3.5.2 Immune response to fused HIV epitopes and gp41

To analyse the antisera for the presence of antibodies directed against the intended HIV E1 and E2 domains and to test if these antibodies are also able to recognise gp41, sera were titrated against synthetic E1 and E2 peptides as well as recombinantly produced gp41 (Beherndt et al., unpublished data). The ELISA protocol was as described in 3.5.1, but this time sera were diluted tenfold to a final dilution of $1:10^5$ only since antibody titres to the smaller HIV-1 domains were expected to be remarkably lower. In all cases the generated antisera contained antibodies that recognised the respective peptides as well as the gp41 antigen but with varying intensity (Figure 44 and Figure 45).

Rat group 364 vaccinated with the FFV Bet-E1 immunogen showed a uniform immune responses against the E1 epitope in a range of 10^5-10^6 with a slight decrease of antibody titers after the third immunisation (Figure 44A). In line with E1 reactivity, these sera also had antibodies binding to gp41, although this protein was recognised to a weaker extent (Figure 44A). One rat developed an extremely strong immune response to gp41 with a permanent increase of its antibody titer from the first to the last boost and reached a final titer of 10^7 (Figure 44A).

Rat group 365 which was immunised with the FFV-Bet-E2 antigen steadily increased antibody titers against the E2 peptide as well as the gp41 protein from 10^3 up to 10^4 until the second immunisation (Figure 45B). Surprisingly after the third boost antibody titers against the E2 peptide decreased dramatically to titers of 10^1-10^2 in two of these four rats (Figure 44B). However, similar to what was observed in rat group 364, titers against gp41 were obviously not affected and further increased with the third immunisation (Figure 44B).

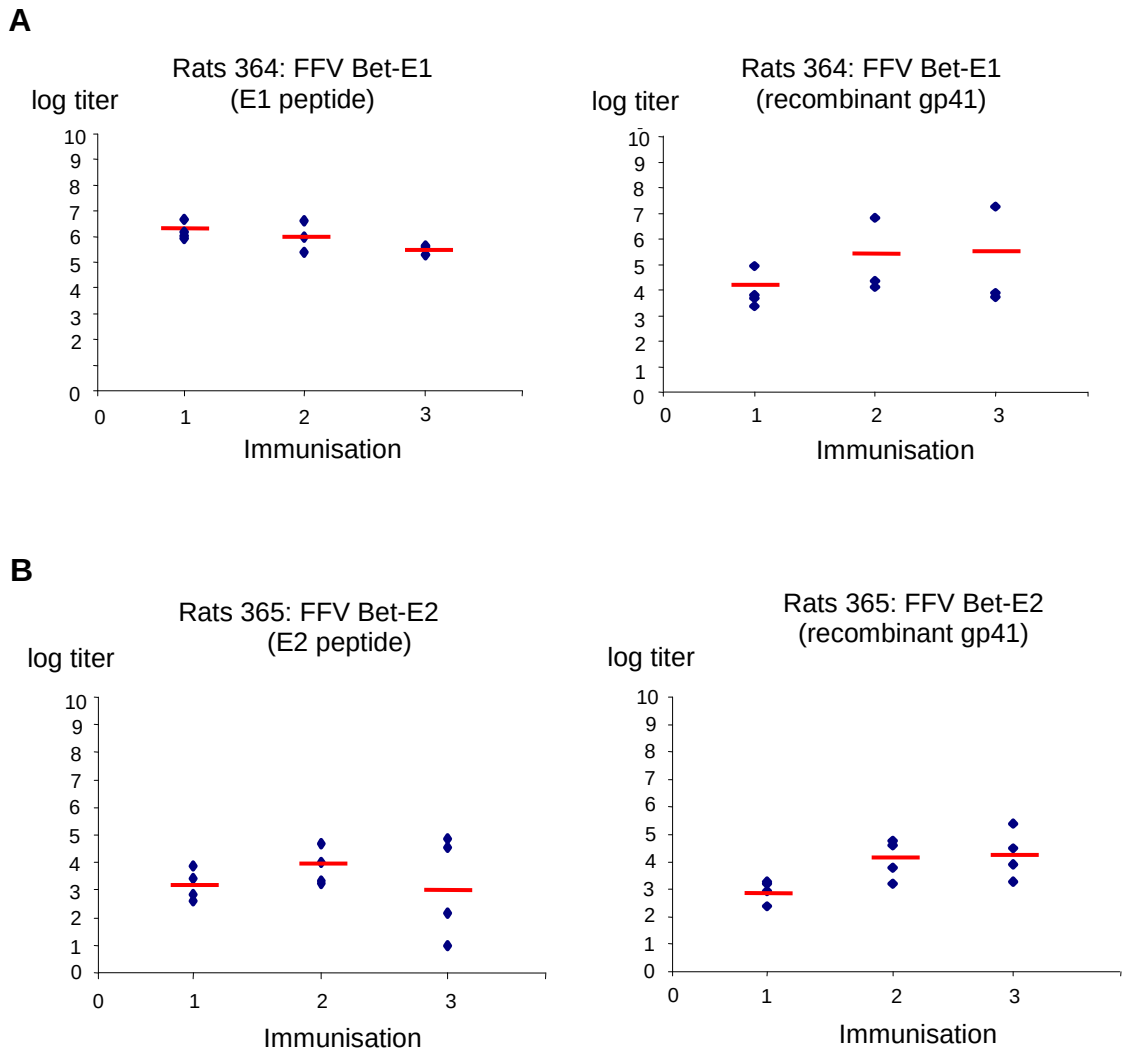

Figure 44: Specificity of antisera to HIV-1 epitopes in the HIV-1 single domain vaccination study.
Sera from immunised rats were serialy diluted in a range of 10^2 to 10^5 and applied on ELISA plates coated with 200 ng of either E1 or E2 peptides or gp41 antigen. After washing, a HRP conjugated anti-rat antibody (1:3000) and OPD was used for development of the plates and absorbance of individual wells measured in an ELISA reader at 492 nm. Diagrams show the antibody andpoint titers of (A) rat group 364 titrated against E1 peptide and gp41 and (B) rat group 365 titrated against E2 peptide and gp41.

Similar to what was found for the previous animals, rat groups 366, 374 and 375 which were immunised with a mixture of both single domain FFV Bet/HIV-1 hybrid antigens (group 366) or with one of the HIV-1 hybrid loop antigens (group 374 and 375) showed in all cases an approximately 2 to 2.5 log higher immune response against the E1 compared to the E2 domain (Figure 45). From these animals, rat group 366 developed the highest antibody response against the E2 peptide with titers up to 10^3 after the second injection (Figure 45A-C). However, this response decreased drastically to titers of 10^1 after the third immunisation (Figure 44A) similar to what was observed for two rats of group 365.

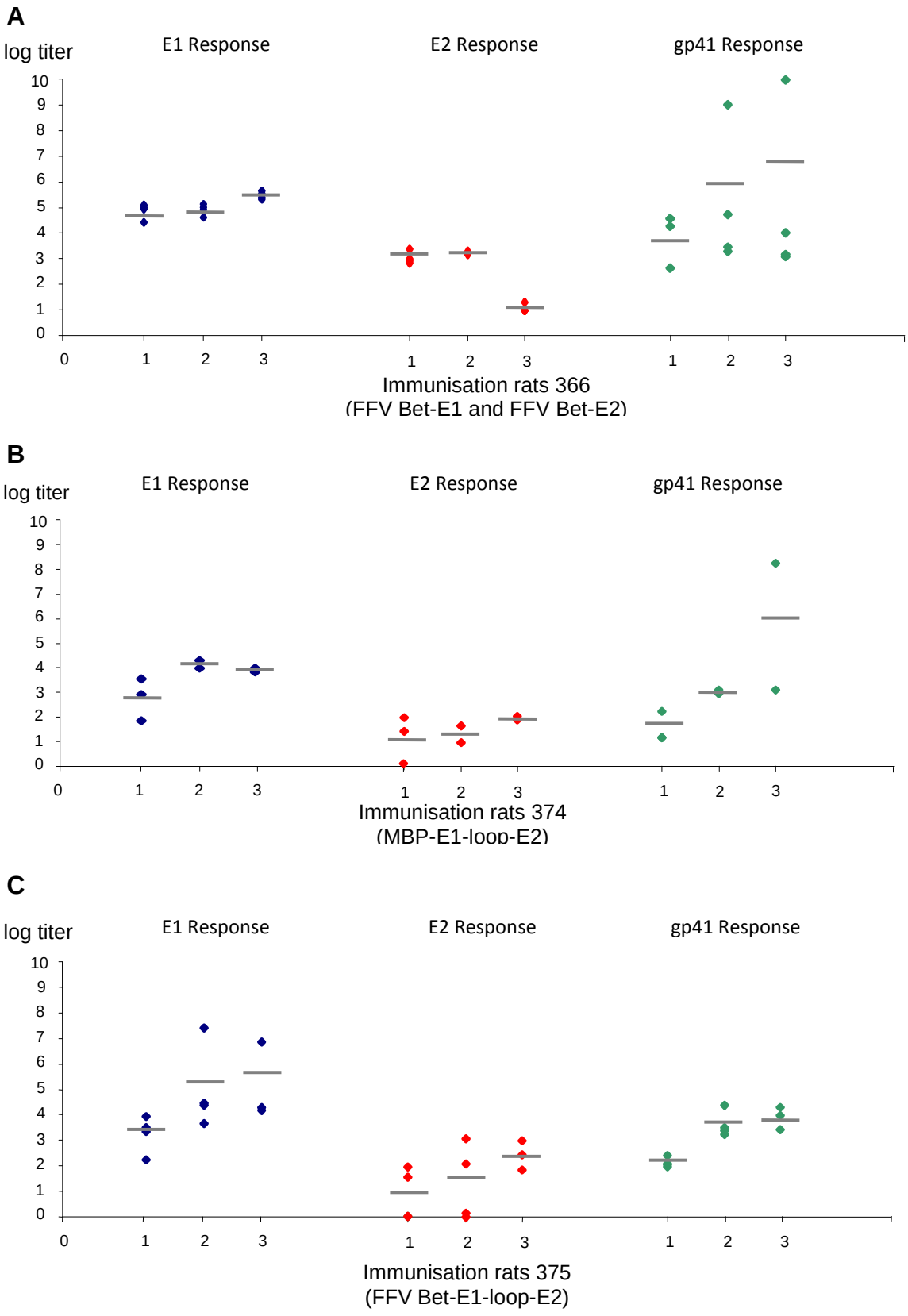

Figure 45: Specificity of antibodies to HIV-1 epitopes after HIV-1 double domain vaccination.
Sera from immunised rats were serialy diluted in a range of 10^2 to 10^5 and applied on ELISA plates coated with 200 ng of either E1 or E2 peptides or gp41 antigen. After washing, a HRP conjugated anti-rat antibody (1:3000) and OPD was used for development of the plates and absorbance of individual wells measured in an ELISA reader at 492 nm. Diagrams show the antibody endpoint titers against the E1 and E2 peptide as well as against gp41 of **(A)** rat group 366, **(B)** rat group 374 and **(C)** rat group 375.

Table 18: Antibody endpoint titers of antisera titrated against their immunogen (Figure 43)

Tested antigen	Rat No.	1st injection	2nd injection	3rd injection
Bet-E1	364-1	1.77×10^6	n.a	n.a
	364-2	9.32×10^5	1.81×10^6	1.64×10^6
	364-3	1.03×10^5	3.41×10^6	$3{,}19 \times 10^6$
	364-5	9.45×10^5	3.08×10^6	2.09×10^6
	Mean	1.17×10^6	2.76×10^6	2.31×10^6
Bet-E2	365-1	4.43×10^6	6.08×10^6	1.96×10^6
	365-2	8.85×10^5	9.77×10^6	1.78×10^6
	365-3	7.14×10^6	1.77×10^7	1.06×10^7
	365-4	2.88×10^6	5.65×10^6	6.10×10^6
	Mean	3.83×10^6	9.81×10^6	5.10×10^6
Bet-E1	366-1	1.32×10^6	3.20×10^6	2.30×10^6
	366-2	1.17×10^6	4.96×10^6	3.83×10^6
	366-3	8.89×10^3	2.64×10^4	2.51×10^4
	366-4	5.35×10^5	1.35×10^6	7.28×10^4
	Mean	7.61×10^5	2.38×10^6	1.56×10^6
Bet-E2	366-1	8.73×10^6	2.90×10^7	3.25×10^7
	366-2	1.10×10^7	3.81×10^7	1.86×10^7
	366-3	1.13×10^4	3.78×10^4	3.80×10^4
	366-4	1.18×10^7	3.62×10^7	2.01×10^7
	Mean	7.89×10^6	2.58×10^7	1.78×10^7
MBP-E1-loop-E2	374-1	3.84×10^5	5.65×10^6	2.80×20^6
	374-2	7.81×10^4	n.a	n.a
	374-3	7.27×10^4	2.44×10^5	2.41×10^6
	374-4	n.a	n.a	n.a
	Mean	1.66×10^5	2.94×10^6	2.61×10^6
Bet-E1-loop-E2	375-1	4.30×10^6	3.49×10^7	1.84×10^7
	375-2	2.32×10^4	1.06×10^6	n.a
	375-3	1.32×10^6	1.01×10^7	4.07×10^6
	375-4	5.98×10^4	8.34×10^5	6.84×10^5
	Mean	1.43×10^6	1.17×10^7	7.72×10^6

Table 19: Antibody endpoint titers of antisera titrated against the specific HIV-1 domains and recombinant gp41 (Figure 44 and Figure 45)

Rat No	E1 peptide			E2 peptide			gp41		
	1st injection	2nd injection	3rd injection	1st injection	2nd injection	3rd injection	1st injection	2nd injection	3rd injection
364-1	1.12×10^{6}	n.a	n.a	-	-	-	5.02×10^{3}	n.a	n.a
364-2	4.71×10^{6}	3.97×10^{6}	3.72×10^{5}	-	-	-	8.67×10^{4}	2.24×10^{4}	7.65×10^{3}
364-3	8.78×10^{5}	9.15×10^{5}	2.03×10^{5}	-	-	-	6.47×10^{3}	6.37×10^{6}	1.81×10^{7}
364-5	1.54×10^{6}	2.37×10^{5}	4.19×10^{5}	-	-	-	2.37×10^{3}	1.30×10^{4}	5.34×10^{3}
Mean	2.06×10^{6}	1.71×10^{6}	3.31×10^{5}	-	-	-	2.51×10^{4}	2.14×10^{6}	6.03×10^{6}
365-1	-	-	-	7.63×10^{3}	1.04×10^{4}	3.51×10^{4}	1.83×10^{3}	6.18×10^{3}	8.10×10^{3}
365-2	-	-	-	3.91×10^{2}	1.69×10^{3}	1.00×10^{1}	8.85×10^{2}	1.61×10^{3}	1.84×10^{3}
365-3	-	-	-	7.12×10^{2}	2.19×10^{3}	1.40×10^{2}	1.61×10^{3}	4.15×10^{4}	2.98×10^{4}
365-4	-	-	-	2.64×10^{3}	5.02×10^{4}	7.34×10^{4}	2.35×10^{2}	6.00×10^{4}	2.43×10^{5}
Mean	-	-	-	2.84×10^{3}	1.61×10^{4}	2.72×10^{4}	1.14×10^{3}	2.73×10^{4}	7.07×10^{4}
366-1	1.07×10^{5}	1.37×10^{5}	4.33×10^{5}	6.54×10^{2}	1.47×10^{3}	1.00×10^{1}	1.83×10^{4}	4.59×10^{4}	9.47×10^{3}
366-2	8.68×10^{4}	1.05×10^{5}	2.55×10^{5}	9.58×10^{2}	1.57×10^{3}	1.00×10^{1}	3.36×10^{4}	8.47×10^{8}	7.28×10^{9}
366-3	2.75×10^{4}	4.02×10^{4}	2.11×10^{5}	8.36×10^{2}	1.97×10^{3}	1.00×10^{1}	4.05×10^{2}	1.78×10^{3}	1.33×10^{3}
366-4	1.24×10^{5}	8.06×10^{4}	3.57×10^{5}	2.27×10^{3}	1.67×10^{3}	2.13×10^{1}	3.56×10^{4}	2.63×10^{3}	1.15×10^{3}
Mean	8.65×10^{4}	9.06×10^{4}	3.14×10^{5}	1.18×10^{3}	1.67×10^{3}	1.28×10^{1}	2.20×10^{4}	2.12×10^{8}	1.82×10^{9}
374-1	8.20×10^{2}	9.12×10^{3}	9.36×10^{3}	1.00×10^{0}	9.97×10^{0}	7.88×10^{1}	1.66×10^{1}	1.38×10^{3}	1.34×10^{3}
374-2	3.34×10^{3}	n.a	n.a	8.82×10^{1}	n.a	n.a	1.97×10^{2}	n.a	n.a
374-3	7.02×10^{1}	1.90×10^{4}	6.63×10^{3}	2.53×10^{1}	4.60×10^{1}	1.11×10^{2}	1.60×10^{1}	9.55×10^{2}	1.70×10^{8}
374-4	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a
Mean	1.41×10^{3}	1.41×10^{4}	8.00×10^{3}	3.82×10^{1}	2.80×10^{1}	9.47×10^{1}	7.65×10^{1}	1.17×10^{3}	8.49×10^{7}
375-1	2.11×10^{3}	2.88×10^{4}	1.51×10^{4}	3.22×10^{1}	1.00×10^{0}	7.00×10^{1}	1.08×10^{2}	2.37×10^{3}	8.44×10^{3}
375-2	1.73×10^{2}	2.43×10^{4}	n.a	8.19×10^{1}	1.17×10^{2}	n.a	1.22×10^{2}	3.12×10^{3}	n.a
375-3	8.27×10^{3}	2.46×10^{7}	6.93×10^{6}	1.00×10^{0}	1.08×10^{3}	9.33×10^{2}	8.82×10^{1}	2.14×10^{4}	1.78×10^{4}
375-4	3.04×10^{3}	4.62×10^{3}	1.71×10^{4}	1.00×10^{0}	1.00×10^{0}	2.54×10^{2}	2.40×10^{2}	1.65×10^{3}	2.60×10^{3}
Mean	3.40×10^{3}	6.16×10^{6}	2.32×10^{6}	2.90×10^{1}	2.99×10^{2}	$4{,}19 \times 10^{2}$	1.40×10^{2}	7.13×10^{3}	9.60×10^{3}

3.5.3 Neutralisation potential of generated antisera to HIV-1

To examine whether the generated antisera exhibit a HIV-1 neutralising activity a neutralisation assay was performed. A standard dilution of 1:20 for all sera was chosen, and these prediluted sera were incubated with an equal volume of virus supernatants containing 200 infectious particles of the HIV-1 clone pNL-4.3. Sera were considered as neutralising when they were able to reduce infection by 50 % in comparison to the pre-immune control. In parallel 2F5 was diluted in a range of 50 µg/µl to 6.25 µg/µl as neutralisation positive control and cells infected with the virus without serum only to assure virus integrity. All samples were tested in triplicates. None of the sera of rats that were vaccinated with one of the FFV Bet/HIV-1 hybrid E1 or E2 antigen (Figure 46A) or the sera produced by immunisation with both domains (Figure 46B) were able to inhibit virus infection with regard to the mentioned criteria.

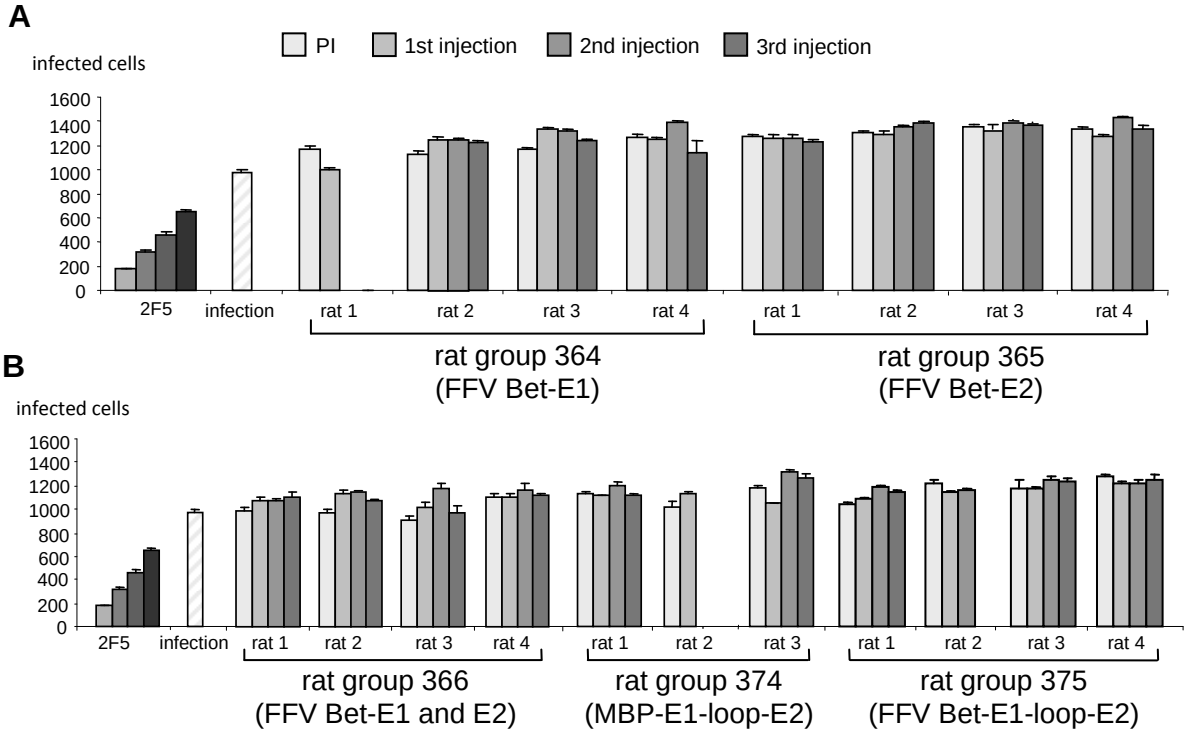

Figure 46: Neutralisation assay with antisera of single or double domain immunisations
1:20 diluted antisera were incubated for 30 min at 37°C with 200 particles of HIV-1 pNL-4.3 prior applying the serum-virus mixture onto 2 x 10^4 adherent TZM-bl cells. In parallel, 2F5 as positive control was used in a concentration of 50, 25, 12.5 and 6.25 µg/µl (from left to right) and a negative "infection" control with virus only. Infected cells were visualised by X-Gal staining 48 h after infection. Positive cells which show distinct blue nuclear staining when infected were counted with an ELISPOT device. Diagrams show the amount of infected cells after two days further culture for **A** rat group 364 and 365, **B** rat group 366, 374 and 375 as well as the negative and positive control.

4. Discussion

4.1. Neutralising antibodies against the MPER of gp41

Induction of HIV-1 broadly neutralising antibodies has been a major goal for HIV vaccine development and potential neutralsising antibodies such as 2F5 and 4E10 and their epitopes in MPER are of particular interest. In this thesis an approach to deliver the less immunogenic MPER domains with the immunogenic foamy viral Bet protein was tested and antigenicity and immunogenicity of such recombinant hybrid fusion proteins characterised. The approach aimed to elicit 2F5 and 4E10 like antibodies with antigens that mimic the prefusion state of gp41 in which the FPPR E1 and MPER E2 domains are in close proximity to each other in order to present the MPER epitope in a conformation that differs from the structure this domain assembles when presented alone [70, 99]. The observation of FPPR and MPER interaction and the influence of the FPPR residues on the MPER structure has been confirmed by other research groups [99, 100]. Indirect evidence that this conformation is linked to neutralisation was provided by Binley et al., 2004, [65] who investigated the interaction of 2F5 and 4E10 antibodies with the gp41 fusion intermediate. In this report the group developed HIV mutants expressing mutated gp160 Env in which gp120 and gp41 were tethered together by a disulfide bridge. These mutants can attach to CD4 but the fusion could only take place after removing the disulfide bridge with DTT. Based on this system Binley et al. [65] created an assay to determine on which stage 2F5 and 4E10 are able to bind and neutralize the virus. Results showed that 2F5 and 4E10 efficiently neutralise in the postattachment assay in which the monoclonal antibodies can bind to the virus after receptor binding. However, a relatively poor neutralisation was seen in the preattachment assay in which the monoclonal antibodies bound to the virus before attachment to the target cell. These observations suggest that the rearrangement and an interaction between the domains of the gp41 are necessary to expose the neutralising epitope which probably also induces these antibodies. Although in this work one antigen was produced that successfully increased the binding for the potential neutralising antibody 4E10 to its epitope, this construct was not able to induce neutralising antibodies in immunisation studies. Potential reasons for the lack of neutralisation of the sera obtained until now are (1) the difficult reconstruction of the trimeric Env spike to allow the presentation of conformational epitopes and the elicitation of 2F5 and 4E10 like antibodies that are able to bind to their epitopes how they are presented on the virus. In addition the

MPER undergoes drastically conformational changes during cell attachment and infection that complicates the reconstruction of the epitopes at a particular time point and to date it is not clear which conformation is responsible for the induction of neutralising antibodies [5, 60], (2) the HIV Env protein masks vulnerable epitopes through protein-protein interactions or by glycosylation [5], (3) other antibodies against gp41 may mask the epitope in the MPER [60] or (4) they represent autoimmune antibodies [54, 101, 102].

4.2. Optimisation of expression and purification

For the performed immunisation studies, several milligrams of all recombinant proteins were needed. The use of bacteria, in comparison to eukaryotic systems associated with low expression levels [103] allows to produce such large amounts in an easy and cost effective way. Therefore, this system is commonly used for research purposes but also the production of therapeutic proteins like human insulin or interferon [103-105]. As a first step of this project prokaryotic FFV Bet and Bet/HIV-1 expression plasmids were therefore generated. The FFV Bet protein was used to establish suitable expression and purification protocols. Next to this rather practical reason, the Bet protein itself is from interest for the characterisation of the molecular mechanisms by which it counteracts cellular antiviral immunity and APOBEC proteins. The availability of large amounts of this protein is thereby a prerequisite for such analysis and could be achieved by this means in parallel. Additionally, the Bet protein has been found useful for serological screening to determine feline foamy virus infections by ELISA [83]. Since no purification protocols were available until now, this ELISA assay relies on non-purified GST-Bet protein coupled to glutathione plates by incubation with induced bacterial lysates [83]. An increased sensitivity and specificity of this assay when using purified Bet instead of whole *E. coli* lysates might be expected and could be tested in future experiments.

During initial optimisation experiments, the FFV Bet expression screen revealed that the engineered SCS-1 and T7-Shuffle expression strains produced the highest amounts of recombinant protein in enriched media in contrast to all other strains (Figure 20) SCS-1 as well as T7-Shuffle encode the lacIq repressor gene that inhibits leaky protein expression prior IPTG induction for protection of bacteria in case of toxic proteins [85]. Although no influence on bacterial growth could be detected (Figure 22) reducing basal expression seemed to be beneficial. Furthermore, in contrast to all other bacteria that are derivatives of

the E. coli BL21 lineage, the SCS-1 strain has a genetic background from E. coli K12 and supports the expression of eukaryotic t-RNAs for the generation larger amounts of eukaryotic proteins by avoiding codon-usage problems what might explain SCS-1's superiority in overexpressing FFV Bet. Sufficient overexpression of a target proteins is essential for successful purification but often correlates with inclusion body formation [106]. Similar result was experienced here for the FFV Bet protein (Figure 20 and Figure 21). Whereas the insoluble protein was well expressed, only minor amounts were detectable in the supernatant by Western blot (Figure 20). All attempts to increase the levels of soluble Bet protein by varying growing temperatures and IPTG concentrations to down regulate expression rates and avoid protein misfolding, adding non-ionic detergents as mild solubilisers during protein extraction or fusing Bet to the maltose binding protein (MBP) as a highly soluble fusion partner were insufficient (Figure 24 and Figure 26). A potential reason for this extreme insolubility could be that the 45 kDa large modified FFV Bet protein contains thirteen cysteines which form disulfide bonds for intramolecular stability. The natural reducing environment in the bacterial cytosol of E. coli inhibits the formation of disulfide bridges resulting in non-native inter- and intramolecular cross-linking of expressed proteins and thereby facilitates their aggregation [107]. The insufficient yields and the degradation problem encountered with the soluble FFV Bet prompted the purification of the target protein under denaturing conditions from inclusion bodies. Inclusion body proteins are devoid of defined tertiary structure and establishing protocols for their solubilisation, purification and refolding to recover their structure and functionality are usually very elaborate to establish. On the other hand purifying proteins from inclusion bodies has also several advantages like easy separation of the particles from residual cell material due to their high density, high concentrations of the target protein in the aggregates and increased resistance to proteolytic degradation [103, 105, 107-109]. Indeed, FFV Bet inclusion bodies purified under denaturing conditions were better protected from proteolytic degradation and could be obtained as a stable and more pure protein with much higher yields compared to its soluble counterpart (Figure 27 and Figure 28). After the isolation of FFV Bet inclusion bodies, they were solubilised by 6M GuHCl. High concentrations of chaotropic agents such as GuHCl or urea are able to solubilise aggregates by disruption the inter- and intramolecular interactions of the secondary structure, leading to their denaturation and solubilisation [108]. GuHCl is compared to urea a stronger denaturant and allows the efficient solubilisation of extremely aggregated inclusion bodies. [105]. Since the interaction between the fused N-terminal His tag of the

recombinant proteins does not depend on tertiary structures, FFV Bet as well as FFV Bet/HIV-1 hybrid proteins could be successfully purified in their denatured form resulting in high yields up to 28 mg/L and purity of more than 90% (Figure 28 and Figure 37). This purity is comparable with purity precondition needed for the generation of therapeutic proteins.

4.3. Renaturation of denatured antigens

In this work, the main challenge for protein production was the transfer of the denatured proteins into a non-denaturing environment that allows reconstitution of their soluble structure. This was a crucial step for the generation of the FFV Bet/HIV-1 hybrid antigens because the more the antigen resemble the structure of the epitope on the virus the higher would be the probability to elicit antibodies that recognise complex conformational epitopes as they exist on gp41 which are then able to bind to the target with high efficiency. Since Anfinsen demonstrated in 1973 the spontaneous *in vitro* refolding of the enzyme ribonuclease A it is well known that refolding after denaturing purification is possible and has become an important tool for recombinant protein production [110]. Although there exist some general rules, hundred of different techniques and conditions for protein renaturing are described in the literature and a refolding protocol has to be individually established for every new protein. Convenient methods for protein refolding are direct dilution, membrane controlled denaturant removal and chromatographic or matrix-assisted methods. An effective refolding protocol is hard to find and usually includes testing of various combinations of methods and conditions such as temperature, pH, ionic strength and additives like sugars, polyols, salts and amino acids as supporters of protein folding [106]. The simplest way and one of the most used methods is to dilute the concentrated protein-denaturant solution by adding it in pulses or continuously in refolding buffer. Such protocols have been successfully applied for the production of the human prourokinase, salmon growth factor, human angiogenin and the arginine deaminase to mention only a few [105, 111-115]. Suitable refolding conditions for FFV Bet were also found by rapid dilution in presence of different buffer conditions and further incubation for 24 h (see 2.3.10). Successful refolding was achieved with 50 mM HEPES, 300 mM NaCl, 100 mM L-arginine at a pH of 9.0 (Figure 29). The strong aggregation suppressing potential of L-arginine is well known and was already used for the refolding of several therapeutic proteins like the human tissue type plasminogen activator (t-PA), Fab antibody

fragments and the interleukin-6 receptor [116-118]. The guanidine head group of arginine interacts with tryptophan residues of proteins and shields hydrophobic regions of partially folded chains what positively influences the solubility and stability of proteins during refolding [105, 119]. The presence of NaCl stabilises protein folding by preventing nonspecific electrostatic interactions between proteins due to an increase in the interfacial tension between the protein surface and the solvent [108]. Proteins with multiple disulfide bonds like the FFV Bet protein are found difficult to refold because only correctly folded disulfide bonds yield a stable and non-aggregated protein. The pre-incubation of the protein-denaturant mixture with the reducing agent ß-mercaptoethanol allows the disruption of interchain and non-native disulfide bonds formed during expression and finally completely linearises the protein. This is a requirement for efficient formation of native disulfide bridges during oxidation of the free cysteines by molecular oxygen in the refolding buffer. The best conditions for refolding of disulfide bonds is commonly achieved at alkaline pH what correlated with the results obtained in the refolding screen (Figure 29) [105]. Based on this data efficient refolding of FFV Bet was performed at a pH of 9.0. Furthermore, HEPES serves as buffer to provide a stable pH optimum. After release of the protein from refolding conditions by dialysis, only minor precipitates were obvious which were removed by centrifugation and filtration. The resulting supernatant contained high concentrations of soluble FFV Bet proteins. By later replacement of the molecular oxygen/thiol redox system to reduced and oxidised glutathione and switching to stepwise dialysis instead of rapid dilution, non-aggregated Bet protein with defined secondary structure that specifically interacts with feline APOBEC could be obtained. Moreover an analysis of the crystal structure of this new protocoled refolded Bet is in preparation to provide a successful reconstitution of the FFV Bet protein (publication in preparation). In summary, protocols for expression, purification and refolding for the generation recombinant FFV Bet and FFV Bet/HIV-1 proteins were successfully established.

4.4. Characterisation of the antigenicity of produced antigens

When the integrity of the produced proteins were analysed using E1 and E2 specific antibodies in Western blot (Figure 41), all antigens containing the MPER or FPPR epitope were recognised by 2F5 and 4E10 or an E1 specific antiserum. For all antigens, the single domain antigens were recognised with the highest intensity by the HIV neutralising antibodies followed by a weaker reactivity to the FFV Bet-E1-loop-E2 antigen and a very

low recognition of the MBP E1-loop-E2 protein in the Western blot analysis (Figure 41B). In Western blot the proteins are denatured and linearised because of the environmental conditions. Therefore, not correctly folded conformational epitopes in the protein structure of the generated antigens could not explain this result. However, the FFV Bet loop constructs exhibit a higher molecular weight (55 kDa) than the FFV Bet-E2 and Bet E1 antigens with a size of 49.9 kDa and 49 kDa. Since equal amounts of protein were loaded onto the gel but not equimolar amounts of the introduced epitopes, this effect might be a result of less antigen presented to the antibody in case of the loop constructs. This size difference results in about 10% less E2 epitope in the larger proteins, what correlates well with the observed intensity difference (Figure 41B). The even lower intensity in which the MBP loop construct was recognised is probably a result of antigen dissociation as mentioned earlier. In all cases, the monoclonal antibody 2F5 recognised its epitope in the E2 sequence much stronger compared to the 4E10 although both epitopes are located in the MPER (QEKNEQELLELDKWASLWNWFNITNWLW). One explanation for this might be that 2F5 binds to the ELDKWA sequence even as simple linear peptide, whereas the 4E10 epitope WFNITNWLW forms an ordered helical peptide structure which is sensitive to denaturation [55]. In line with this, Zwick et al., 2001 and 2005, also showed in a previous study that the affinity of 4E10 to denatured recombinant gp41 is strongly decreased compared to native gp41 and the reverse phenomena was seen for 2F5 [64, 120]. One rationale to use Bet as carrier protein in foamy viruses was its ability to be secreted from infected cells and present introduced epitopes to the immune system. An important question was therefore if the HIV epitopes fused to the Bet protein were surface exposed or hidden within the generated antigens under physiological conditions. Therefore binding of antibodies was also investigated with antigens presented in solution in an ELISA setting. In this experiment 2F5 recognised all antigens in a similar pattern as in the Western blot but with slight background reactivity to the Bet E1 protein. However, 4E10 bound in contrast to the Western blot experiment with an about threefold stronger intensity to the Bet loop antigen when compared to the single domain Bet E2. This suggests that the presence of the FPPR domain in this construct strongly improves binding of 4E10 and might be a result of altered MPER confirmation as has been anticipated during design of this protein. The construction of the loop antigens was a consequence of the apprehension that Bet E1 and Bet E2 antigens might not allow an interaction of both domains due to sterical constraints caused by the large Bet fusion partner. In line with this, no increased

interaction to monoclonal antibodies could be detected when both antigens were applied together in this assay.

4.5. Antisera characterisation

When the immunogenicity of produced antigens, antisera were analysed for antibodies to their immunogen and HIV epitopes by ELISA titration, results showed that all antigens seemed to be highly immunogenic and induced titres in a range of 10^5 up to 10^7 (Figure 43). In a next step, the level of antibodies specific for the HIV E1 or E2 domains was analysed. Therefore sera were titrated against equal amounts of a synthetic E1 or E2 peptide as well as gp41 coated on ELISA plates (Figure 44 and Figure 45). These experiments revealed that antibodies against the introduced HIV parts are generated with moderate titres and that the E1 domain is more immunogenic than the E2 domain resulting in 2 to 2.5 logs higher E1 titres. This finding correlates with the low immunogenicity of the MPER domain described in the literature [26]. The higher immunogenicity of the E1 domain could be an explanation for the lower antibody response induced against the E2 peptide in rats that were immunised with both single domain antigens and the loop constructs compared to rats that were immunised with the FFV Bet-E2 antigen only. Notably, in all groups that developed high antibody titres to E2 the immune response to this epitope decreased drastically for two rats of group 365 and for all rats in group 366 that were immunised with a mixture of FFV Bet-E1 and Bet-E2, but not for the loop constructs. The E2 response induced by HIV-1 loop antigens with titers of 10^1 for MBP/HIV-1 loop and 10^2 or FFV Bet/HIV-1 loop antigen were extremely low, but showed no reduction in antibody titers during the course of immunisation compared to animals of group 365 and 366 with high E2 titers even after the first immunisation (Figure 44B and Figure 45). Some authors reported that the induction of 2F5 and 4E10 like antibodies might be prevented by the immune system since they are self-reactive and represent autoimmune antibodies which will be deleted by immunologic tolerance mechanisms [102, 121]. It could be hypothesised that such a downregulation of E2 specific B cells is also the reason for the reduction in antibody titres in the mentioned rat groups. By ELISA using recombinant gp41 it could be confirmed that the generated antibodies were not just able to recognise the synthetic peptides but also reacted with their epitopes when presented in the whole molecule context. Interestingly, gp41 titers of rat group 365 and 366 were obviously not affected by the decreased course of E2 titers and further increased with the third

immunisation (Figure 44B and Figure 45A). Dependent on the immunised antigen, the gp41 response reached mean enpoint titers with three logs difference in a range from 10^3 to 10^6 (Figure 44 and Figure 45). The highest gp41 titres were induced by vaccination with the FFV Bet-E1 single domain antigen with mean titers up to 10^6 what can be a result of the higher immunogenic potential of the E1 domain (Figure 44A). For the double domain vaccination highest gp41 titers were achivied if the animals were immunised with both FFV Bet/HIV-1 single domain antigens (Figure 45A). The lowest gp41 antibody titer was obtained for the FFV Bet E1-loop-E2 antigen (group 375) with an average value of 10^4 (Figure 45C). In the group 374 which was immunised with the MBP/HIV-1 hybrid antigen, average titers were in the range of 5×10^5 (Figure 45B). In case of rat group 364 (FFV Bet-E1), 366 (FFV Bet-E1 and FFV Bet-E2) and 374 (MBP-E1-loop-E2) one rat developed an incredible increased immune response against gp41 with titers of 10^7 up to 10^9. However this high gp41 immune response did not correlate with a higher E1 or E2 response. A reason therfore can be that the produced antibodies in these animals recognise conformational epitopes on the recombinant gp41 but not on the linear E1 or E2 peptide.

In conclusion, these ELISA experiments showed that also the produced FFV Bet-E1 and FFV Bet-E2 antigens as well as the loop constructs induced antibodies against their respective immunogen and also with specificity to the introduced HIV-1 domains with moderate titers.

Preliminary data from immunofluorescence experiments on HIV-1 infected cells further showed that these sera also recognise gp41 when presented as native trimeric gp160 env molecule on the cell surface (Mühle et al. unpublished data). Further analysis to investigate the binding of serum antibodies also to viral particles by electron microscopy is currently in preparation.

4.6. Neutralising potential of generated antisera

Antisera were also examined for their neutralising capacity by challenge with HIV-1 pNL-4.3 in a TZM-bl based neutralisation assay (Figure 46). Despite the fact that produced sera strongly recognised their immunogen and contained HIV-1 E2 specific antibodies they were not able to prevent HIV-1 infection. Since the ELISA experiments were performed with the full length E2 domain, it is unclear to which exact amino acids antibodies were

generated against. It would be interesting to see by epitope mapping if this sera recognise the intended ELDKWAS and WNWFNITLW epitopes and if not or only partially, might explain the lack of neutralisation. The antigenicity data obtained in this study showed that the FFV Bet loop construct is able to increase the binding of 4E10, suggesting that a beneficial conformation for epitope presentation is present in this antigen. However, in this most promising construct the titres of induced E2 antibodies was relatively low (mean titre 3-4x10^2). The lack of neutralisation might therefore be a consequence of insufficient amounts of E2 specific antibodies. It is currently investigated if concentration of MPER specific antibodies by affinity chromatography will allow virus neutralisation. Interestingly, Wang et al., 2011, reported recently the elicitation of MPER specific neutralising antibodies with a similar loop construct. They developed three trimeric antigen consisting of the HIV gp41 NHR and CHR domains connected by a loop in which the CHR domain has a protruding MPER domain. One antigen was modified by a deletion of two single amino acids, one in the NHR and the other in the recognition epitope of 4E10 located in the MPER. These deletions have been shown to allow a better exposure of the conserved neutralising epitopes of 2F5 and 4E10 and enhance immunogenicity. They also observed no neutralisation when they analysed their antisera or IgGs affinity purified with the immunised immunogen. However, when they isolated the MPER specific antibody population, these IgGs showed inhibitory activity against HIV-1 mediated syncytium formation and infection with an HIV-1 pseudovirus as well as some primary HIV-1 isolates [122]. Introducing such mutations might also be an interesting improvement for the Bet loop antigens created in this work. Next to this, it also shows that minor changes in these domains can have a dramatic influence on antigenicity and immunogenicity. In this regard, expression in *E. coli* and the applied denaturing purification strategy might also influence the outcome of the immunisation study. The use of a replication competent foamy virus would circumvent this problem because the antigen would be produced by the host after integration of the provirus into the genome and guarantee a correctly folded antigen. The combination of a better exposed neutralising epitope and the use of a replication competent virus to deliver such a native antigen permanently to the immune system to allow affinity maturation of B-cells will be a useful approach for the generating of an effective vaccine. The results of this thesis thereby support the strategy of using the foamy viral Bet protein for this purpose.

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