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Construction of antibody containing vectors for transfection into RMCE competent CHO cell line

Master Thesis

submitted by
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1 Acknowledgements

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Thank you all…

2 Abstract

The Chinese hamster ovary CHO cell is one of the most used mammalian cells for biotechnological production of drugs. The primary use lies in the production of antibodies. The traditional method in development of such a CHO cell line mostly starts with constructing a vector, which comprises the sequence of the antibody. Next step is the transfection of the recombinant DNA into the mammalian host cell, which will be integrated at a random genome locus and with an uncertain gene copy number (GCN). These factors lead to a tremendous time taking screening procedure to find the best antibody producing clone. Nowadays it is very important to gain safe antibodies in a short period. To undergo that huge screening cost the recombinase mediated cassette exchange (RMCE) system was developed. These system works with specified recognition sequences which are integrated at an active genomic locus of the host organism. These signal sequences called FRT flipase recognition target will be recognized by the recombinase flipase and are used to integrate recombinant DNA into this genomic active locus.

Within this work, two vectors for transfection into a RMCE competent CHO cell line had to be constructed. The two vectors should contain the sequence of two different antibodies. Therefore a super humanized SH3H6 and a chimeric CH3H6 antibody was created. The two sequences of antibodies only differ in their variable light and variable heavy chain domain. Rest of the vector DNA must be identically. Therefore in future works it should be possible to make comparably studies with regard to productivity.

3 Zusammenfassung

Die CHO Zelle ist die am häufigsten verwendete Säugetierzelle zur biotechnologischen Produktion von Arzneimitteln. Hierbei liegt ein großes Augenmerk auf der Produktion von Antikörpern. Beim traditionellen Verfahren einer solchen Zelllinie, erfolgt zunächst meist die Erstellung eines Plasmids, welches die nötige Information des Antikörpers trägt. Als nächster Schritt erfogt die Transfektion der rekombinanten DNA in den Wirtsorganismus, welche an zufälliger Stelle und in unbestimmter Anzahl in das Genome integriert wird. Dies führt zu einem massiven Screeningverfahren das viele Ressourcen und vor allem viel Zeit in Anspruch nimmt. In der heutigen Zeit ist es sehr wichtig schnell und sicher Antikörper herzustellen. Um dieses aufwändige Screeningverfahren zu minimieren, wurde das Rekombinase vermittelte Kassettenaustausch (RMCE) System entwickelt. Dabei handelt es sich um ein System bei welchem bestimmte Signalsequenzen an einem aktiven Genlocus in den Wirtsorganismus eingebracht werden. Diese Signalsequenzen genannt FRT sites können von einer Flipase erkannt und dazu genutzt werden zielgerichtet DNA in diesen aktiven Genlocus zu integrieren.

Ziel dieser Masterarbeit war es zwei Vektoren mit unterschiedlichen Antikörpersequenzen herzustellen, die in weiterer Folge in eine RMCE kompetente CHO Zelllinie transfiziert werden können. Hierbei wurden ein super humanized SH3H6 und ein chimärer CH3H6 Antikörper hergestellt. Diese beiden Antikörper sollen sich nur in den variablen Domänen der leichten und schweren Ketten des Antikörpers unterscheiden. Die konstanten Domänen der Antikörper, Signalsequenzen und der Rest der Vektoren müssne ident sein. Dadurch ist es später möglich diese beiden Vektoren in jeweils CHO Zellen zu tranfizieren und Vergleichsstudien durchzuführen.

4 Table of Contents

5 Abbreviations and Units

5.1 Abbreviations

AmpR	ampicilin resistence gene
ApE	a plasmid Editor
BAC	bacterial artificial chromosome
BLAST	Basic Local Alignment Search Tool
BSA	bovine serum albumin
CDR	complementarity determining region
CHO	chinese hamster ovary
CH3H6	chimeric protein model 3H6
CIP	calf intestine phosphatase
CMV	cytomegalovirus
ori	origin of replication
DHFR	dihydrofolate reductase
DNA	deoxyribonucleic acid
dH2O	autoclaved water
dNTP	deoxynucleotide
E.coli	Escherichia coli
EDTA	ethylenediaminetetraacetic acid
FASTA	“FAST-ALL” DNA and protein sequence alignment software package
FDA	Food and Drug Association

FRT	flipase recognition target
GFP	green fluorescent protein
GOI	gene of interest
GCN	gene copy number
HC	heavy chain
IRES	internal ribosome entry site
KanR	kanamycin resistence
KDp	Klenow DNA polymerase
LB	lysogeny broth
LC	light chain
loxP	locus of crossing over (x) derived from bacteriophage P1
MCS	multiple cloning site
NEB	New England Biolabs
NeoR	neomycin resistence
neo	neomycin
ori	origin
PCR	polymerase chain reaction
rDNA	recombinant DNA
RMCE	recombinase mediated cassette exchange
SH3H6	super humanized protein model 3H6
SV40	siamin vacuolating virus 40
Taq	*Thermus aquaticus*
TAE	tris base, acetic acid and EDTA

TRIS	tris(hydroxymethyl)aminomethane
UV	ultraviolett
vH	variable heavy chain
vL	variable light chain

5.2 Units

kb	kilo base
bp	base pair
g	gram
mg	milligram (10^{-3}g)
µg	mikrogram (10^{-6} g)
L	liter
mL	milliliter (10^{-3} L)
µL	microliter (10^{-6} L)
M	molarity (mol/L)
mM	millimolar (10^{-3} M)
µM	mikromolar (10^{-6} M)
mm	milimeter (10^{-3} m)
nm	nanometer (10^{-9} m)
µF	microfarad (10^{-6} F)
°C	degree celsius
Ω	ohm

U	units
h	hours
min	minutes
OD	optical density
% w/w	percentage weight of weight
% w/v	percentage weight of volume

6 Introduction

The first approval of a therapeutic protein, derived from recombinant mammalian cells, took place in 1986 (**WALSH 2010**). Since then Chinese hamster ovary (CHO) cells are the first choice as host for producing biopharmaceuticals. More than 2/3 of all recombinant therapeutic proteins are produced from CHO cells (**JAYAPAL et al. 2007**). There are few main reasons why CHO cells are the predominant hosts for producing proteins. Firstly CHO cells are considered as safe hosts for more than 20 years of research, therefore it is considerably easier to get approval to market from the FDA for a recombinant protein derived from CHO cells **(KIM LY 2012**). Secondly powerful gen amplification methods are available such as dihydrofolate reductase (DHFR) selection system or glutamin synthetase (GS) selection (**KINGSTON 2002**). Thirdly one of the main reasons why CHO cells are used tremendously as production host is its post-translational modification ability and also to produce recombinant protein with glycoforms.

To improve productivity, yield and increased security requirements the production of recombinant proteins in CHO cells has to be continuously developed. This can be done by improving cell line development, up-stream process or down-stream process development. Every process starts with the development of suitable high producer cell lines which is also linked with considerable investment of resources and time (**KIM LY 2012**).

6.1 Traditional development of high-producing CHO cell lines

The common procedure to gain recombinant proteins starts by cloning the desired gene of interest into a vector, such as a plasmid which can be transferred successfully into the mammalian host cell. In these first few steps the functionality and properties of the recombinant protein can be customized by protein-engineering. After cloning of the transgene into the vector this recombinant protein has to be introduced into CHO host cell

line such as DHFR-deficient CHO mostly by lipofection (**REHM 2009**). To save time this CHO host cell lines have been adopted for growth in serum free SF suspension.

The strength of expression of the gene is determined by various factors. The construct of the vector itself, the used promoter, poly A signal and the gene for selection. A significant influence on expression level is defined by the genomic locus where the recombinant DNA is integrated into the host chromosome. In principal the integration of the rDNA happens at a random place. Only a small amount of the genome is active, therefore very few of transfected cells do have the GOI at an active genomic locus (**GORMAN 2000**), (**LITTLE 1993**). This interrelationship between level of transcription and locus of integrated GOI on the genome is also known as the position effect (**KARPEN 1994**). Because of this random integration to find the best producing clone does need good selection system and makes it elaborate. A vast number of clones have to be screened to find the best producing clone. Because of this random integration and the fact that more genes got integrated into one cell makes it hard to compare different studies which were made in focus on productivity (**MAYRHOFER 2013**).

Nowadays there are some approaches to undergo this position effect to gain better producing CHO cells. One of them is to make a transfection with a vector, which comprises of the gene of interest and also an own active transcription gene locus (see bacterial artificial chromosome system (BAC)) (**MADER 2013**).

Another approach is with the help of a recombinase mediated cassette exchange RMCE system. Because of RMCE it is possible to integrate the gene of interest at a predetermined position at the host chromosome. Precondition therefore is a highly transcriptionally active genomic locus identified with a reporter gene which is placed within two heterospecific RMCE recognition sites (**SCHLAKE 1994**), (**QIAO 2009**). The exchange of DNA will be done by the enzyme recombinase. In the last years most used recombinase were Cre and Flp recombinase. Cre recombinase was extracted from *bacteriophage P1* whereas Flp derived from *Saccharomyces cervisiae*. Both of them are well examined recombinases and a lot of studies were made to improve them for better catalytic reaction (**TAKATA 2011**). First used

recombinase was Flp in the Flp/FRT system which had a temperature optimum of 30°C. Flp got later replaced by a recombinase called Cre, which had a better catalytic activity and also a better temperature optimum (37°C) by working in situ. The Cre recombinase is used in the Cre/lox system. It recognizes a 34 bp long sequence called loxP (locus of crossing over (x) from P1). The structure and direction of this loxP sites determines the changes of the genetic material (**MÄGDEFRAU 2007**). Later reports showed that Cre recombinase does have a toxic effect on cells. Because of the development of mutant Flp recombinase with a better temperature optimum and the fact that Cre does have a negative effect on cells made the Flp recombinase again the best choice for working with (**TURAN 2011**).

6.2 Flp/FRT RMCE system

The Flp recombinase is derived from a 2 µm plasmid out of *Saccharomyces cerevisiae* and catalyzes the recombination between inverted sequence repetitions.

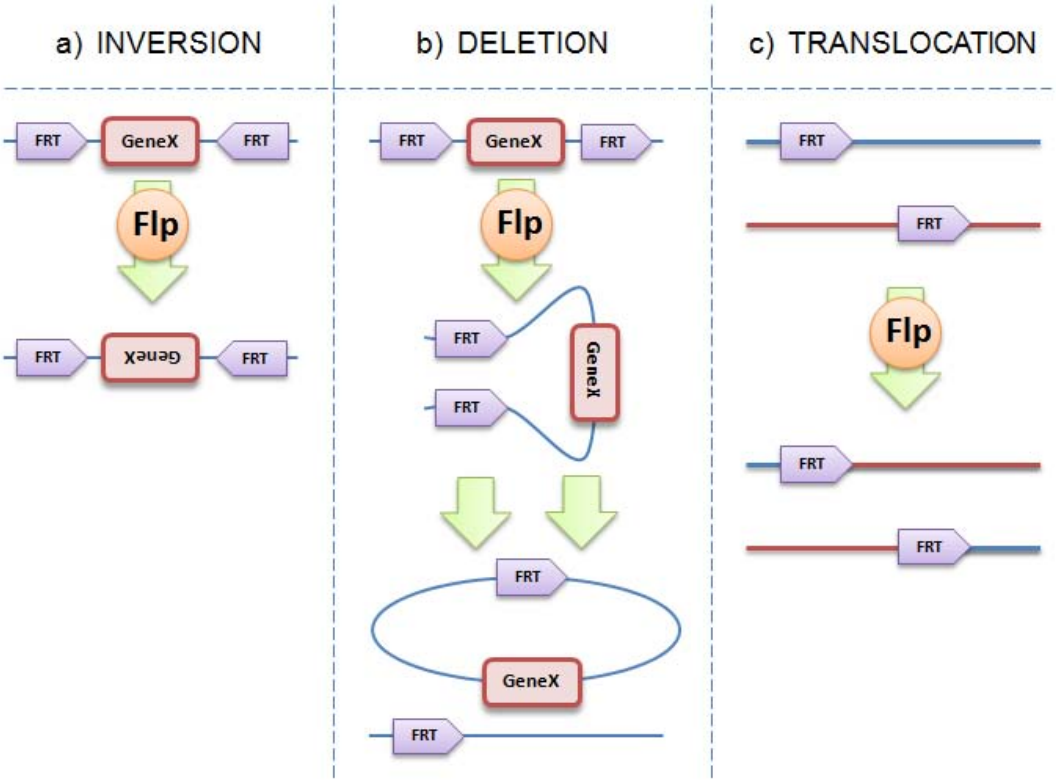


Figure 6.1: Flp/FRT System: **(a)** If two FRT sites are on the same DNA strand and are in opposite orientations, Flp catalyzes the inversion of DNA between the loxP sites. **(b)** If two FRT sites are on the same DNA strand and are facing the same direction, Flp excises the DNA between the FRT sites and creating a circular piece with it

outside of the DNA strand. **(c)** If the sites are on separate DNA molecules, Flp catalyzes the translocation of DNA at the FRT sites.

Figure 6.1 shows the three types of rearrangements which can be done with identical FRT sites. The position and relative orientation of these two FRT sites determine the outcome of the FLP-mediated recombination. This variation of options could be extended significantly by the generation of FRT mutants.

By the use of two heterospecific FRT sites and the Flp recombinase it is possible to exchange DNA within the FRT sites, if the DNA which will be integrated is also flanked by the same FRT sites. Figure 6.2 shows the standard concept of the recombinase mediated cassette exchange RMCE with Flp/FRT system. At a beneficial genomic locus (in the host genome) after a CMV promoter two heterospecific FRT sites are placed. In between this FRT sites are placed reporter and selection genes. Therefore green florescent protein GFP (for flow cytrometric analysis), thymidine kinase TK (as negative selection) and neomycin phosphatase neo (as positive selection) is used.

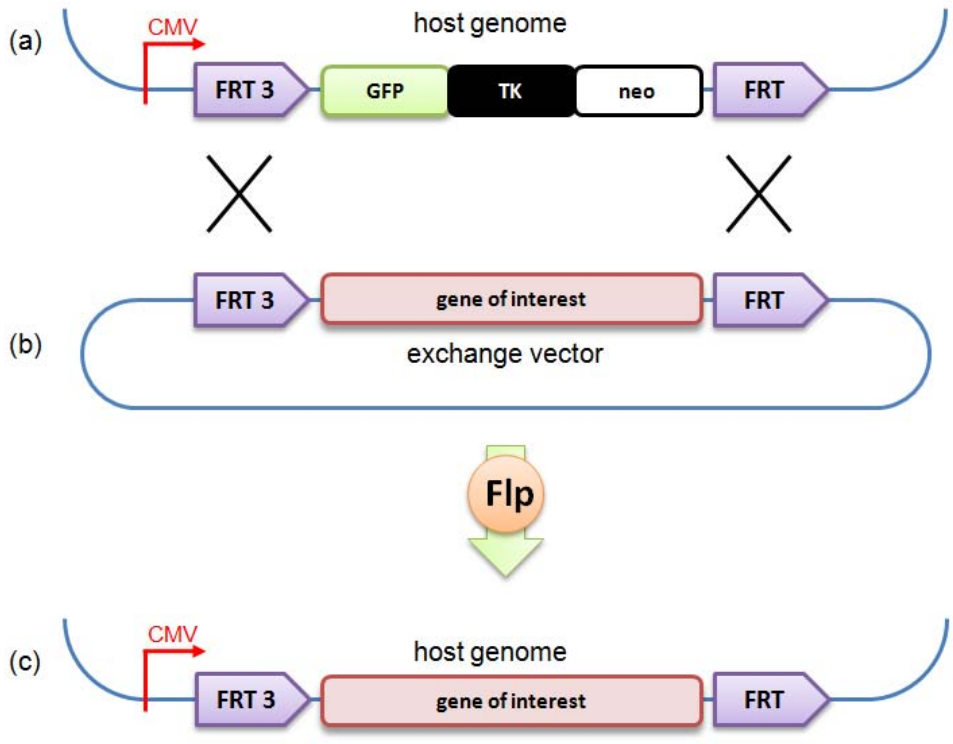


Figure 6.2: standard concept of Flp/FRT system with heterospecific FRT sites. **(a)** Symbolizes the host genome where after a promoter CMV 2 heterospecific FRT and FRT3 sites are located. In between are reporter gene

cassette (GFP, TK, neo). **(b)** The exchange vector consist of the same two FRT sites but in between of them is located the gene of interest (GOI). **(c)** After recombination with Flp enzyme the gene reporter cassette has been exchanged by GOI, which now is located in the host genome after the CMV promoter.

In **Figure 6.3** the FRT and FRT3 sequences are shown. Both FRT sites contain three palindromic sequences (except for a single bp highlighted in a red box) of 13 bp each (yellow boxes), separated by an 8 bp sequence called spacer (green box). The orientation is defined by this asymmetric spacer sequence. The binding mechanism of Flp recombinase is linked to the palindromic sequences, whereas the DNA break, exchange and ligation takes place at the spacer sequence (**Lacroix 2011)**.

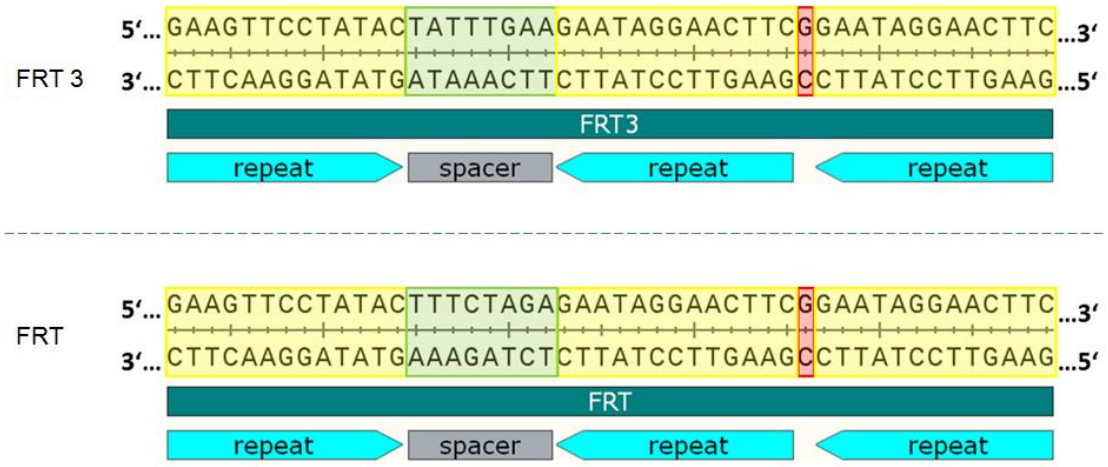


Figure 6.3: FRT and FRT3 sequences.

If a host genome with these specific attributes is available it is possible to integrate the gene of interest GOI always at the same genomic locus. What leads to less screening effort by searching for best producing clones as well as it makes it easier to make comparable studies.

6.3 IgG antibody structure

The IgG antibody is composed of two light chains and two heavy chains (**Figure 6.4**). The light chain is built up from one variable and one constant domain, whereas the heavy chain is composed of one variable and three constant domains joined by the hinge region. For one antibody the super humanized protein model SH3H6 and for the other the chimeric protein model CH3H6 was used. They differ only in their variable regions of heavy and light chain.

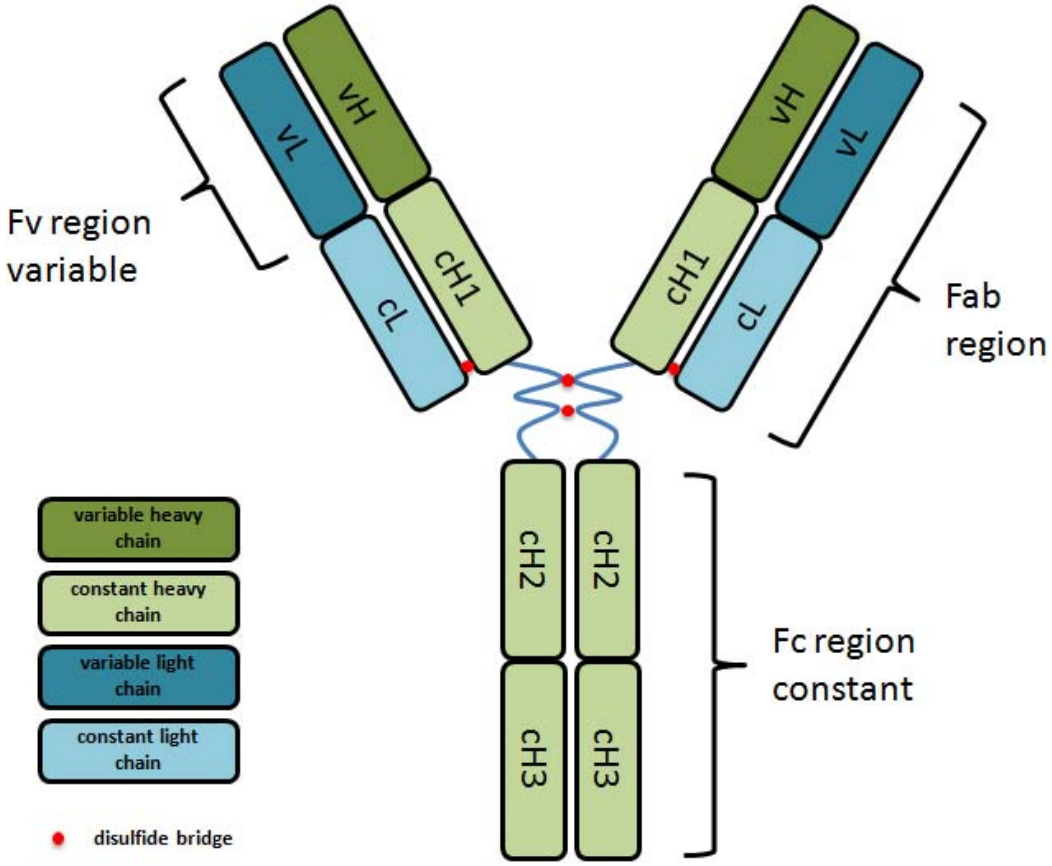


Figure 6.4: schematic of IgG antibody

7 Project Proposal

Within this project, two Plasmids, each of them carrying the sequence of a unique antibody, were constructed. The only differences in these two plasmids are bearing the variable regions of the antibodies (super humanized SH and chimeric version CH). The plasmids are constructed in a way that it should be possible to introduce them into CHO DUKX-B11 cells which are harboring a fully functionally Flp/FRT RMCE system, so in later trials it should be tested if a slight difference in the gene sequence of the variable region of antibodies does have an effect on its productivity in CHO cells, when it gets introduced always at the same genomic locus. These RMCE competent DUKX-B11 cells were constructed in an earlier work done by DI Mayrhofer Patrick (**MAYRHOFER 2013**).

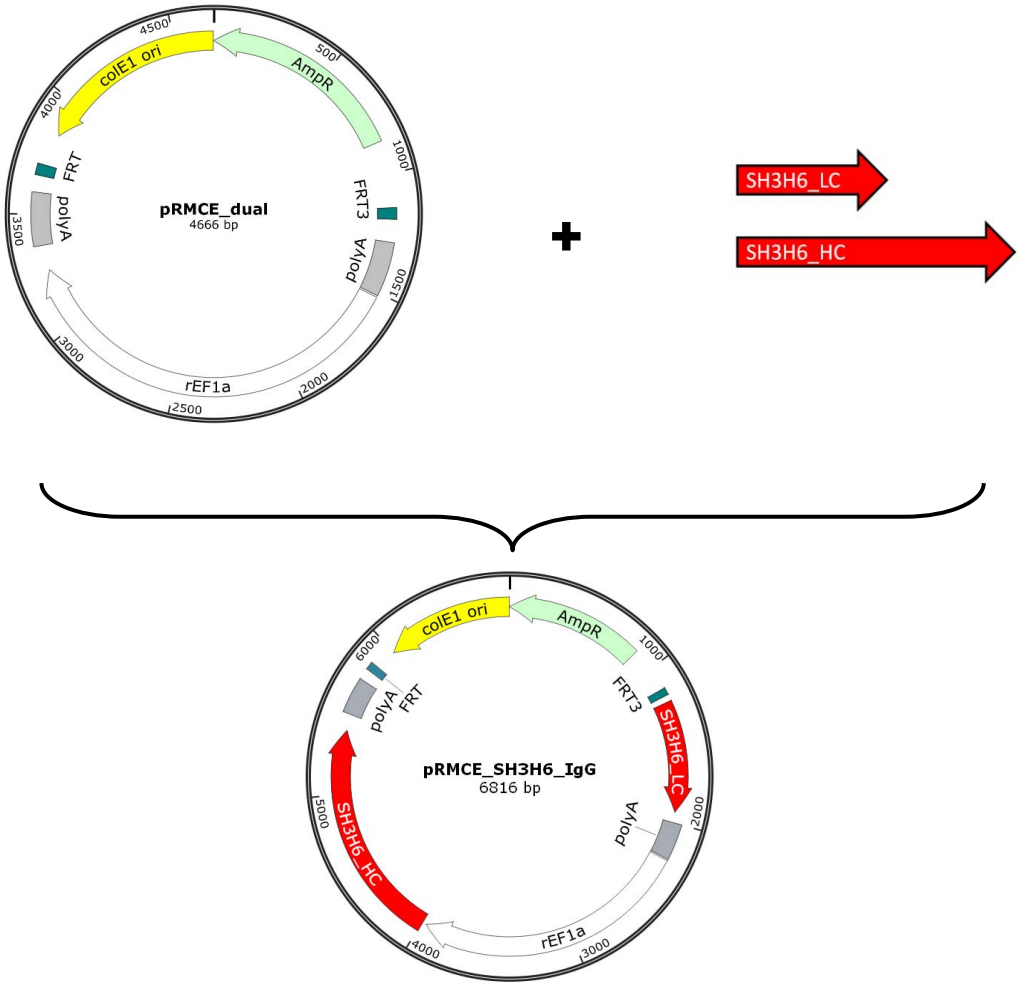


Figure 7.1: simplified schematic of pRMCE_SH3H6_IgG construction.

Therefore both antibodies had to be constructed and cloned into the same RMCE compatible vector (**Figure 7.1**). As DNA vehicle the plasmid pRMCE_dual (**Figure 8.7**) was used. It comprises all features which are necessary for a successful transfection into CHO cells, like two different FRT sites (FRT3 and FRT), poly A signal in between these FRT sites, promoter rEF1α (rat elongation factor 1 alpha) for mammalian cells and as well all required genes for expression in *E.coli*. **Figure 6.2 Figure 7.2** shows the concept of used Flp/FRT system with pRMCE_XX3H6_IgG vector as DNA exchange vector. First the heavy chain (XX3H6_HC) was cloned into the pRMCE_dual vector in between the mammalian promoter rEF1α and the poly A sequence. For completing the vector the light chain (XX3H6_LC) has to be cloned in between the FRT3 and the promoter rEF1a. The promoter for the light chain lies outside the FRT sites on the mammalian host genome as it is described from **Mayrhofer 2013**. This promoter strategy is called “promoter trap” and should prevent the expression of any randomly integrated gene.

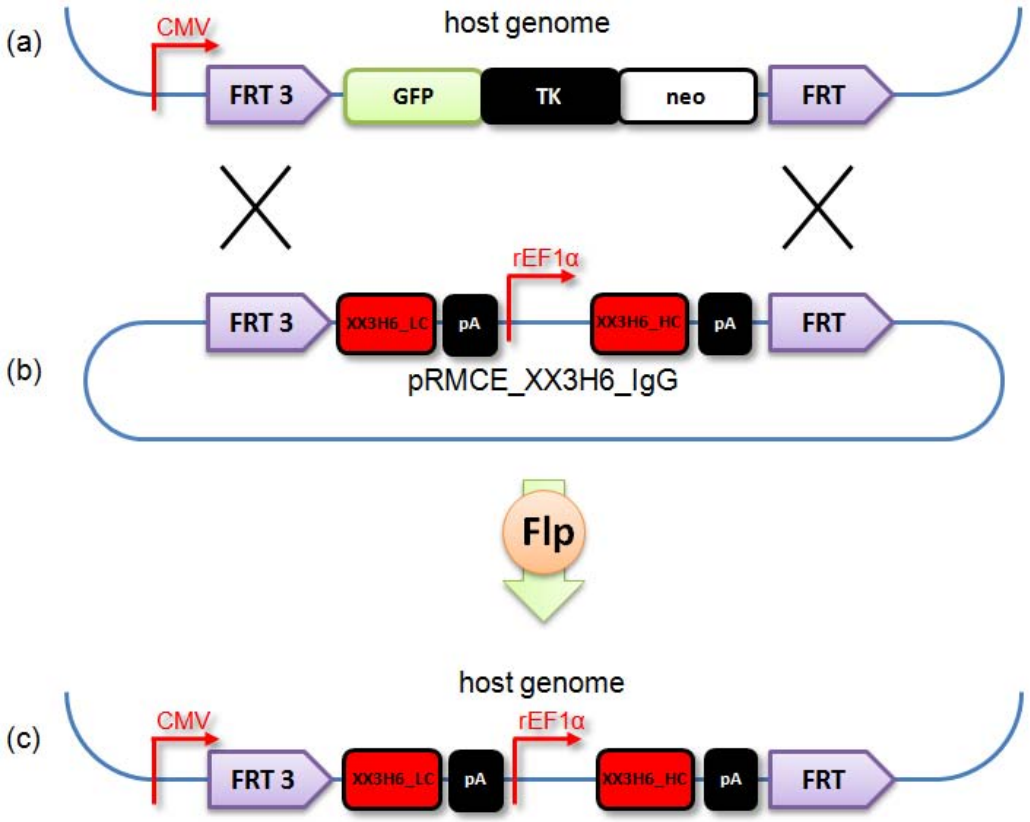


Figure 7.2: concept of Flp/FRT system with heterospecific FRT sites and “promoter trap” strategy. XX stands for either SH or CH version of this plasmid.

Because heavy and light chains of these two antibodies came from different vectors the hard work was to ensure after all cloning steps that there are no differences between these two plasmids except of the variable regions. **Figure 7.1** shows the simplified procedure.

8 Materials and Methods

8.1 Equipment

Photometer	Implen NanoPhotometer P-300
Microscope	Leica DM IL LED microscope, Leica Microsystems GmbH, Type 11090137001
Pipette	Pipetman Neo P1000N, Gilson, Cat. F144566
	Pipetman Neo P200N, Gilson, Cat. F144565
	Pipetman 1000 µL, Gilson, Cat. 51990
	Pipetman 200 µL, Gilson, Cat. DK54448
	Pipetman 100 µL, Gislon, Cat. CN61618
	Pipetman 20 µL, Gilson, Cat. CM57486
	Pipetman 10 µL, Gilson, Cat. P58933A
multipipette	Gilson, 8x200 µL, Ref. F14403
centrifuge	Megafuge 16 Heraeus, Thermo scientific, Cat. 75004230
	Megafuge 40 Heraeus, Thermo scientific, Cat. 41017119
	Eppendorf centrifuge 5424
	Eppendorf centrifuge 5415R
	DownSpin PVC-2400 Grant-bio
autoclav	Varioklav, H+P Labortechnik GmbH
vortex mixer	Vortex genie® 2, Scientific industries, Model. G560E
electroporator	Gene Pulser XcellTM, Bio-Rad

thermoblock	Thermomixer Comfort , Eppendorf
	Thermomixer Compact 5350, Eppendorf
thermocycler	Bio-Rad C1000 Thermal cycler serial CC008738
Centrifuge rotor	TX-400 Swinging Bucket Rotor Thermo scientific 75003629
Thermomixer	VWR VS-C4-2
balance	Sartorius balance AW-4202 24407903
agarose gel analyzer	Gel DocTM XR, BioRad
gel electrophoresis chamber	BioRad
power supply for electrophoresis	Power Pac Basic, BioRad

8.2 Material

8.2.1 Chemicals and reagents

Agar-agar	Merck, Cat.1.01614.1000,
Agarose	peqGOLD Universal-Agarose, peQLab, Cat. 35-1020
Ampicillin	100 mg/mlAgar-Agar: Merck, Cat No. 1.01614.1000
BSA (100X)	Cat. B9001S, New England Biolabs
Buffer NEB1 (10X)	Cat. B7001S, New England Biolabs
Buffer NEB2 (10X)	Cat. B7002S, New England Biolabs
Buffer NEB3 (10X)	Cat. B7003S, New England Biolabs
Buffer NEB4 (10X)	Cat. B7004S, New England Biolabs

DNA gel loading dye (6X)	Cat. R0611, Fermentas
Cutsmart buffer	Cat. B7204S, New England Biolabs
Klenow	Cat. M0210S, New England Biolabs
dNTPs (10mM stocks)	Cat. N0447L, New England Biolabs
EDTA	500 mM, New England Biolabs, Cat. No. B0255A
Ethanol	Emplura Ethanol absolute, Merck, Cat. No.8.18760.2500
Ethidium Bromide	Sigma Aldrich, Cat. No. E1510, 10 mg/ml
Generuler ladder (0,5 µg/µL)	Cat. SM0331, Fermentas
Glycerol Merck	Cat. 1.04092.2500
Liquid Nitrogen	
SOC Medium	
T4 DNA Ligase buffer	Cat. B0202S, New England Biolabs
Thermopol buffer (10X)	Cat. B9004S, New England Biolabs
Ultra-pure Water	Fluka Cat. No. 14211, autoclaved

8.2.2 Consumables

Pipette tips	Gilson 1000 µL, greiner bio-one, Cat.740290
	Gilson 200 µL, greiner bio-one, Cat.739290
	10µL, Cat. K138.1, Roth
Hemocytometer cover glasses	Marienfeld, 22x22x0.4 mm, Ref. 0351000
Cryo-tubes	NuncTM cryotubeTM vials, Cat. 375418
Petri dishes	Petri dish 94 × 16 mm Greiner Bio-One, Austria

Serological pipettes	Costar stripette 2 mL, Cat. 4486
	Costar stripette 5 mL, Cat. 4487
	Costar stripette 10 mL, Cat. 4488
	Costar stripette 25 mL, Cat. 4489
	Costar stripette 50 mL, Cat. 4490
Microtubes	Microtube 2 mL Sarstedt, Ref. 72.694.005
Centrifuge tubes	Nunc centrifuge tubes, Cat. 347856
	Eppis VWR Cat. 20170-038 micro-centrifuge tubes for high G-forces
Glas vials	Perkin Elmer OVE0100
PCR-tubes	Quiagen 0.2 mL, Cat. 981005

8.2.3 Kits

Wizard SV Gel and PCR Clean-up system, Promega,	Cat. A9282
NucleoBond Xtra Midi EF,	Cat. 740420.50
PeqGOLD Plasmid Miniprep Kit I	Cat. 12-6942-02
Nucleo Spin® Gel and PCR Clean-up Kit I	Cat. 74060950

8.2.4 Enzymes

Restriction enzymes:

AgeI-HF:	New England BioLabs, Cat. No. R3552S

ApaI:	New England BioLabs, Cat. No. R0114S
BsiWI:	New England BioLabs, Cat. No. R0553S
BspEI:	New England BioLabs, Cat. No. R0540S
BstZ17I:	New England BioLabs, Cat. No. R0594S
KpnI:	New England BioLabs, Cat. No. R0142S
MfeI-HF:	New England BioLabs, Cat. No. R3589S
NotI-HF:	New England BioLabs, Cat. No. R3189S
XhoI:	New England BioLabs, Cat. No. R0146S

Alkaline phosphatase Calf intestinal (CIP):	New England BioLabs, Cat. No M0290S
DNA Polymerase I Large (Klenow) fragment:	New England BioLabs, Cat. No. M0210S
T4 DNA Ligase:	New England BioLabs, Cat. No. M0202S
Taq DNA polymerase:	New England BioLabs, Cat. No. M0321S
Peqlab, Kapa Hifi Polymerase,	Cat. No. KK2101

8.2.5 Buffers and solutions

50x TRIS Acetate-EDTA (TAE) Buffer:

2 M	Tris(hydroxymethyl)-aminomethan (TRIS)
1 M	acetic acid (glacial)
50 mM	EDTA (0.5 M stock adjusted to pH 8)

TAE running buffer:

1x	TAE buffer
300 µg/L	Ethidium bromide

6x Gel loading Buffer (BX buffer):

0.25% (w/v)	Bromophenol blue
0.25% (w/v)	Xylene cyanol FF
30%	Glycerol in water

LB-media:

10 g/L	Bakto Trypton or Peptone out of Casein
5 g/L	Yeast-Extract
170mM	NaCl; pH 7.0

LB_{amp} media:

500 mL	LB-media
500 µL	100 mg/µL ampicillin

SOC media:

20 g/L	Bacto Trypton
5 g/L	Yeast-Extract
10 mM	NaCl
3 mM	KCl
10 mM	$MgCl_2.6H_2O$
20 mM	Glucose
10 mM	$MgSO_4.7H_2O$

LB-Agar Plate with Ampicillin:

LB-media	
1.5 %	Agar-Agar
100 µg/mL	Ampicillin 13

BSA (100X):

20 mM	Tris-HCl
100mM	KCl
0.1 mM	EDTA
50%	Glycerol

BX loading buffer 1X (DNA gel loading dye):

10 mM	Tris-HCl (pH 7.6)
0.03%	bromophenol blue
0.03%	xylene cyanol FF
60%	glycerol 60 mM EDTA

8.2.6 Oligonucleotides

All primers listed in **Table 8.1** were purchased from Sigma-Aldrich. For getting a yield of 100 µM stock solution of every lyophilized primer, dH_2O were added according to manufacturer's instructions. For further experiments like PCR and sequencing the stock solution was diluted 1:10 with dH_2O for getting a 10 µM working solution.

Table 8.1: list of all used oligonucleotides

nomenclature	sequence (5' > 3')	Tm [°C]
13H5A5_HC_987_as	CAGCCAGTCCTGGTGCAG	66.1
AgeI_lead_3H6vH_s	GCCACCGGTGTCCACAGCGAGGTCCAACTGCAGCAGTCTG	90.9
ApaI_3H6vH_CH1_as	GATGGGCCCTTGGTGGAGGCTGC	78.8
BsiWI_ckappa_as	ACCGTACGCTTGATCTCCAGGTT	68.7
BspEI_lead_3H6vL_s	TATCCGGAGCCTACGGCGAGACAACCGTGACC	83.3
for L:F3:[29](GAC)D>Y(TAC)	GACGTGGCCTACTACTACTGC	60.4
HC_lead_sense_opt	ATGGACTGGACCTGGCGGAT	70.1

nomenclature	sequence (5' > 3')	Tm [°C]
huIgG_CH1_as_opt	GTGCCGCCGCTGGTGCTC	75.2
kan/neo_NheI_as	GTGCTAGCGGCAGGTATTAGGGATAATCCTAGC	60.2
KpnI_SH3H6_LC_as	ATGGTACCTCAGCACTCGCCCCTGT	74.9
KpnI_SH3H6_LC_s	ATGGTACCACCATGGCCCTGCAGAC	76.0
MCS_AgeI_s	AACCACCGCTAATTCAAAGCA	58.8
MCS_AgeI_s	AACCACCGCTAATTCAAAGCA	58.8
pCEP4_gagpol_as7	GCAATAGCATCACAAATTTCACA	63.6
pCIneo_976sense	GGCACCTATTGGTCTTACTGA	57.0
pIRESdhfr_CGA_578_sense	GCAGTACATCTACGTATTAGTC	48.0
pIRESdhfr_CGA_760_sense	CCCCATTGACGCAAATGGGC	59.0
pMG433_3D6IgA_LC_1983s	TGTGCCTGCTGAACAACTTC	64.0
pMG433_FSHB_2465_as	GGAAATGTTGAATACTCATACTC	47.0
pROM111_1314as	GCCGCTCTGCAGGGCGTTGT	66.0
pROM112_1687s	GAGGAGCAGTACAACAGCACC	57.0
rEF1a_PacI_a	TTTTGCTGGCCTTTTGCTCA	59.2
rev L:F3:[29](GAC)D>Y(TAC)	GCAGTAGTAGTAGGCCACGTC	60.4
rev_seq pCEP4	CTGCATTCTAGTTGTGGTTTGTC	62.4

8.3 Plasmid maps

8.3.1 pROM108

The 6.3 kb plasmid pROM108 (**Error! Reference source not found.**) was used for the integration of the chimeric variable domain (CH3H6_vH) of heavy chain in between heavy chain leader and the constant domain (3H6_cH). It consists of a bacterial origin of replication (ColE1 ori) and the ampicillin resistance gene (AmpR) for plasmid amplification and selection in E.coli. The essential feature of this plasmid is a 1 kb long gene, which is also controlled by SV40 promoter, and decodes for a leader and the three constant regions of chimeric heavy chain of IgG1. Between leader and constant region this plasmid can be digests by two enzymes (*ApaI*/*AgeI-HF*), which allows to insert the variable domain.

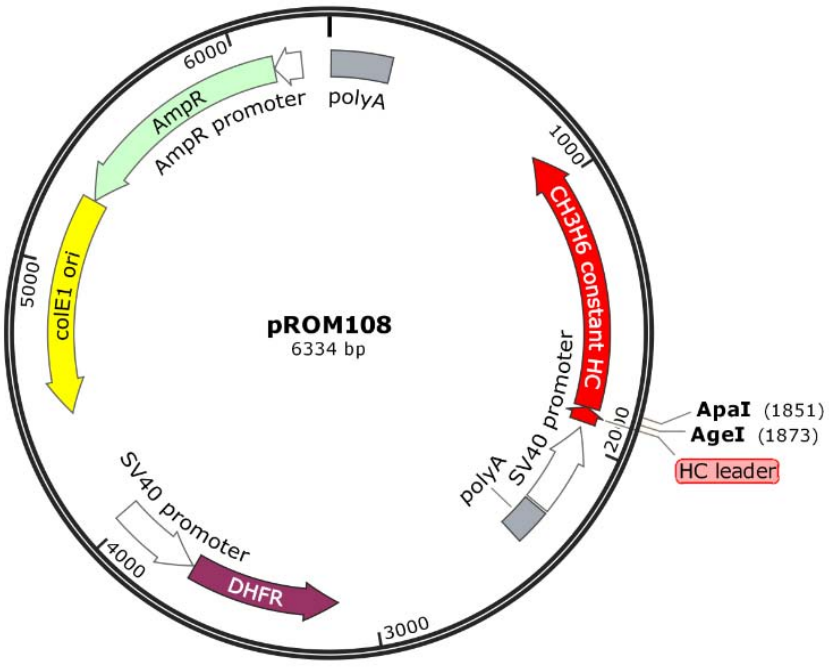


Figure 8.1: plasmid map of pROM108 with highlighted restriction enzyme for integration of the variable domain of heavy chain CH3H6.

8.3.2 pROM111

The 5.8 kb plasmid pROM111 (**Figure 8.2**) was designed by Roman Marvan (working group of Prof. Kunert) and was used for the integration of the chimeric variable domain (CH3H6_vL) of light chain in between light chain leader and the constant domain (3H6_cH). It consists of a bacterial origin of replication (ori) and the ampicillin resistance gene (AmpR) for plasmid amplification and selection in E.coli. The essential feature of this plasmid is a 0.4 kb long gene (controlled by the CMV promoter), which decodes for the leader and the constant regions of the light chain of the antibody 3H6. Between leader and constant region this plasmid can also be digests by two enzymes (*BspEI/BsiWI*), which allows to insert the variable region for the light chain.

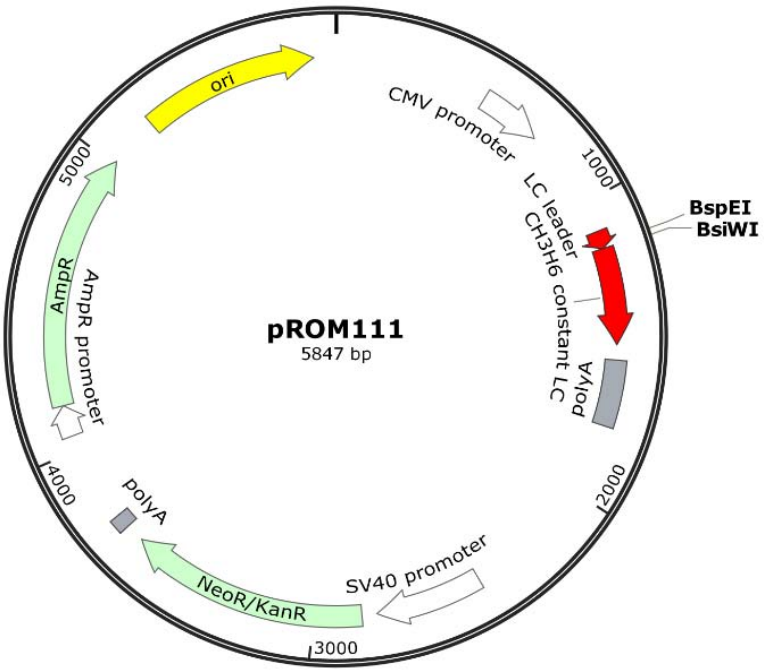


Figure 8.2: plasmid map of pROM111 highlighted restriction enzyme for integration of the variable domain of light chain 3H6.

8.3.3 pROM108_SH3H6_HC

The 6.7 kb plasmid pROM108_SH3H6_HC (**Figure 8.3**) was developed by Alexander Mader (working group of Prof. Kunert) and was used for the construction of pRMCE_SH3H6_IgG plasmid. It consists of a bacterial origin of replication (ColE1 ori) and the ampicillin resistance gene (AmpR) for plasmid amplification and selection in E.coli. For gene amplification and selection in CHO cells the plasmid comprises the gene of DHFR which lies under the control of the SV40 promoter. The essential feature of this plasmid is a 1.4 kb long gene (controlled by a SV40 promoter) which decodes for a leader, the variable region and the three constant regions of the heavy chain of an antibody SH3H6. The gene sequence of the antibody lies between 2 different MCS's (multiple cloning sites).

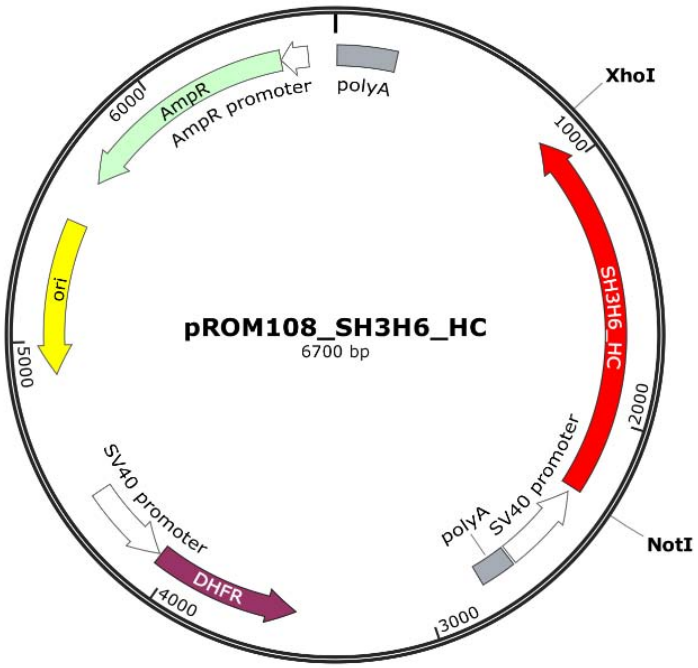


Figure 8.3: plasmid map of pROM108_SH3H6_LC with highlighted restriction enzyme which are relevant for cutting out the heavy chain of SH3H6 antibody.

8.3.4 pROM111_SH3H6_LC

The 6.2 kb plasmid pROM111_SH3H6_LC (**Figure 8.4**) was also designed by Alexander Mader and was used for the construction of pRMCE_SH3H6_IgG plasmid. It offers all the same features as pROM111 (see chapter 8.3.2), but with the variable domain of the light chain of antibody SH3H6, which is flanked upstream (5') by the LC leader and downstream (3') by the constant region of the light chain. For creating SH3H6_LC light chain construct with *KpnI* restriction sites on both ends two primers for amplification were designed.

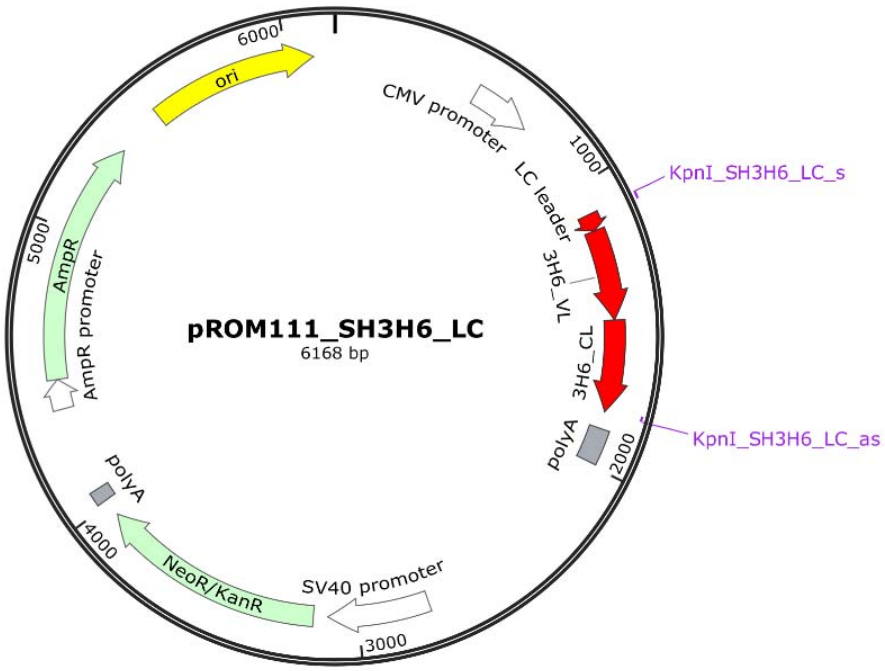


Figure 8.4: plasmid map of pROM111_SH3H6_LC with highlighted primer for amplification of SH3H6_LC to gain light chain construct with *KpnI* restriction sites on both ends.

8.3.5 pIRES_3H6_HC

The 7.5 kb plasmid pIRES_3H6HC (**Figure 8.5**) was used for amplification of the chimeric variable domain of heavy chain CH3H6_vH. It consists of a bacterial origin of replication (ori) and the ampicillin resistance gene (AmpR) for plasmid amplification and selection in *E.coli*.

The essential feature of this plasmid is the 1.4 kb long gene 3H6_HC, which decodes for heavy chain. The gene lies under the control of promoter CMV. For construction the vector pRMCE_CH3H6_IgG only the variable domain CH3H6_vH is needed. To gain this variable domain PCR amplification with two predesigned primers were done.

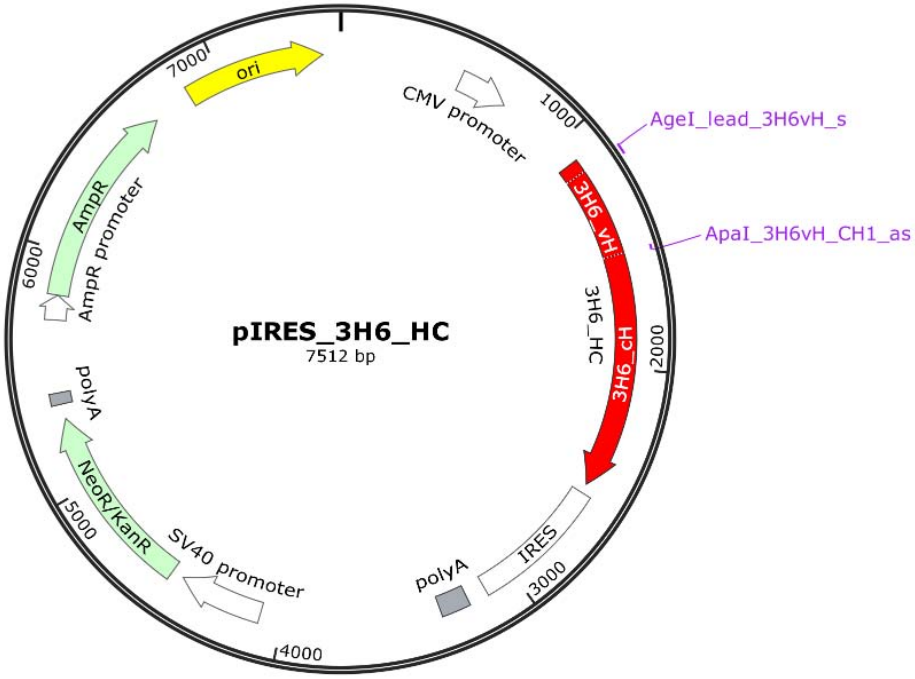


Figure 8.5: plasmid map of pIRES_3H6_HC with highlighted primer for amplification of CH3H6_vH to gain variable heavy chain construct with two specific restriction sites on both ends.

8.3.6 pIRESdhfr_3H6_Fab_LC

The 5.9 kb plasmid pIRESdhfr_3H6_Fab_LC (**Figure 8.6**) was designed by Alexander Mader and was used for amplification of the chimeric variable domain of light chain CH3H6_vL. It consists of a bacterial origin of replication (ori) and the ampicillin resistance gene (AmpR) for plasmid amplification and selection in E.coli. The essential feature of this plasmid is the 340 base long gene, which is controlled by a CMV promoter. This sequence decodes for the chimeric variable region of the light chain of an antibody CH3H6_vL. To gain this variable domain PCR amplification with two predesigned primers were done.

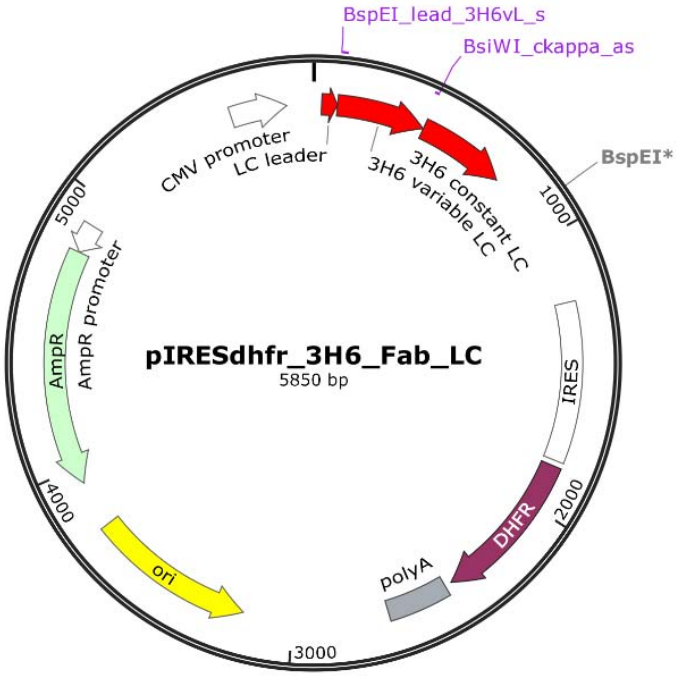


Figure 8.6: plasmid map of pIRESdhfr_3H6_Fab_LC with highlighted primer for amplification of CH3H6_vL to gain variable light chain construct with two specific restriction sites on both ends.

8.3.7 pRMCE_dual

The 4.7 kb plasmid pRMCE_dual (**Figure 8.7**) was designed by Alexander Mader and was used as exchange vector for the Flp/FRT system in CHO cells, in which the final sequences of the heavy and the light chain had to be cloned. It consists of a bacterial origin of replication (ori) and the ampicillin resistance gene (AmpR) for plasmid amplification and selection in *E.coli*. The key features of this plasmid are the two heterospecific FRT sequences (FRT and FRT3). A rEF1α promoter is located in between these two FRT-sides. This promoter will be used to regulate the transcription of the heavy chain of the antibody, which will be cloned between rEF1α and poly A sequence. The light chain will be cloned between FRT3 and poly A signal and will be regulated by a promoter outside the FRT sites on the CHO cells itself.

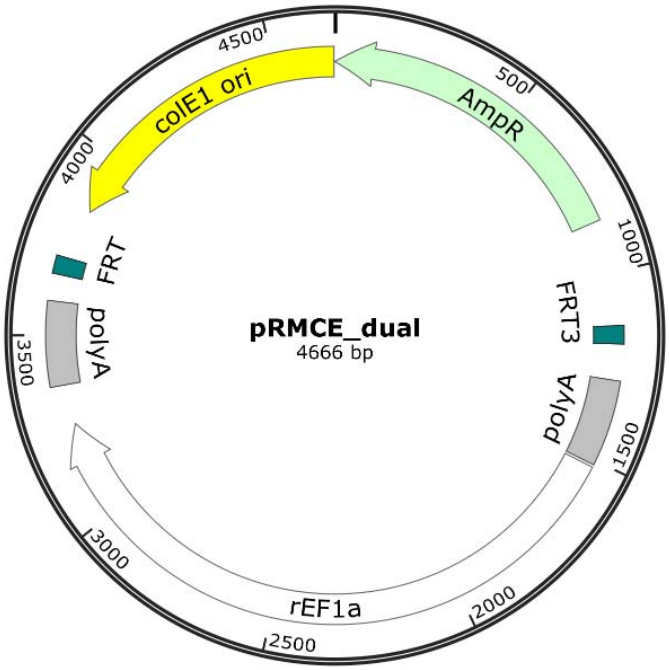


Figure 8.7: plasmid map of pRMCE_dual with all relevant features.

8.4 Molecular biology methods

8.4.1 Overnight culture from cryopreserved cells for Miniprep

As starting point for Miniprep 10 mL overnight culture of *E.coli* with desired plasmid was used. Therefore two 50 mL Greiner centrifuge tubes were used. Each of this tube was filled with 5 mL LB_{amp} medium. Before starting the overnight culture the desired culture was picked with a sterile toothpick and inserted into the flask. This flask was put into an incubator at 37°C / 200 rpm for 16 h. This procedure were done with cultures from cryotubes (-80°C) as well as for cultures from colony plates.

8.4.2 Mini-scale plasmid preparation for molecular cloning (Miniprep)

To get enough amount of plasmid DNA to work with (>6µg plasmid) a Miniprep Kit (PeqGOLD Plasmid Miniprep Kit I) was used. Therefore 10 mL overnight culture (see chapter 8.4.1) of *E.coli* carrying the respective plasmid was taken to start with. First the cells were harvested by centrifugation at 5000 rpm for 10 min. With this gained pellet all steps in the manufacturer's protocol were worked off. After all these purification steps the cleaned plasmid DNA was eluted with 50 µL sterile H_2O.

8.4.3 Midiprep

Unlike for molecular cloning where only a small amount of plasmid DNA is needed, much more purified plasmid DNA is needed for transfection. Therefore the NucleoBond Xtra Midi EF kit was used. For better yield 10 mL starter *E.coli* culture, carrying the respective plasmid, were prepared and incubated for 4 h at 37°C / 200 rpm. 100 µL of this starter culture suspension was added to 100 mL LB_{amp} medium. This bacteria suspension was incubated overnight at 37°C / 200 rpm (for 10 - 16h). With this suspension all steps from the

manufacturer's protocol were worked off. In the last step the plasmid DNA-pellet was solubilized in 500 µl of H2O-EF (supplied by manufacturer).

8.4.4 Enzymatic digestion (restriction)

All used DNA endonuclease enzymes were purchased from New England Biolabs (NEB) (https://www.neb.com/), where all enzymes are listed with their corresponding buffers. Information if the restriction endonuclease can be heat inactivated, at which temperature and how long this has to be done is also provided via homepage of NEB. Digestions of DNA were performed in a preparative or analytical scale.

In case of analytical digestion the gel plot was used to confirm the right DNA pattern after digestion. A typical analytical digestion consists of the following reagents listed in Table 8.2. Unless otherwise described the analytical restriction reaction where incubated for 1.5 h at 37°C.

Table 8.2: composition of a typical analytical digestion assay

component	amount
dH2O	filled up to 50 µL
buffer (NEB 1-4 10X, Cutsmart 10X)	5µL
DNA template	300 ng
enzyme*	1 U
BSA (100X)**	0.5 µL

* one or two depending on digestion setup

** addition of BSA depended on used restriction enzyme

In case of preparative digestion the gel plot was used to confirm the right DNA pattern after digestion and to isolate the desired DNA fragment. A typical preparative digestion consists of the following reagents listed in **Table 8.3**. Unless otherwise described the preparative restriction reaction where incubated for 3 h at 37°C.

Table 8.3: composition of a typical preparative digestion assay

component	amount
dH2O	filled up to 50 µL
buffer (NEB 1-4 10X, Cutsmart 10X)	5µL
DNA template	3 µg
enzyme*	10 U
BSA (100X)**	0.5 µL

* one or two depending on digestion setup

** addition of BSA depended on used restriction enzyme

8.4.5 CIP

In order to prevent religation of a linearized plasmid the enzyme calf intestine phosphatase CIP was used. CIP catalyzes the dephosphorylation of 5´ and 3´ ends of DNA phosphomonoesters. If the phosphate group is missing at the DNA-end it can no longer religate by itself. After preparative digestion (see chapter 8.4.4) of the plasmid the CIP step were done under following condition see **Table 8.4**.

Table 8.4: composition of a typical CIP reaction

component	amount
digested DNA sample	50 µL
CIP	0.5 U / µg vector*

* unless otherwise described for 3 µg plasmid DNA 0.5 µL CIP enzyme was added

After adding of CIP enzyme the solution was incubated at 37 °C for 60 min. To purify the DNA sample an agarose gel purification step was done.

8.4.6 Generating blunt end by Klenow DNA polymerase (KDp)

After some digestions it was necessary to create blunt ends for further molecular cloning. This was done by using the large fragment of the DNA polymerase I from *E.coli* called Klenow fragment. It retains its 3' to 5' polymerase activity, but has lost its 5' to 3' exonuclease activity. With its polymerase activity it fills in the 3' overhangs of restricted DNA fragments and creates in this way blunt ends. In **Table 8.5** is the standard reaction assay for KDp.

Table 8.5: standard reaction assay for Klenow fragment

component	amount
DNA fragment in NEB buffer	50 µL
dNTP (10 mM)	33 µM*
Klenow DNA polymerase I	1 U / µg DNA**

* unless otherwise described 3 µL dNTP were added to the reaction

** unless otherwise described 1 µL enzyme was added to the reaction

After adding KDp the assay was incubated at room temperature (25°C) for 15 min. To inactivate the enzyme 1 µL of 500 mM EDTA was added to the assay and incubated at 75°C for 20 min.

8.4.7 Agarose gel electrophoresis

For all electrophoresis gels a concentration of 1% (w/v) agarose was used. First 4 g agarose was dissolved in 400 mL TAE buffer (392 mL RO-water + 8 mL (50X) TAE buffer). Under frequently shaking the flask had to be heated up in a microwave until the whole liquid appeared clear. After cooling down the liquid under approximately 55°C 12 µL ethidium bromide (final concentration 200 ng/mL) were added to the buffer and gently shaked. Then it was poured in prepared gel chambers where it stayed until it went solid. Two different gel

pockets size were used, small ones (approx. 25 µL) for analytical samples and bigger ones (approx. 60 µL) for preparative samples. Before using the gels were stored at 4°C in TAE buffer supplemented with 200 ng/mL ethidium bromide. A standard preparation of the sample before applying on the gel is described in **Table 8.6**.

Table 8.6: standard preparation of sample before applying on analytical or preparative gel

component	**amount**	
	analytical	preparative
DNA sample	5 µL [50 ng*]	50 µL [3 µg*]
BX buffer (6X)**	1 µL	5µl

* amount of DNA applied on gel, unless otherwise described

** final concentration of BX buffer in loading sample should be 1X, the amount of BX buffer had to be adjusted to the amount of used DNA sample

The DNA sample were separated in gel electrophoresis chamber (BioRad), filled with TAE buffer containing ethidium bromide, at 100 V (Power Pac Basic, BioRad) for approx. 60 min. For visualization of the separated DNA fragments the gel was put in the Illumination chamber (Gel doc, Biorad) there the gel bands could be visualized under UV illumination at 254 nm. In case of preparative gels the band of interest had to be cut out under illumination. This had to be done with lower intensity and as fast as possible because excessive illumination can damage the DNA by nicking. If it was not possible to clean the gel piece instantly it can either be stored at 4°C for some days before further process. Because of its mutagen and carcinogen effect, all steps where ethidium bromide was involved had to be carried out by using nitrile gloves, eye protection and wearing a lab coat all the time. As marker for all agarose gel electrophoresis the Generuler ladder 6X (0.5 µg/µL) of Fermentas (**Figure 8.8**) was used. In all assays 6 µL of Generuler ladder mix was used, in it is shown how much ng DNA per lane was applied.

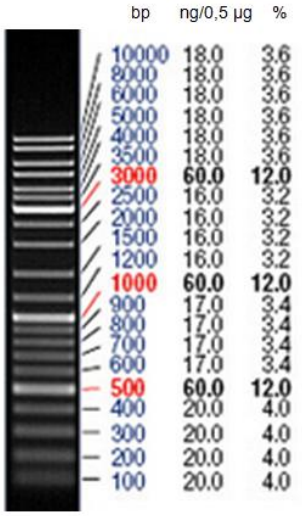


Figure 8.8: 0,5 µg / lane DNA ladder mix, smallest band length is 100 bp the largest 10000 bp, bands with length of 500 bp, 1000 bp and 3000 bp do have 60 ng DNA in its band, all other bands consists of less than 20 ng per band.

8.4.8 Cleaning and isolation of DNA fragments from PCR assay or agarose gel

To isolate the DNA fragments from agarose gel or from a PCR assay, the Wizard SV Gel and PCR Clean-up system from Promega was used. This was done following the manufacturer's protocol. Except the last step where the DNA sample was eluted with 30 µL instead of 50 µL nuclease-free water supported by manufacturer. For better yield the gel slice has to be thin as possible when cutting out of agarose gel.

8.4.9 DNA quantification with photometer

To calculate the amount and purity of Plasmid DNA or genomic DNA all samples were measured by NanoPhotometer^{TM} P-300 (Implen). To gain the amount of DNA 0.3 µL of the sample was put on centre of the measuring window of the Submicroliter Cel. To determine the concentration in solution the absorbance at 260 nm wavelengths was measured and applied on following Equation 8.1.

Equation 8.1: function describing the concentration to absorbance relation

$$c_{nuc} = Abs.\ 260 * factor_{nuc} * lid\ factor * dilution\ factor$$

c_{nuc}	nucleic acid concentration [ng/µL]
Abs. 260	absorbance of nucleic acid
$factor_{nuc}$	substance specific factor for nucleic acid (ds DNA = 50 [ng*cm/µL])
lid factor	virtual dilution factor (5, 10, 50, 100 or 250)
dilution factor	used dilution

Proteins do have the absorption maximum at wavelength 280 nm. Contamination by proteins can therefore be measured by the ratio A_{260} /A_{280}. If this ratio is around 1.8 the sample is considered as pure, if the ratio appreciably lower it may indicates the presence of proteins, phenols or other contaminants.

8.4.10 Ligation

Unless otherwise described in results all ligation were performed with three different molar ratio from vector to insert (1:0, 1:1, 1:3). In all experiment 50 ng of vector DNA was used. How much insert was needed was calculated with Equation 8.2.

Equation 8.2: equation to calculate the amount of needed insert, excess factor *ef* is determined by the ratio vector to insert [0, 1 or 3]

$$m_{insert}[ng] = \frac{m_{vector}[ng] * l_{insert}[bp]}{l_{vector}[bp]} * ef$$

A typical ligation assay consists of ligase buffer, vector DNA, Insert DNA, the enzyme T4 DNA ligase and was filled up to a total volume of 20 µL with dH_2O. In Table 8.7 you can see a typical ligation assay.

Table 8.7: A typical ligation assay

component	**ratio**		
	1:0	1:1	1:3
dH_2O	16 µL	15.5 µL	14.5 µL
ligase buffer	2 µL	2 µL	2 µL
vector DNA [50 ng] *	1 µL	1 µL	1 µL
insert DNA **	**-**	**0.5 µL**	**1.5 µL**
T4 DNA ligase	1 µL	1 µL	1 µL
total volume	20 µL	20 µL	20 µL

* c(vector)= 50 [ng/µL], l(vector) = 5000 bp

** c(insert)= 20 [ng/µL], l(insert) = 1000 bp

Incubation time depends on the mode of ligation, if sticky or blunt ends need to be ligated. In case of sticky ends the incubation time was 10 min at room temperature for blunt ends the time increased to 120 min. To inactivate the T4 DNA ligase the sample was heated up to 65 °C for 10 min.

8.4.11 Transformation

After ligation of plasmid DNA with insert DNA the newly developed plasmid had to be transferred into a host. In all transformations electrocompetent *E.coli* TOP 10 bacteria were chosen as host. First 40µL of the electrocompetent cells where thawed up on ice and shacked carefully. 3 µL newly generated vector was mixed with E.coli cells. The total volume was transformed into a pre-cooled cuvette. For a successful transformation no bubbles had

to be visible at the bottom of the cuvette. For transformation the Gene Pulser Xcells from Bio-Rad was used with following parameter: 1.8 kV / 2.5 µF / 200 Ω. Immediately after transformation 250 µL of SOC medium were transferred to the bacteria suspension and were put into a shaker with 400 rpm for 50 min at 37 °C. After incubation 50 µL and 150 µL of these cells were plated on two LB_{amp}-agar plates and incubated over night at 37 °C. These steps were done with all three different molar ratios. To ensure a credible result of these transformations all steps had to be done by same time intervals. Next day the transformation plates where checked for positive transformants.

8.4.12 Cryopreservation

To store cells for other experiments in the future, cells had to be cryopreserved. Therefore a single positive colony was picked with a pipette tip from LB_{amp}-plates and transferred into 5 mL of LB_{amp} media. This suspension was incubated at 37 °C / 200 rpm overnight. Next day two cryotubes were filled with 375 µL 80 % glycerol and 625 µL bacteria suspension. This cryotubes had to be stored immediately at minus 80 °C.

8.4.13 PCR

8.4.13.1 PCR amplification for preparative DNA fragment

To create DNA fragments with specific restriction sites at both ends we used PCR amplification. Therefore a master mix with all components was prepared. In **Table 8.8** you can see a standard master mix.

Table 8.8: Standard PCR assay

component	amount	
	volume / 25 µL	concentration
sterile dH2O	fill up to 25 µL	-
10X ThermoPol Reaction Buffer	2.5 µL	1X
10 mM dNTPs	0.5 µL	200 µM
10 µM primer sense	0.75 µL	300 nM
10 µM primer antisense	0.75 µL	300 nM
DNA template	X µL	~100 ng
Taq polymerase	0.125 µL	25 U/mL

With two PCR-tubes, which were filled with 25 µL of this master mix, a standard PCR program, with conditions listed in **Table 8.9**, was carried out.

Table 8.9: standard cycling parameter for PCR amplification

Cycling steps		temperature	time	
1	initial denaturation	95 °C	2 min	
2	denaturation	98 °C	20 sec	30 cycles
3	primer annealing	57 °C	15 sec	
4	extension	72 °C	20 sec	
5	final extension	75 °C	5 min	
6	cooling	12 °C	forever	

8.4.13.2 Colony PCR

After ligation and transformation of E.coli cells to find positive clones colony PCR was used. Similar to a normal PCR as in chapter 8.4.13.1 a slightly different master mix was prepared as it shows in **Table 8.10**.

Table 8.10: Master mix of colony PCR for one PCR-tube

component	**amount**	
	volume	concentration
10X ThermoPol Reaction Buffer	2.5 µL	1X
10 mM dNTPs	0.5 µL	200 µM
10 µM primer sense	0.5 µL	200 nM
10 µM primer antisense	0.5 µL	200 nM
Taq polymerase	0.125 µL	25 U/mL
total volume	**4.125 µL**	

First 20.9 µL of sterile dH_2O was pipetted into a PCR-tube. Next a pipette tip was used to transfer one positive colony from LB_{amp}-plate to the PCR-tube. If a second primer pair was used to identify positive clones the pipette tip was also transferred into a second PCR-tube. After enough cells were in solution (by shaking the PCR-tube softly) some cells where streaked onto a master plate by using the pipette tip. Then 4.125 µL of the prepared master mix was added to the PCR-tube and was mixed thoroughly. This was done with 8 or 16 clones. Finally a normal PCR with conditions according to was carried out.

Table 8.11: standard cycling parameter for colony PCR

Cycling steps		temperature	time	
1	initial denaturation	95 °C	5 min	Because of whole cells
2	denaturation	95 °C	30 sec	30 Cycles *
3	primer annealing	55 °C	30 sec	
4	extension	68 °C	60 sec	
5	final extension	68 °C	5 min	
6	cooling	12 °C	forever	

* step 2-5 were repeated 30 times

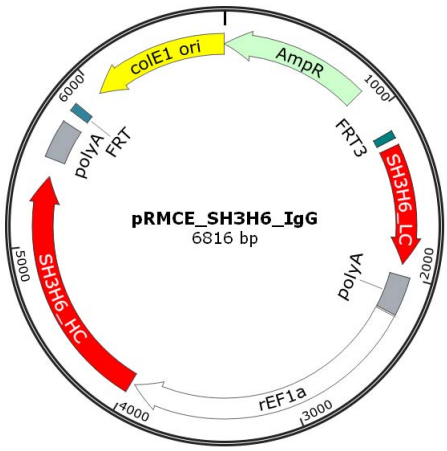
8.4.14 Sequencing

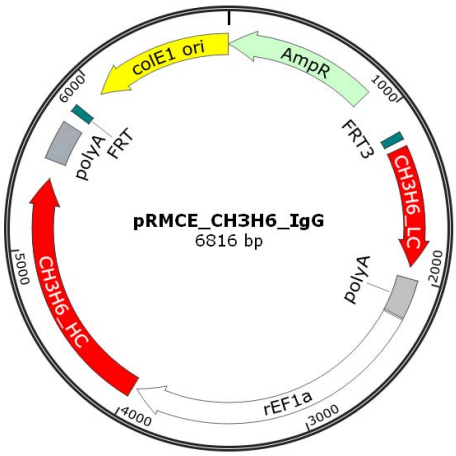
To check for positive cloning methods parts of plasmid DNA was checked by sequencing. This was done by an overnight DNA sequencing service of company Mycrosynth Austria GmbH (http://www.microsynth.at/). Therefore 1.2 ng plasmid DNA was mixed with 3 µL [10 µM] primer and filled up to 15 µL with sterile dH_2O. The received FASTA file was then checked with ApE (A plasmid Editor Freeware version 2.0.48_pre23) and also by SnapGene Version 2.6.2 (free test version).

9 Results

9.1 Verification of the constructed vectors

The objectives of this work was to construct two plasmids which comprise of genes for plasmid production in E.coli, sequences for successful homologous integration into RMCE competent CHO cells and for sure within these RMCE-system sequences the sequence of the antibody which should be produced by the CHO cells including regulatory sequences.





AmpR	…for selection in *E.coli*…	AmpR
ColE1 ori	…origin of replication in *E.coli*…	ColE1 ori
FRT3	…1st signal sequence for RMCE system…	FRT3
FRT	…2nd signal sequence for RMCE system…	FRT
poly A	…poly A signal for light chain…	poly A
poly A	…poly A signal for heavy chain…	poly A
rEF1α	…promoter for heavy chain…	rEF1α
SH3H6_HC	…sequence for super humanized heavy chain…	-
SH3H6_LC	…sequence for super humanized light chain…	-
-	…sequence for chimeric heavy chain…	**CH3H6_HC**
-	…sequence for chimeric light chain…	**CH3H6_LC**

Figure 9.1: Plasmid maps of pRMCE_SH3H6_IgG and pRMCE_CH3H6_IgG as well as a list of all relevant gene sequences.

9.2 Construction of vector

9.2.1 Construction of vector pRMCE_SH3H6_IgG

9.2.1.1 Ligation of vector pRMCE_dual with super humanized heavy chain insert

For constructing the vector pRMCE_SH3H6_IgG, the pRMCE_dual vector with pre-existing FRT sites, proper restriction sites and already containing poly A signal was used. The first step in constructing the final pRMCE_SH3H6_IgG vector was to insert the super humanized (SH) heavy chain (HC) by ligation into the pRMCE_dual vector. The SH_HC was already cloned on the ROM108_SH3H6_HC (Figure 9.3). To get this plasmid an overnight culture was grown with cryopreserved cells. After overnight culture and Mini-prep the concentration of plasmid [244 ng/µl] was determined with Implen Nano-Photometer P-300. The sequence of SH_HC is flanked by two single cutting restriction sites. *NotI* on the leader site and *XhoI* on downstream site. By cutting with these two restriction enzymes we got the desired SH_HC. This sequence consists of a human leader sequence followed by the variable and constant domain of the heavy chain (Figure 9.2).

(a)
MDWTWRILFLVAAATGVHSEVQLVQSGAEVKKPGATVKISCKVSGYTFTDYYMHWVQQAPGKGLEWMGLVDP
EDGETIYAEKFQGRVTITADTSTDTAYMELSSLRSEDTAVYYCATTSIGYGSSPPFPYWGQGTLVTVSSASTKGPSVF
PLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVN
HKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYV
DGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSR
DELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHE
ALHNHYTQKSLSLSPGK*
(b)
MALQTQVFISLLLWISGAYGETTLTQSPAFMSATPGDKVNISCITSTDIDDDMNWYQQKPGEAAIFIIQDGNTLRP
GIPPRFSGSGYGTDFTLTINNIESEDAAYYFCLQSDNLPYTFGQGTKLEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLL
NNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNR
GEC*

Figure 9.2: a) sequence of SH3H6_HC, **b)** sequence of SH3H6_LC, leader sequence, variable chain, constant chain, red letters showing the three complementarity determining regions CDRs

After the enzymatic digestion we got two sticky ends. For further ligation with pRMCE_dual vector DNA polymerase I, Large (Klenow) Fragment was used to generate blunt ends. After

the digestion 50µl sample were applied on an electrophoresis gel to separate the SH3H6_HC from the rest of the plasmid and the enzymes. The agarose gel electrophoresis plot (**Figure 9.3**) showed two fragments with an expected length of 1432 bp and 5268 bp. The smaller fragment (SH3H6_HC) was cut out under fluorescent light and cleaned with Nucleo Spin^{R} Gel and PCR Clean-up Kit. The concentration of the cleaned fragment [26 ng/µl] was determined by measuring with the Implen Nano-Photometer P-300.

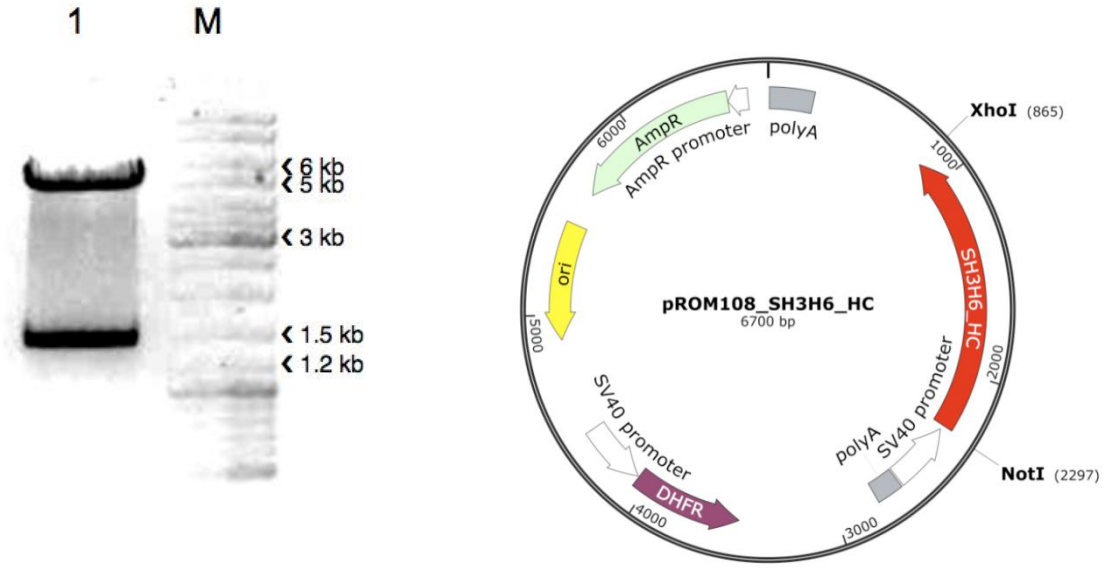


Figure 9.3: Left figure: gel plot 1: digestion of vector pROM108_SH3H6_HC [3 µg] with *NotI* and *XhoI* two visible bands at the length of 1432 bp and 5268 bp, M: 0.5 µg generuler DNA ladder; right figure plasmid map of pROM108_SH3H6_HC with *XhoI* and *NotI* restriction sites

For inserting the fragment SH3H6_HC in the pRMCE_dual vector between the rEF1α promoter and the poly A region which lies between the two significant FTR sites (**Figure 9.4**) the plasmid was digested by the single cutting restriction site enzyme *BstZ17I*. Cutting with this enzyme creates two blunt ends. To prevent religation of this linearized plasmid, dephosphorylation with Alkaline Phosphatase Calf Intestinal (CIP) was performed. To separate the linearized plasmid from all enzymes an agarose gel electrophoresis using 1% agarose was done (**Figure 9.4**). After this procedure the concentration of linearized pRMCE_dual was 134 ng/µl.

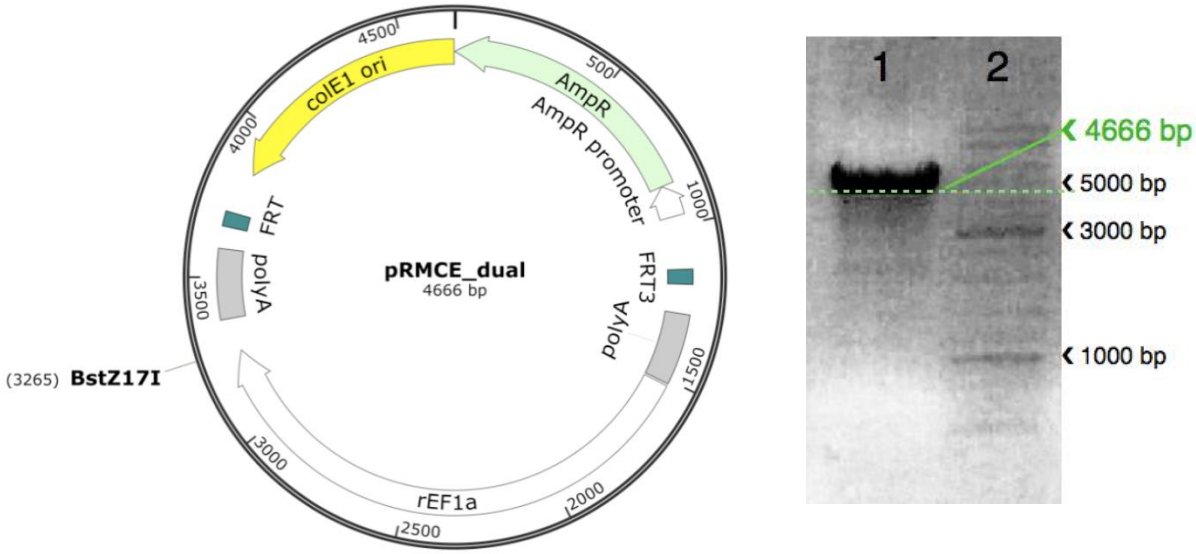


Figure 9.4: left figure: plasmid map of pRMCE_dual with restriction site *BstZ17I*; **right figure:** electrophoresis plot of digestion of pRMCE_dual [3 µg] with *BstZ17I*, 1: Row 1 shows a visible band at the length of 4666 bp, 2: 0.5µg generuler DNA ladder;

The ligation, of pRMCE_dual vector with SH3H6_HC insert, was performed with three different molar ratios from vector to insert (1:0, 1:1, 1:3). The transformation in electrocompetent *E.coli* TOP 10 bacteria was done with each ratio.

Table 9.1: colonies counted after overnight culture of transformation pRMCE_dual with SH3H6_HC

ratio	colonies
1:0	0
1:1	0
1:3	69

For further cloning, clones from the ratio 1:3 were used because no clones appeared on ratio 1:0 or 1:1. This new vector was called pRMCE_SH3H6_HC. By doing a colony PCR with 16 clones the transformed bacteria were checked for positive clones. For colony PCR the primer pROM112_1687sense and primer pEF1a_PacI_s were chosen. If ligation was successful this primer pair should create a 1079 bp long DNA fragment. 8 of 16 tested clones showed a visible band at the desired length (**Figure 9.5**).

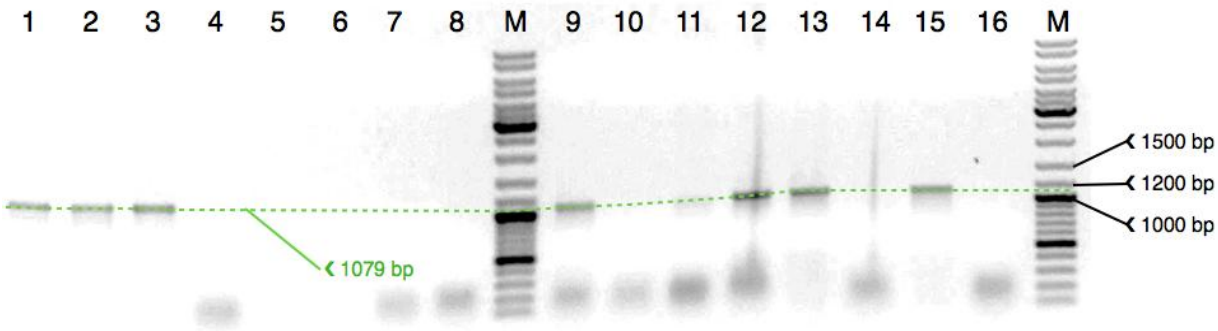


Figure 9.5: colony PCR of ligation pRMCE_dual with insert SH3H6_HC, positiv bands can be seen at clones 1, 2, 3, 9, 11, 12, 13 and 15, M: 0.5µg generuler DNA ladder;

For further vector construction clone 12 were picked for growing in LB-Media with antibiotics in an overnight culture. A mini prep kit was used to isolate the plasmid DNA from the bacteria. A control digestion with *KpnI* showed a visible band at 6102 bp which indicates only 1 inserted fragment taken up during ligation. The picture of this control digestion can be seen in **Figure 9.6**. The amount of plasmid in the sample after mini prep was measured with a photometer and results in 218 ng/µl.

Figure 9.6: control digestion of pRMCE_SH3H6_HC [60 ng] with *KpnI*, 1: digestion with *KpnI*, 2: undigested vector [50 ng] M: 0.5 µg generuler DNA ladder;

The next step in constructing the final vector pRMCE_SH3H6_IgG was to insert super humanized (SH) light chain (LC) by ligation into the pRMCE_SH3H6_HC vector. This vector we constructed by ligation into the *KpnI* restriction site in between a FRT signal and a poly A

signal. In cutting this vector at this site and inserting the light chain at this spot will lead to a light chain which can be produced in compatible CHO cell (promotor trap + FRT sites) seen in **Figure 9.7**. Therefore the light chain was adapted with *KpnI* restriction sites on both ends. To prevent religation of the linearized plasmid a dephosphorylation step was performed using the enzyme calf intestine phosphatase (CIP). After cutting the vector pRMCE_SH3H6_HC with *KpnI* and CIP treatment the sample had to be cleaned from the enzymes. The main reason was because the restriction enzyme *KpnI* could not be heat inactivated by temperature. This clean up was done with the Nucleo Spin^{R} Gel and PCR Clean-up Kit. Afterwards a concentration of 134 ng/µl was measured with the photometer.

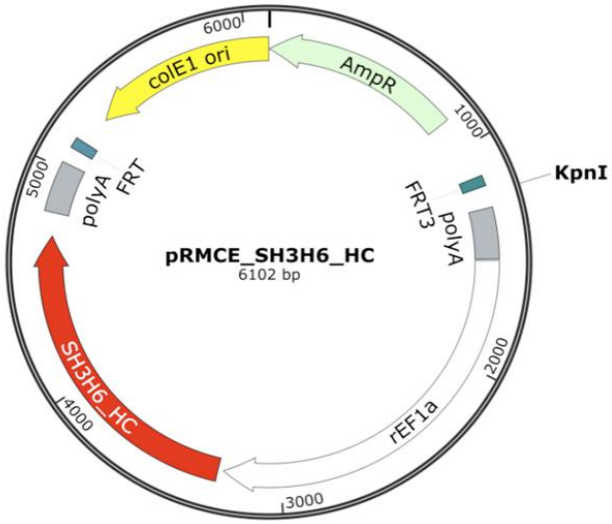


Figure 9.7: plasmid map of pRMCE_SH3H6_HC with *KpnI* restriction site

9.2.1.2 PCR amplification of super humanized light chain

Before ligation the next step was to construct the light chain fragment with *KpnI* restriction sites on both ends. This was done by amplifying the super humanized (SH) light chain (LC) from the plasmid pROM111_SH3H6_LC **Figure 9.8**. These two primers (KpnI_SH3H6_LS_s and KpnI_SH3H6_LS_as) were developed in a way that after PCR the fragment implies *KpnI* restriction sites on both ends (**Figure 9.9**).

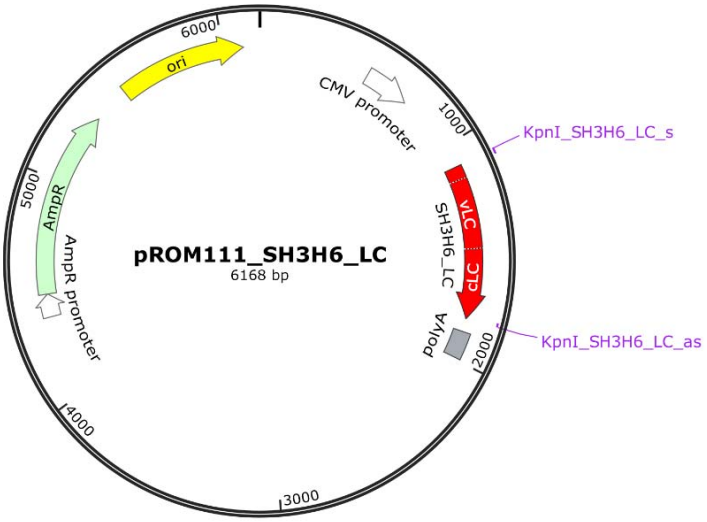


Figure 9.8: plasmid map pROM111_SH3H6_LC with highlighted primer pair for PCR amplification

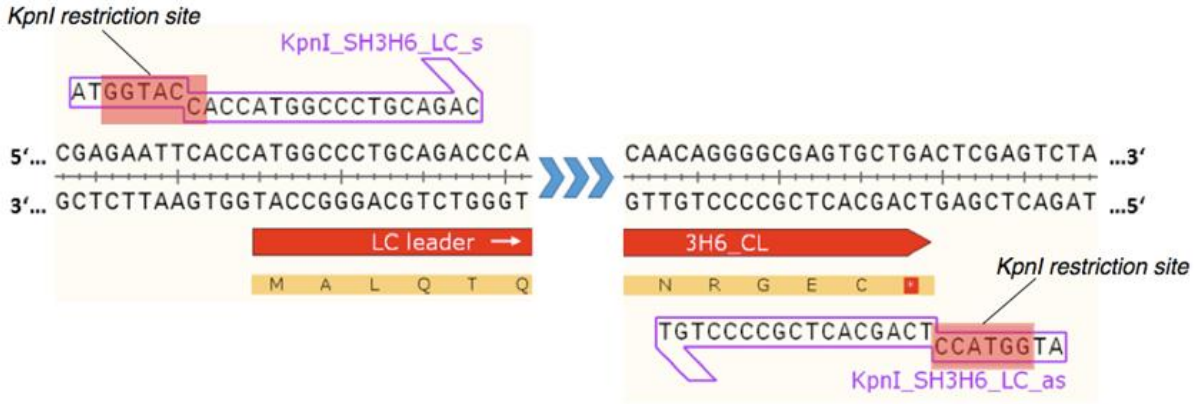


Figure 9.9: PCR amplification, primer KpnI_SH3H6_LC_s binds to the leader signal and integrates a *KpnI* restriction site before the light chain sequence, whereas primer KpnI_SH3H6_LC_as binds to the end of the sequence and integrates another *KpnI* restriction site after the coding sequence.

After PCR an agarose gel electrophoresis was done (**Figure 9.10**) and the band at length 724 bp were cut out and cleaned with Nucleo Spin^{R} Gel and PCR Clean-up Kit. As a last step before ligation the fragment had to be cut with the enzyme *KpnI* and also as in the previous step where *KpnI* was used it had to be cleaned with Nucleo Spin^{R} Gel and PCR Clean-up Kit. In the end the concentration of SH3H6_LC insert reaches 34 ng/µl, which was quite enough for more than one ligation.

Figure 9.10: preparative agarose gel of amplified DNA fragment SH3H6_LC, 1: amplified fragment, 2: 0.5 µg generuler DNA ladder;

9.2.1.3 Ligation of pRMCE_SH3H6_HC with insert super humanized light chain

The Ligation, of pRMCE_SH3H6_HC vector with SH3H6_LC insert, also was performed with three different molar ratios from vector to insert (1:0, 1:1, 1:3). After transformation in electrocompetent *E.coli* TOP 10 bacteria and selection on LB agar plates supplemented with ampicillin, 8 clones were picked for colony PCR.

Table 9.2: colonies counted after overnight culture of transformation pRMCE_SH3H6_HC with SH3H6_LC

ratio	colonies
1:0	6
1:1	119
1:3	>300

Ratio plate 1:3 was overgrown by transformed colonies so all clones were picked from ratio plate 1:1. For colony PCR two primer-pairs were chosen. If only one copy of the insert was transformed into the desired place of vector pRMCE_SH3H6_HC for each primer-pair one visible band should be identified in the following agarose gel electrophoresis. With primer-pair pMG433_FSHB_2465_as // KpnI_SH3H6_LC_as the length of the band should be 1072 bp long and for primer-pair pCEP4_gagpol_as7 // KpnI_SH3H6_LC_s the length should be

849 bp long (**Figure 9.11**). **Figure 9.12** shows the agarose gel electrophoresis plot of the colony PCR. 5 out of 8 clones were tested positive. For further vector construction clone 2 and clone 6 were chosen and were grown in LB-Media with antibiotics in an overnight culture. Before any further steps these two clones were transferred in cryotubes and stored at -80°C.

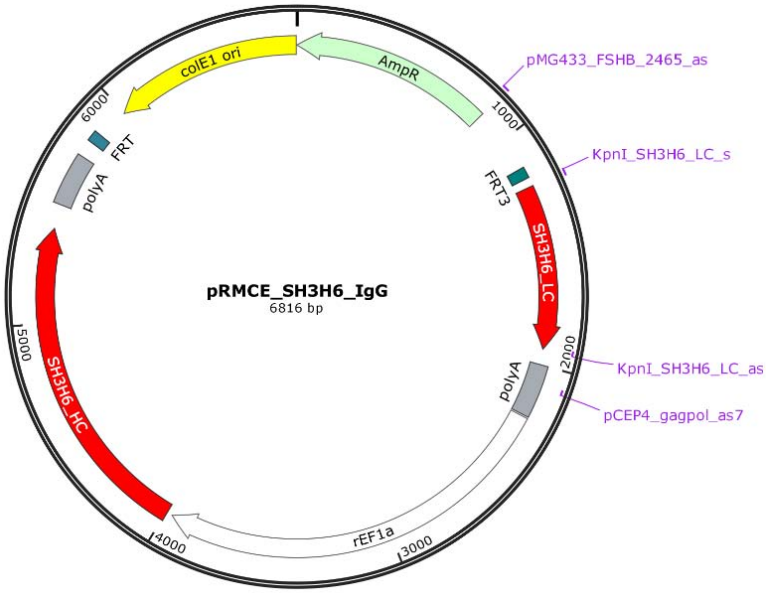


Figure 9.11: plasmid map of vector pRMCE_SH3H6_IgG with highlighted primer pairs for colony PCR

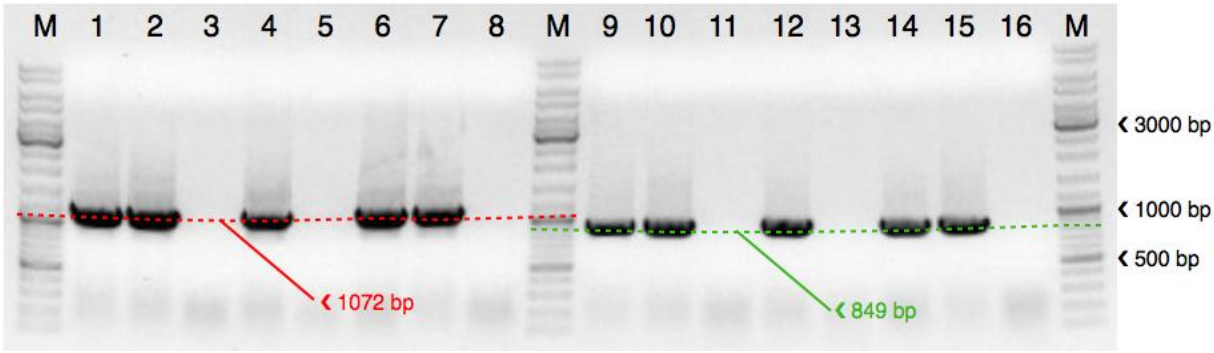


Figure 9.12: colony PCR of ligation pRMCE_SH3H6_HC with insert SH3H6_LC, row 1 - 8 were amplified with the primer-pair pMG433_FSHB_2465_as // KpnI_SH3H6_LC_as whereas row 9 - 16 were amplified with the primer-pair pCEP4_gagpol_as7 // KpnI_SH3H6_LC_s, positiv bands can be seen at clones 1, 2, 4, 6 and 7, M: 0.5µg generuler DNA ladder;

A mini prep kit was used to isolate the plasmid DNA (pRMCE_SH3H6_IgG) from clone 2 from the grown bacteria. The amount of plasmid in the sample after mini prep was measured with a photometer and resulted in 208 ng/µl. Before a midi prep was performed, this vector

pRMCE_SH3H6_IgG were checked by analytical digestion and sequencing. As control digestion the restriction enzyme *BsiWI* was chosen. *BsiWI* cuts this plasmid two times (**Figure 9.13**). One restriction site is located between SH3H6_HC and poly A signal, the other one cuts in between the SH3H6_LC insert. **Figure 9.13** shows the agarose gel electrophoresis plot of this digestion. Two bands were visible one with a length of 3000 bp and one with a length of 3816 bp.

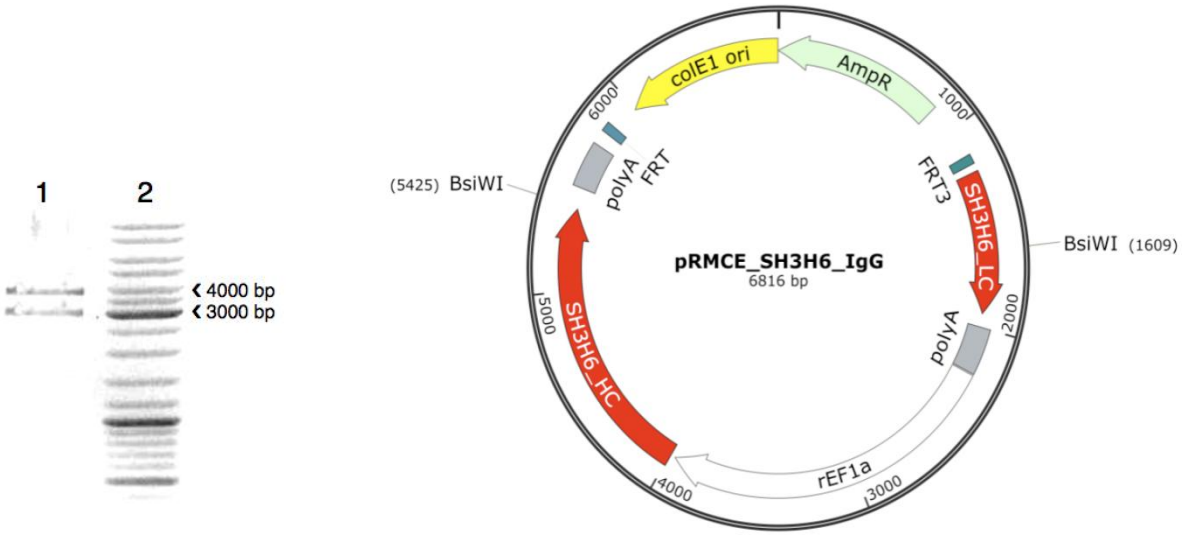


Figure 9.13: left figure: 1:digestion of pRMCE_SH3H6_IgG [50 ng] with *BsiWI* results in 2 visible bands at 3816 and 3000 bp, 2: 0.5µg generuler DNA ladder; right figure: plasmid map of pRMCE_SH3H6_IgG with two restriction site *BsiWI*;

9.2.1.4 Sequencing of final vector pRMCE_SH3H6_IgG

Five different primers were used for sequencing the vector as shown in **Figure 9.14**. To confirm the SH3H6_LC light chain sequence and the FRT3 signal two primers were used as shown in **Figure 9.15**. Primer pMG433_FSHB_2465_as is located before the FRT3 signal sequences upstream whereas primer pCEP4_gagpol_as7 is located after the light chain SH3H6_LC and sequences downstream. The obtained sequences were aligned to the sequence of the plasmid map by using the program SnapGene Version 2.6.2 (free testversion). No deletion or exchange of bases was observed in the coding region of light

chain nor in the FRT3 signal. One insertion of a base was observed in the region between FRT3 signal and light chain. One exchange of a base in the region between AmpR gene and FRT3 signal and also 3 exchanges in the region between light chain and poly A signal. All disparities can be ignored because they are located in non-relevant spots.

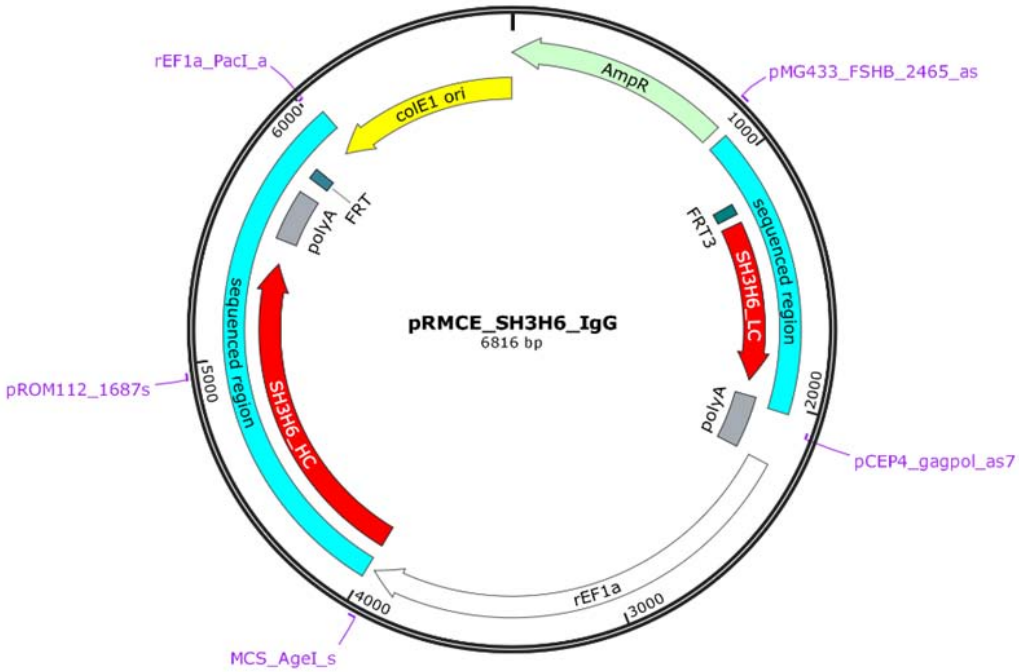


Figure 9.14: plasmid map of pRMCE_SH3H6_IgG with primers which were used for sequencing and highlighted sequenced region

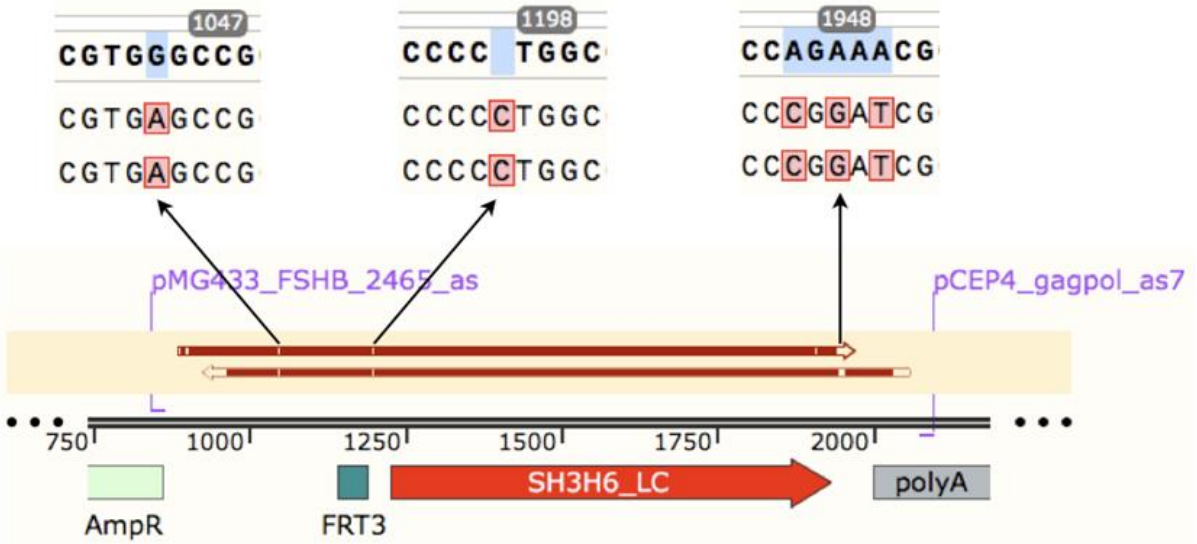


Figure 9.15: alignment of SH3H6_LC was done with SnapGeneR, red arrows symbolizes the alignment, white spots in this red arrows are showing irregularities with the original plasmid map.

To confirm the SH3H6_HC heavy chain sequence and the FRT signal three primers shown in **Figure 9.16** were used. Primer MCS_AgeI_s is located upstream the heavy chain sequence, primer pROM112_1687s is located in the middle of the heavy chain whereas primer rEF1a_PacI_as is located downstream of the FRT signal. Also here no deletion or exchange of bases where observed in the coding region of heavy chain nor in the FRT signal. Still a few insertions and deletions were detected in between FRT and poly A signal. As before also this disparities can be ignored because they are located in non-relevant spots.

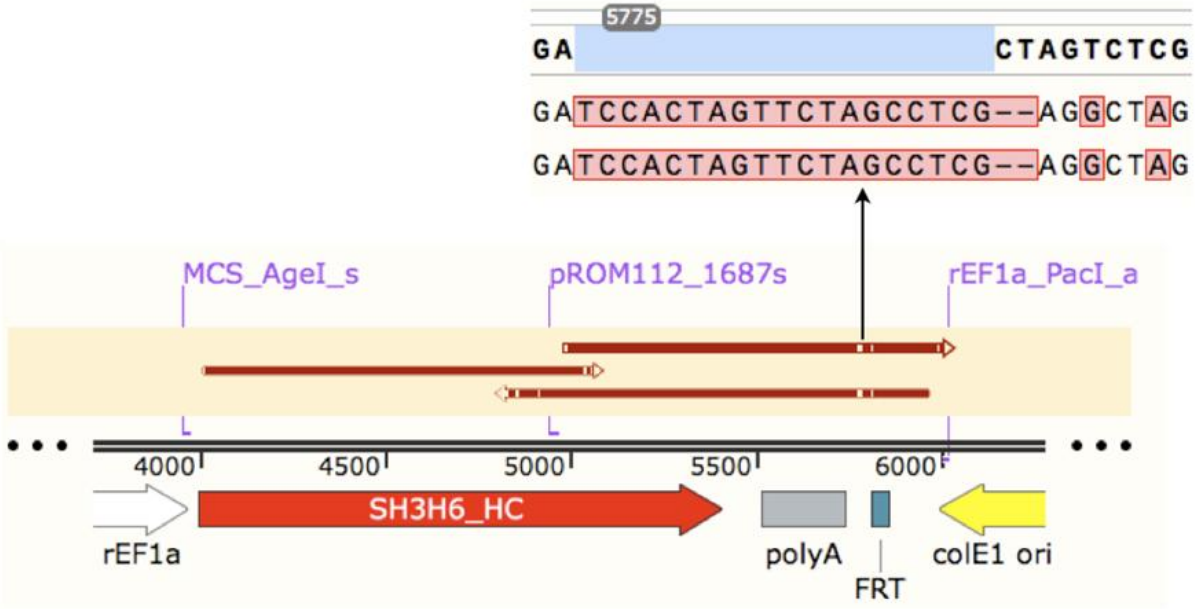


Figure 9.16: alignment of SH3H6_HC were done with SnapGene[R], red arrows symbolizes the alignment, white spots in this red arrows are showing irregularities with the original plasmid map.

After verification of the final plasmid a midiprep preparation was done to gain enough plasmid DNA. After this procedure the concentration of finally cleaned plasmid was measured with photometer and results in 950 ng/µl.

9.2.2 Construction of vector pRMCE_CH3H6_IgG

For constructing the vector pRMCE_CH3H6_IgG, which only differs significant from vector pRMCE_SH3H6_IgG at the complementarity determining regions (CDRs) (**Figure 9.17**), the same pRMCE_dual vector was used as in construction of SH vector. In contrast to pRMCE_SH3H6_IgG vector, where HC and LC already were complete (leader sequence + variable domain + constant domain) and only had to be transfered at the right position of the pRMCE_dual vector, for pRMCE_CH3H6_IgG both chains had to be constructed. For both chains the variable domain had to be cut out of a vector and cloned in between the leader sequence and the constant domain of another vector.

(a)
MDWTWRILFLVAAATGVHSEVQLQQSGPELVKTGASVKISCKASGYSFTDYFMHWVKQSHGKSLDWIGYINCYT
GATNYSQKFKGKATFTVDTSSNTAYMQFNSLTSEDSAVYYCARTSIGYGSSPPFPYWGQGTLVTVSAASTKGPSVF
PLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVN
HKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYV
DGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSR
DELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHE
ALHNHYTQKSLSLSPGK*

(b)
MALQTQVFISLLLWISGAYGETTVTQSPASLSMSIGEKVTIRCITSTDIDDDMNWYQQKPGEPPRLLISDGNTLRPG
VPSRFSSSGYGTDFVFTIENMLSEDVADYYCLQSDNLPYTFGGGTNLEIKRTVAAPSVFIFPPSDEQLKSGTASVVCL
LNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNR
GEC*

Figure 9.17: leader sequence, variable chain, constant chain, red letters showing the three complementarity determining regions CDRs; **a)** sequence of CH3H6_HC; **b)** sequence of CH3H6_LC;

9.2.2.1 Construction of CH3H6_HC

The first step in constructing the pRMCE_CH3H6_IgG vector was to construct the chimeric (CH) heavy chain (HC). Leader and constant domain where located on the vector pROM108 (**Figure 9.18**). The variable domain which should be cloned into pROM108 is located on vector pIRES_3H6HC (**Figure 8.5**). With Vector pROM108 a digestion was done with two

enzymes *ApaI* and *AgeI-HF*. They cut the vector in between the leader and the constant HC so the variable HC can be cloned into the right position. After digestion with the two enzymes *AgeI-HF* and *ApaI* the sample was applied on a electrophoresis gel to separate the pROM108 from enzyme and undigested plasmid. The agarose gel electrophoresis plot (**Figure 9.18**) showed a band at expected length of 6312 bp. This band was cut out under fluorescent light and cleaned with Nucleo Spin^{R} Gel and PCR Clean-up Kit.

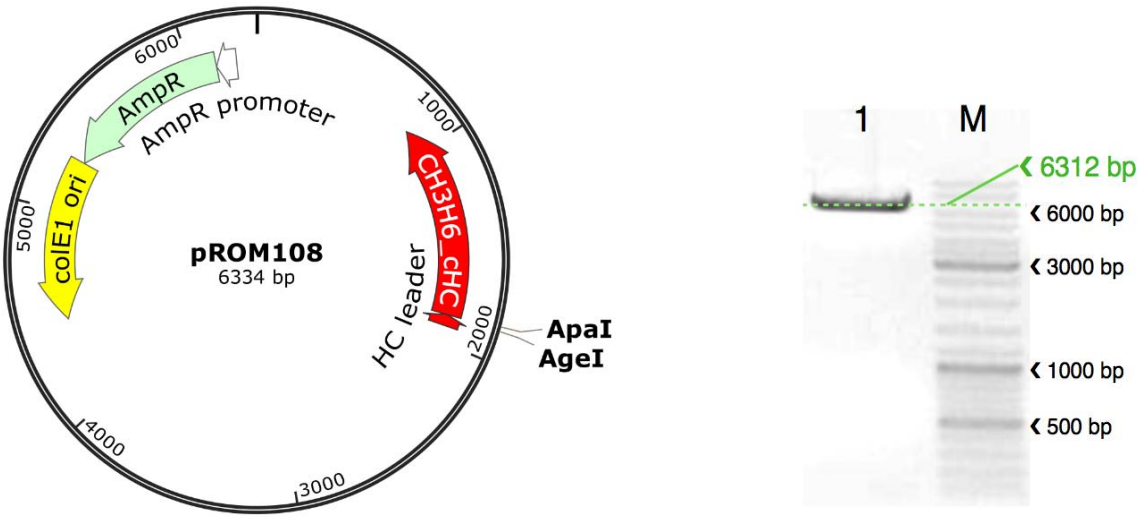


Figure 9.18: left figure: plasmid map of pROM108 with highlighted restriction sites; **right figure:** agarose gel plot of digested pROM108, 1: plasmid pROM108 [3 µg] digested with *ApaI* and *AgeI-HF*, shows a visible band at the length of 6312 bp, M: 0.5µg generuler DNA ladder;

For ligation the variable HC had to be amplified with the two primer AgeI_lead_3H6vH_s and ApaI_lead_3H6vH_as. This results in a CH3H6vH amplicon which is flanked by two restriction sides *AgeI-HF* and *ApaI* (**Figure 9.19**). This PCR amplicon was then cleaned with Nucleo Spin^{R} Gel and PCR Clean-up Kit. After digestion like with pROM108 agarose gel electrophoresis was done. The agarose gel electrophoresis plot (**Figure 9.19**) showed a band at expected length of 404 bp. This band was cut out under fluorescent light and cleaned with Nucleo Spin^{R} Gel and PCR Clean-up Kit. Before ligation the concentration of cleaned pROM108 [15 ng/µl] and CH3H6vH [80,5 ng/µl] were determined with Implen Nano-Photometer P-300.

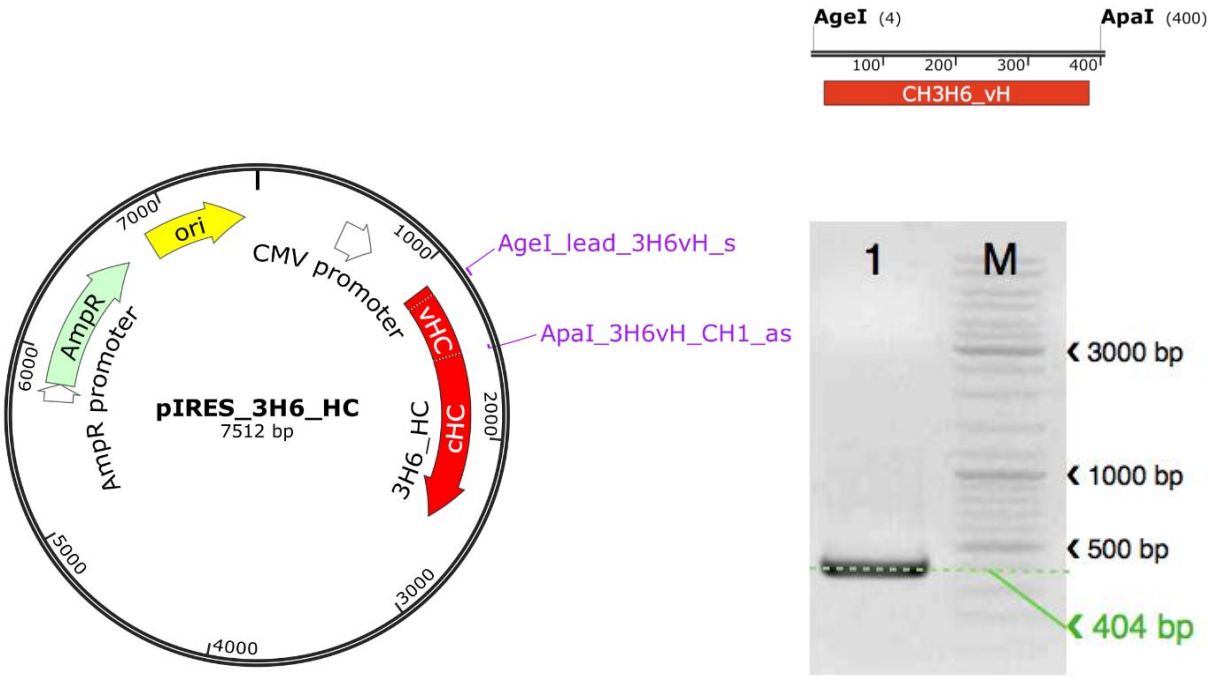


Figure 9.19: left figure: plasmid map of pIRES_3H6_HC with highlighted primer; **right top figure:** amplicon with *AgeI-HF* and *ApaI* restriction sites; **right bottom:** gel agarose plot of PCR amplicon, 1: PCR amplicon, M: 0.5µg generuler DNA ladder;

The ligation, of vector pROM108 with CH3H6_vH insert, was also performed with three different molar ratios from vector to insert (1:0, 1:1, 1:3). After transformation in electrocompetent *E.coli* TOP 10 bacteria and selection on LB agar plates supplemented with ampicillin, 16 clones were picked for colony PCR.

Table 9.3: counted after overnight culture of transformation pROM108 with CH3H6_vH

ratio	colonies
1:0	35
1:1	130
1:3	>300

All clones were picked from the ratio 1:1, because ratio 1:3 was overgrown. This new vector was called pROM108_3H6_HC (**Figure 9.20**). By doing a colony PCR with 16 clones the transformed bacteria were checked for positive clones. For colony PCR the primer huIgG_CH1_as_opt and primer HC_Lead_sense_opt were chosen. If ligation was successful

this primer pair should create a 487 bp long DNA fragment. 12 out of 16 tested clones showed a visible band at the desired length (**Figure 9.20**).For further vector construction clone 1 and 9 was picked for growing in LB-Media with antibiotics in an overnight culture. Before any further steps these two clones were transferred in cryotubes and stored at -80°C. All further cloning was done with clone 9. A mini prep kit was used to isolate the plasmid DNA from the bacteria. The amount of plasmid in the sample after mini prep was measured with a photometer and results in 195 ng/µl.

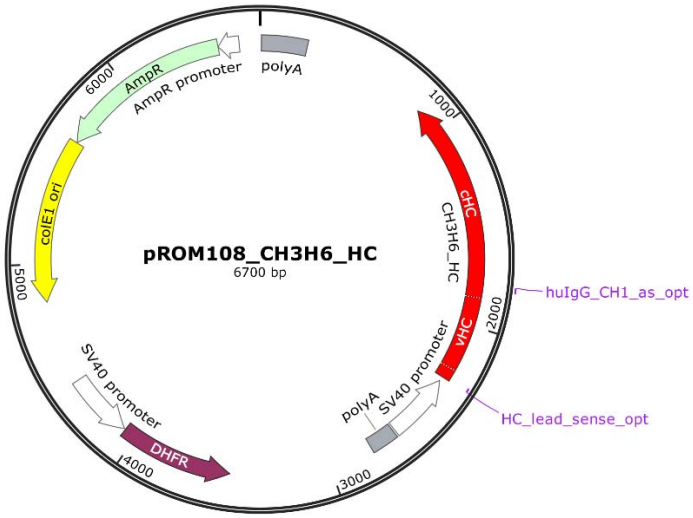


Figure 9.20: plasmid map of pROM108_CH3H6_HC with highlighted primer pair

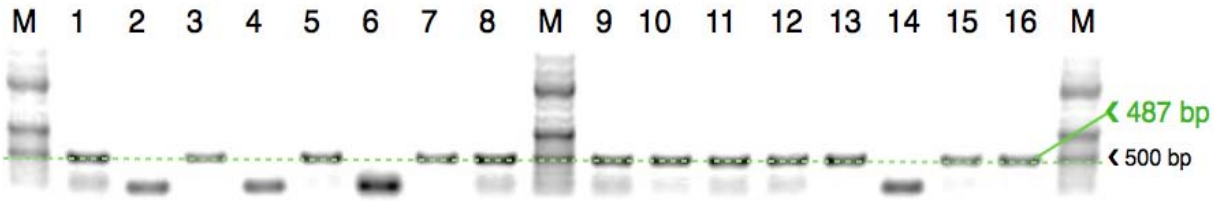


Figure 9.21: colony PCR of pROM108_3H6_HC, positive clones showing a band at 487 bp, M: 0.5µg generuler DNA ladder;

9.2.2.2 Ligation of vector pRMCE_dual with CH3H6_HC insert

The next step in constructing the final pRMCE_CH3H6_IgG vector was to insert this previously constructed CH3H6_HC heavy chain by ligation into the pRMCE_dual vector, same as it was done with SH3H6_HC for vector pRMCE_SH3H6_IgG. As the CH3H6_HC is

flanked by the same two single cutting restriction sites *NotI* and *XhoI* as SH3H6_HC (), the next few steps are identical to them. This steps are digestion with *NotI* and *XhoI*, creating blunt ends with Large (Klenow) Fragment, applying sample on agarose electrophoresis gel, cutting out the fragment which comprises the CH3H6_HC insert, cleaning it with Nucleo Spin^{R} Gel and PCR Clean-up Kit and finally determining the final concentration with Implen Nano-Photometer P-300. The agarose gel electrophoresis plot (**Figure 9.22**) showed two fragments with an expected length of 1432 bp and 5268 bp. The smaller fragment CH3H6_HC (**Figure 9.22**) was cut out under fluorescent light. Cleaned fragment concentration results in 23.5 ng/µl.

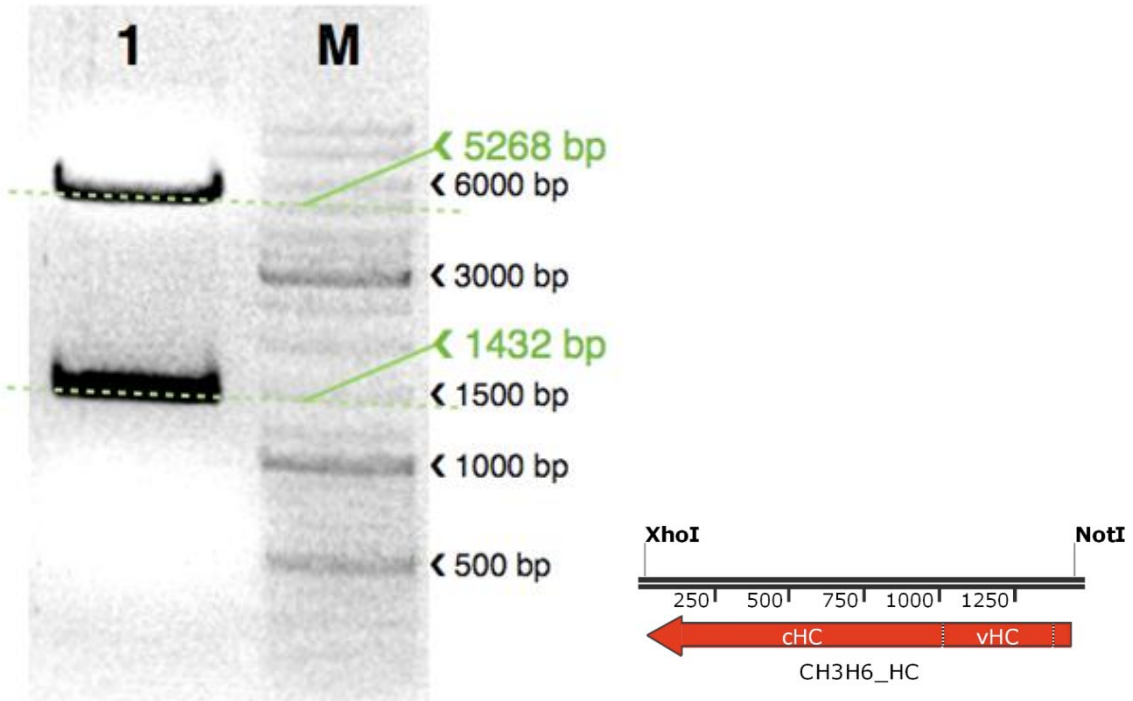


Figure 9.22: left figure: digestion of vector pROM108_CH3H6_HC [3 µg] with *NotI* and *XhoI* 1: two bands at the length of 1432 bp and 5268 bp are visible, M: 0.5µg generuler DNA ladder; **right figure:** CH3H6_HC fragment;

Next step was the ligation of CH3H6_HC with pRMCE_dual vector. Same as for the SH vector this should create a new vector, where the inserted fragment lies between the rEF1α promoter and the poly-A region which lies between the two significant FTR sites (**Figure 9.23**).

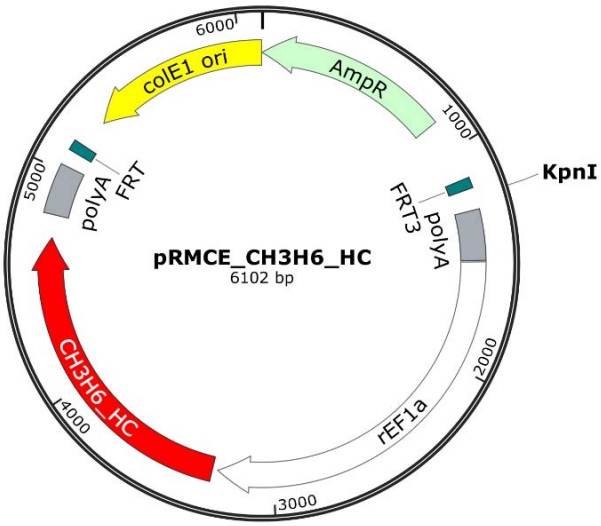


Figure 9.23: plasmid map of pRMCE_CH3H6_HC with highlighted restriction site *KpnI*

Because we had not enough digested pRMCE_dual vector from previous ligation, we had to redo all steps like cutting with *BstZ17I*, dephosphorylation with Alkaline Phosphatase Calf Intestinal (CIP), agarose gel electrophoresis using 1% agarose (**Figure 9.24**), cut out the fragment from gel under fluorescent light and cleaning with Nucleo Spin^{R} Gel and PCR Clean-up Kit. After this procedure the concentration of linearized pRMCE_dual was 80.2 ng/µl.

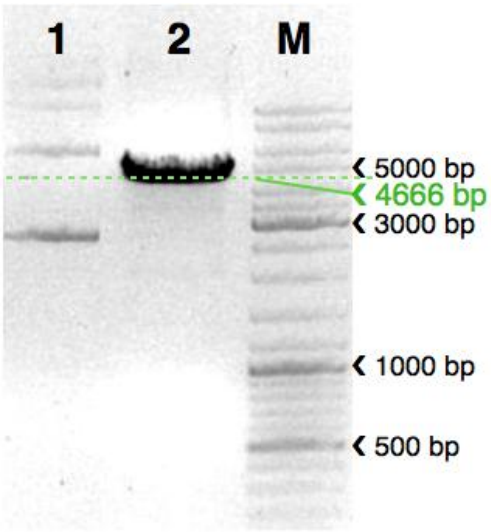


Figure 9.24: Gel plot of *BstZ17I* digested pRMCE_dual, 1: undigested pRMCE_dual [200 ng] vector, 2: digested pRMCE_dual vector [3 µg] shows a visible band at the length of 4666 bp, M: 0.5µg generuler DNA ladder.

The ligation of pRMCE_dual vector with CH3H6_HC insert, was also performed with three different molar ratios from vector to insert (1:0, 1:1, 1:3).

Table 9.4 Colonies counted after overnight culture of transformation pRMCE_dual with CH3H6_HC.

ratio	colonies
1:0	7
1:1	93
1:3	118

For further cloning, clones from the ratio 1:1 and 1:3 were used. By doing a colony PCR with 16 clones (clone 1 to 8 from ratio 1:1, clone 9 to 16 from ratio 1:3) the transformed bacteria were checked for positive clones. For this colony PCR two primer pairs (pROM112_1687s / pEF1a_PacI_a and MSC_AgeI_s / 13H5A5_HC_987_as) were used (**Figure 9.25**).

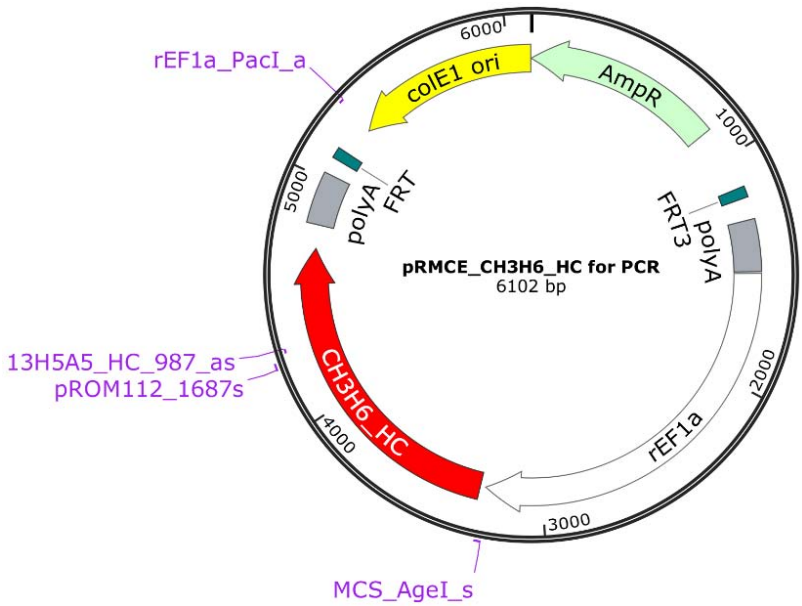


Figure 9.25: Plasmid map of pRMCE_CH3H6_HC with all used primers.

If ligation was successful this should create with the first primer pair a 1079 bp and with the second primer pair a 1058 bp long DNA fragment. Only 2 out of 16 tested clones showed visible bands at the desired length (

Figure **9.26**). Before any further steps these two clones were preserved in cryotubes at -80°C. For further vector construction clone 11 were picked for growing in LB-Media with antibiotics in an overnight culture. Again a mini prep kit was used to isolate the plasmid DNA from the bacteria. The amount of plasmid in the sample after mini prep was measured with photometer and results in 148 ng/µl. A control digestion with *AgeI-HF* (**Figure 9.27**) showed two visible bands at 2072 bp and 4030 bp which indicates only 1 inserted fragment taken up during ligation. The gel electrophoresis plot of this control digestion can be seen in **Figure 9.27**.

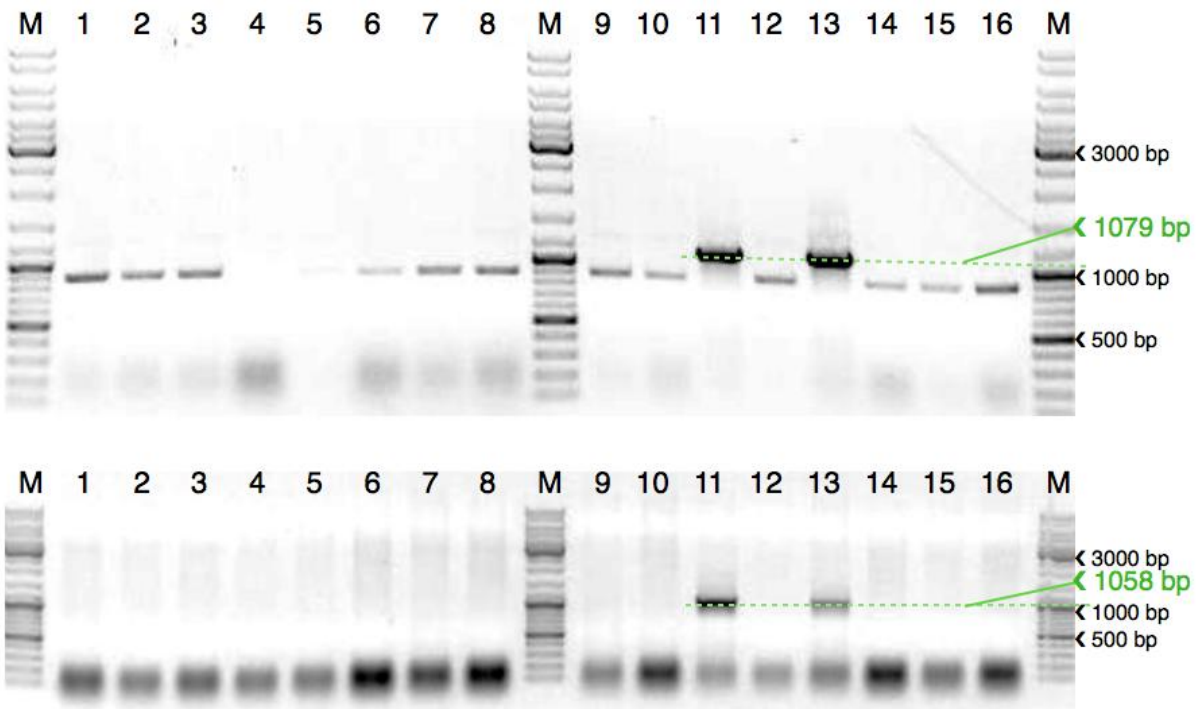


Figure 9.26: Colony PCR of ligation pRMCE_dual with insert CH3H6_HC, positiv bands can be seen at clones 11 and 13, M: 0.5µg generuler DNA ladder, upper plot visualizes the amplification with primerpair pROM112_1687s // pEF1a_PacI_a two bands at the desired length 1079 bp are visible, bottom plot visualizes the amplification with primerpair MSC_AgeI_s // 13H5A5_HC_987_a at the same clones two bands at the desired length of 1058 bp are visible.

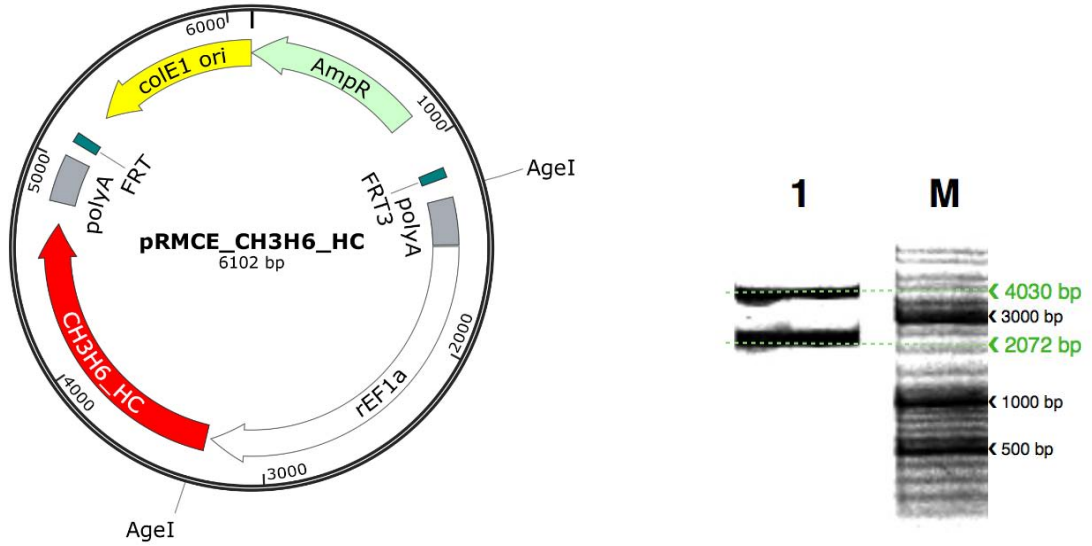


Figure 9.27: Left figure: plasmid map of pRMCE_CH3H6_HC with *AgeI-HF* restriction site. **right figure:** control digestion of pRMCE_CH3H6_HC [500 ng], 1:digestion with *AgeI-HF*, M: 0.5µg generuler DNA ladder.

The next step in constructing the final vector pRMCE_CH3H6_IgG was to insert CH3H6_LC (light chain) by ligation into this newly constructed vector pRMCE_CH3H6_HC. Same as for pRMCE_SH3H6_IgG the promoter for LC lies outside of the FRT sides on the DNA of compatible CHO cell (promoter trap). After cutting the vector pRMCE_CH3H6_HC (**Figure 9.23**) with KpnI and CIP treatment the sample had to be cleaned from all enzymes. This clean up was done with the Nucleo SpinR Gel and PCR Clean-up Kit. Afterwards a concentration of 48 ng/µl was measured with the photometer.

Figure 9.28: Control digestion of pRMCE_CH3H6_HC [50 ng], 1: undigested vector [50 ng], 2: digestion with *KpnI*, M: 0.5µg generuler DNA ladder.

To confirm the digestion agarose gel electrophoresis plot was done with the digested pRMCE_CH3H6_HC vector. If digestion with KpnI is successful only one band should be visible at 6102 bp length. **Figure 9.28** confirms the correct digestion of pRMCE_CH3H6_HC.

9.2.2.3 Sequencing of vector pRMCE_CH3H6_HC

Before working further with this vector, FRT site and CH3H6_HC insert had to be checked for cloning errors. The FRT sequence and the last 600 bp of inserted fragment were checked by sequencing with primer pEF1a_PacI_a (**Figure 9.29**). No deletion or exchange of bases where observed in the coding region of heavy chain nor in the FRT signal. Still a few deletions and an insertion of 17 bases were detected in between FRT and poly A signal. As before this disparities can be ignored because they are located in non-relevant spots (**Figure 9.30**).

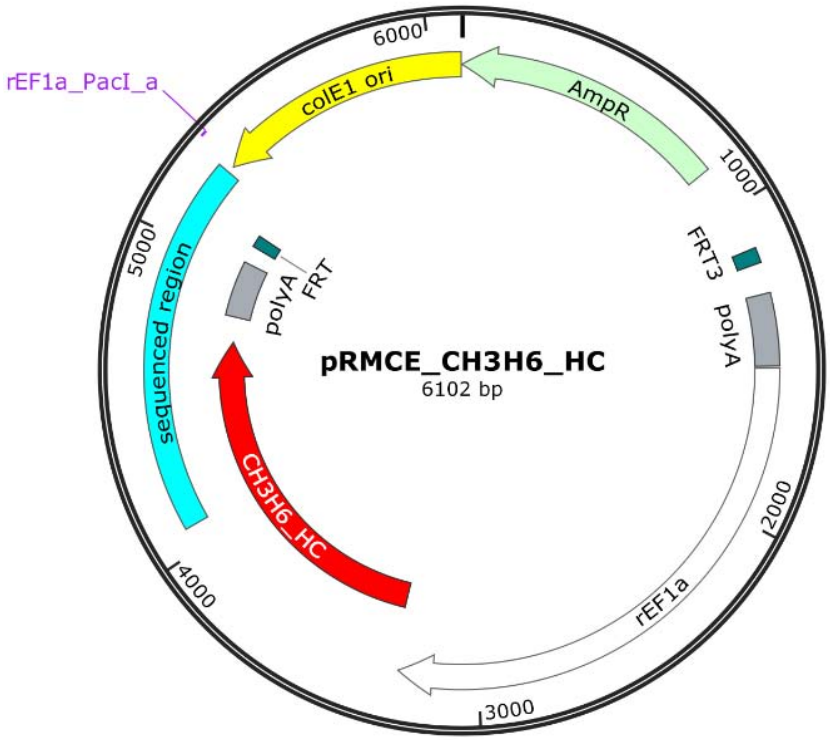


Figure 9.29: pRMCE_CH3H6_HC with primer for sequencing and highlighted sequenced region.

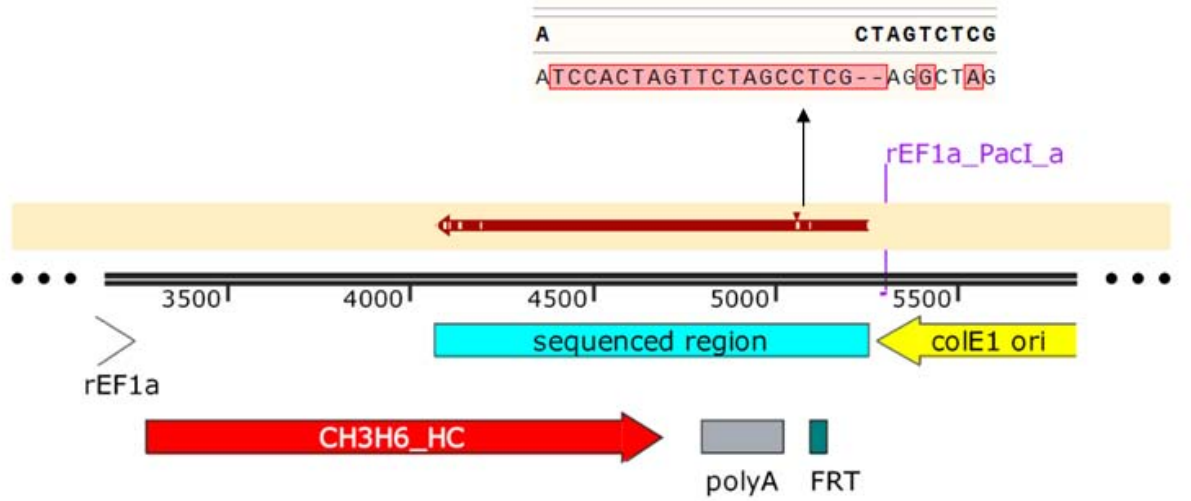


Figure 9.30: Alignment of CH3H6_HC were done with SnapGeneR, red arrows symbolizes the alignment, white spots in this red arrows are showing irregularities with the original plasmid map.

9.2.2.4 Construction of CH3H6_LC

Unlike for vector pRMCE_SH3H6_IgG where LC already exists on vector pROM111_SH3H6LC, for vector pRMCE_CH3H6_IgG the LC had to be constructed out of two different vectors. Leader and constant LC were located on vector pROM111 whereas variable LC was located on vector pIRESdhfr_3H6_Fab_LC. The next step was to construct the variable light chain fragment with *BspEI* restriction site on 3'-end and *BsiWI* restriction site on 5'-end. This was done by amplifying the light chain from the vector pIRESdhfr_3H6_Fab_LC with the primer pair BspEI_lead_3H6vL_s and BsiWI_ckappa_as (**Figure 9.31**). These two primers were designed in a way that after PCR the fragment implies *BspEI* and *BsiWI* restriction sites at the ends (**Figure 9.32**).

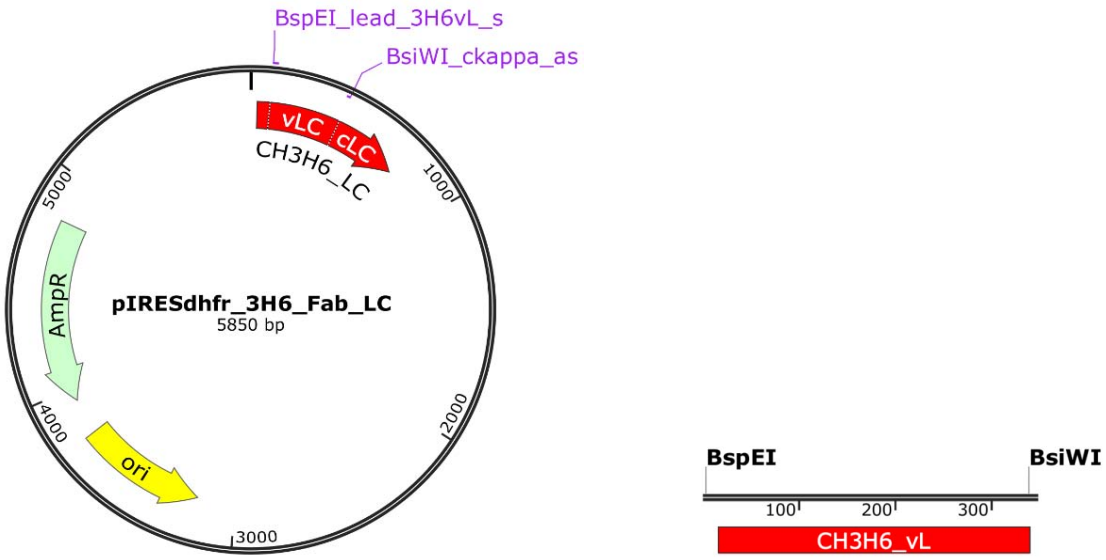


Figure 9.31: Left figure: plasmid map of pRMCE_CH3H6_HC with primer pair BspEI_lead_3H6vL_s and BsiWI_ckappa_a. **right figure:** amplified fragment

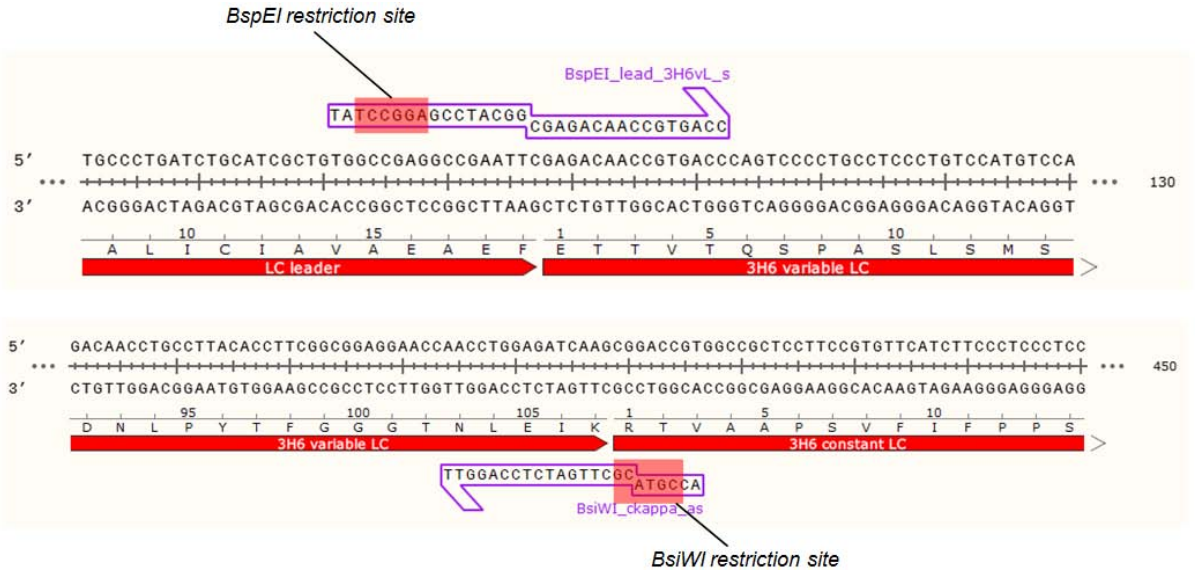


Figure 9.32: PCR amplification, primer BspEI_lead_3H6vL_s binds to the leader signal and integrates a *BspEI* restriction site befor the light chain sequence, whereas primer BsiWI_ckappa_as binds to variable and constant region of LC and integrates a *BsiWI* restriciton site on the constant LC.

After PCR amplification this sample was cleaned with Nucleo Spin^{R} Gel and PCR Clean-up Kit. To complete the variable LC the sample was digested with the two restriction enzymes *BspEI* and *BsiWI*. Like before the sample was cleaned with Nucleo Spin^{R} Gel and PCR Clean-up Kit. In the end the concentration of variable LC reaches 83.5 ng/µl. As control a gel

electrophoresis plot was done with the sample. One visible band at 336 bp confirms that PCR amplification had worked (**Figure 9.33**).

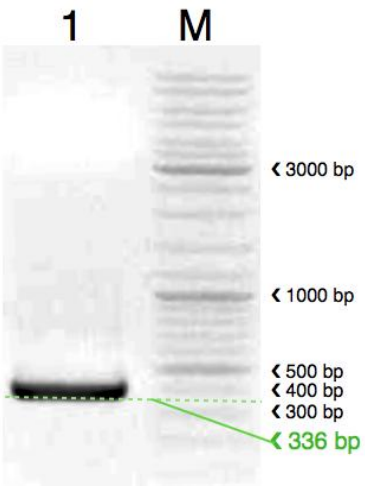


Figure 9.33: Electrophoresis plot of variable LC, 1: variable LC a visible band at 336 bp, M: 0.5µg generuler DNA ladder;

For working with vector pROM111 (**Figure 9.34**), which comprises leader and constant 3H6_LC, an overnight culture with cryopreserved cells was done. After overnight culture and Miniprep the concentration of plasmid [409 ng/µl] was determined with Implen Nano-Photometer P-300.

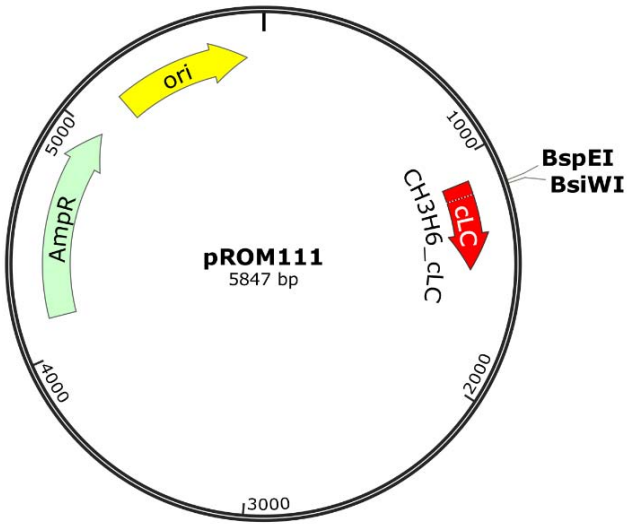


Figure 9.34: Plasmid map of pROM11 with highlighted restriction sites *BspEI* and *BsiWI.*

Next this vector was digested with the two single cutters *BspEI* and *BsiWI*, which were also used in construction of the variable LC. To prevent religation of this linearized plasmid, dephosphorylation with Alkaline Phosphatase Calf Intestinal (CIP) was performed. To separate the linearized plasmid from all enzymes a gel electrophoresis using 1% agarose was done (**Figure 9.35**). The band at 5832 bp was cut out under UV light and cleaned with Nucleo Spin^{R} Gel and PCR Clean-up Kit. After this procedure the concentration of digested pROM111 was 66.4 ng/µl.

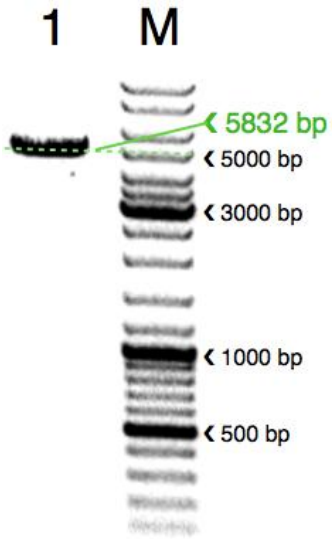


Figure 9.35: Electrophoresis plot of digested pROM111, 1: digested vector pROM111 [3 µg] with *BspEI* and *BsiWI* shows a visible band at 5832 bp, M: 0.5µg generuler DNA ladder;

9.2.2.5 Ligation of pROM111 with CH3H6 variable LC

The ligation, of vector pROM111 with CH3H6_vL insert, was also performed with three different molar ratios from vector to insert (1:0, 1:3 and 1:5). After transformation and selection on LB agar plates supplemented with ampicillin, 16 clones were picked for colony PCR.

Table 9.5: Colonies counted after overnight culture of transformation pROM111 with CH3H6vLC

ratio	colonies
1:0	0
1:3	11
1:5	49

All clones were picked from the ratio 1:3 and 1:5, because both ratios showed less growth. This new vector was called pROM111_CH3H6_LC (Figure 9.36).

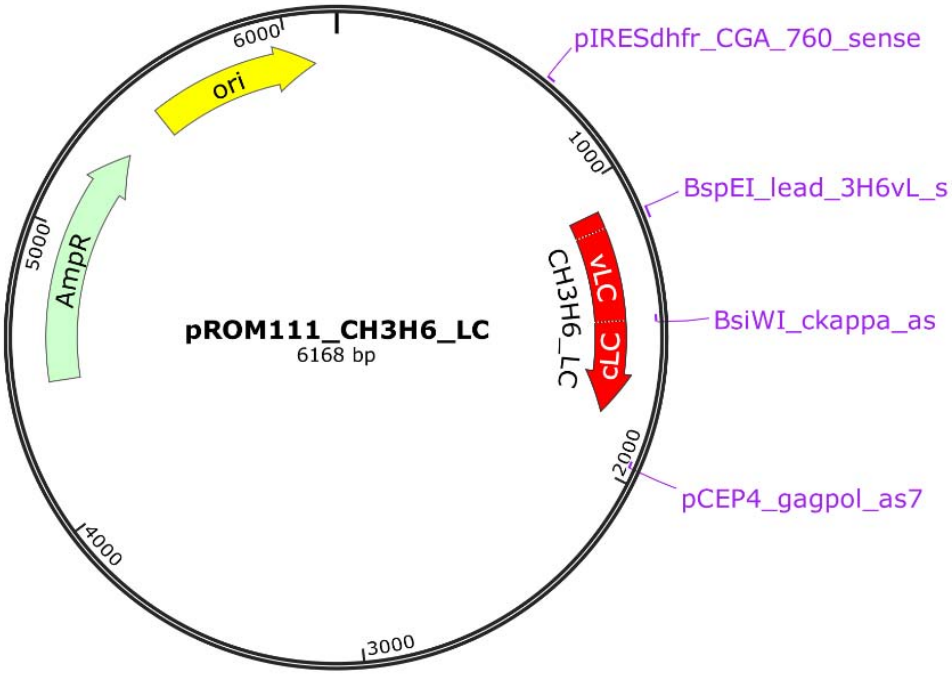


Figure 9.36: Plasmid map of pROM111_CH3H6_LC with highlighted primer pairs, primer pair 1 pIRESdhfr_CGA_760_sense and BsiWI_ckappa_as, primer pair 2 BspEI_Lead_3H6vL_s and pCEP4_gagpol_as7.

By doing a colony PCR with this 16 clones the transformed bacteria were checked for positive clones. For colony PCR the primer pair pIRESdhfr_CGA_760_sense and BsiWI_ckappa_as and primer pair BspEI_Lead_3H6vL_s and pCEP4_gagpol_as7 were chosen. In case of successful ligation this primer pairs should create bands at 823 bp and 807 bp. 3 out of this 16 tested clones showed a visible bands at the desired length (**Figure 9.37**). For further vector construction clone 9 and 14 were picked for growth in LB-Media with

antibiotics in an overnight culture. Before any further steps these two clones were transferred in cryotubes and stored at -80°C. All further cloning was done with clone 14. A Miniprep kit was used to isolate the plasmid DNA from the bacteria. The amount of plasmid in the sample after mini prep was measured with a photometer and results in 217 ng/µl.

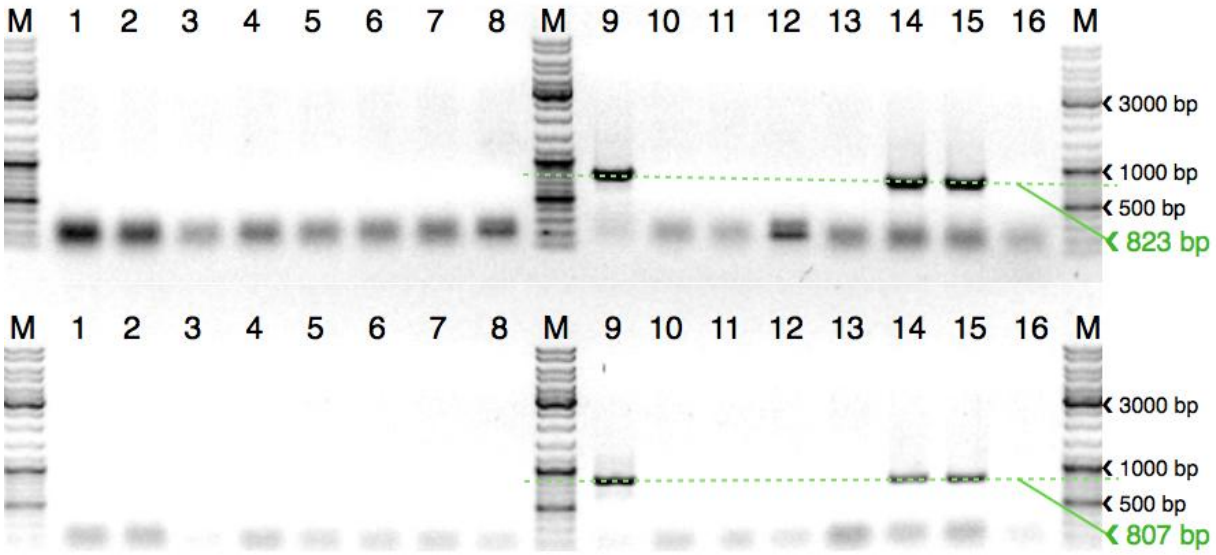


Figure 9.37: Colony PCR gel electrophoresis plot of pROM111_CH3H6_LC clones, clone 9, 14 and 15 were tested positive, M: 0.5µg generuler DNA ladder, upper plot visualizes the amplification with primer pair pIRESdhfr_CGA_760_sense and BsiWI_ckappa_as positive clones showing a band at the desired length of 823 bp, bottom plot visualizes the amplification with primer pair BspEI_Lead_3H6vL_s and pCEP4_gagpol_as7 at the same clones bands are visible at the desired length of 807 bp.

9.2.2.6 PCR amplification of CH3H6 light chain

For ligation with *KpnI* digested pRMCE_CH3H6_HC the insert CH3H6_LC needs to have *KpnI* restriction sites on both ends. This was done by amplifying the CH3H6_LC from the plasmid pROM111_CH3H6_LC in the same way as it was done for vector pRMCE_SH3H6_IgG (see chapter 9.2.1.2). For PCR amplification the same two primers were used to create *KpnI* restriction sites on both ends (**Figure 9.38**). After PCR amplification a gel electrophoresis (**Figure 9.38 right bottom figure**) was done and the band at length 724 bp were cut out and cleaned with Nucleo Spin^{R} Gel and PCR Clean-up Kit. As last step before ligation the fragment had to be cut with the enzyme *KpnI* and also as in the previous step where *KpnI* was used it had to be cleaned with Nucleo Spin^{R} Gel and PCR

Clean-up Kit. In the end the concentration of CH3H6_LC insert reaches 27,5 ng/µl, which was quite enough for more than one ligation.

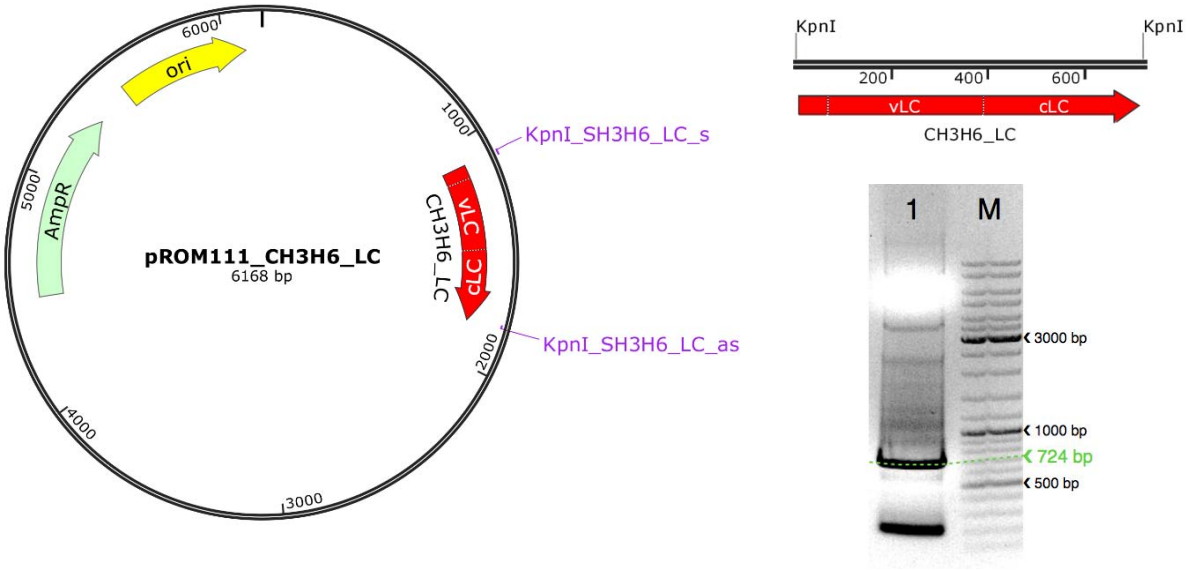


Figure 9.38: Left figure: plasmid map of pROM111_CH3H6_LC with primer pair KpnI_SH3H6_LC_s and KpnI_SH3H6_LC_as; **right top figure:** amplified fragment CH3H6_LC with *KpnI* restriction sides on both ends; **right bottom figure:** electrophoresis plot of amplified CH3H6_LC, a band can be spotted at 724 bp length which is the length of desired fragment.

9.2.2.7 Ligation of vector pRMCE_CH3H6_HC with CH3H6_LC insert

The Ligation, of vector pRMCE_CH3H6_HC with CH3H6_LC insert, was done in the same way as for ligation of vector pRMCE_SH3H6_HC with SH3H6_LC. After ligation and transformation (1:0, 1:3, 1:5) 16 clones were picked for colony PCR. 8 clones were picked from plate 1:3 and also from 1:5.

Table 9.6: Colonies counted after overnight culture of transformation pRMCE_CH3H6_HC with CH3H6_LC.

ratio	colonies
1:0	2
1:3	47
1:5	67

For colony PCR same primer pair was used as for vector pRMCE_SH3H6_IgG (**Figure 9.39**).

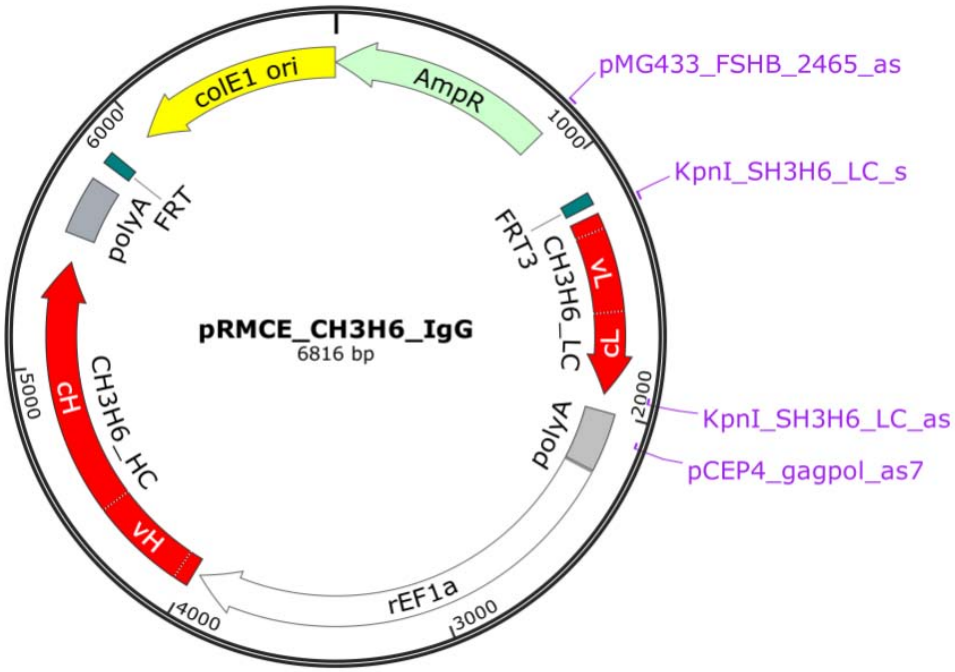


Figure 9.39: plasmid map of vector pRMCE_CH3H6_IgG with highlighted primer pairs

In case of correct ligation with primer pair pMG433_FSHB_2465_as and KpnI_SH3H6_LC_as the length of the amplicon should be 1072 bp long. For primer pair pCEP4_gagpol_as7 and KpnI_SH3H6_LC_s the length should be 849 bp long (**Figure 9.40**). 8 out of 16 clones were tested positive. For further vector construction clone 3 and clone 11 were chosen and were grown in LB-Media with antibiotics in an overnight culture. Before any further steps these two clones were transferred in cryotubes and stored at -80°C. A mini prep kit was used to isolate the plasmid DNA (pRMCE_SH3H6_IgG) from clone 3 from the grown bacteria. The amount of plasmid in the sample after mini prep was measured with a photometer and resulted in 472 ng/µl.

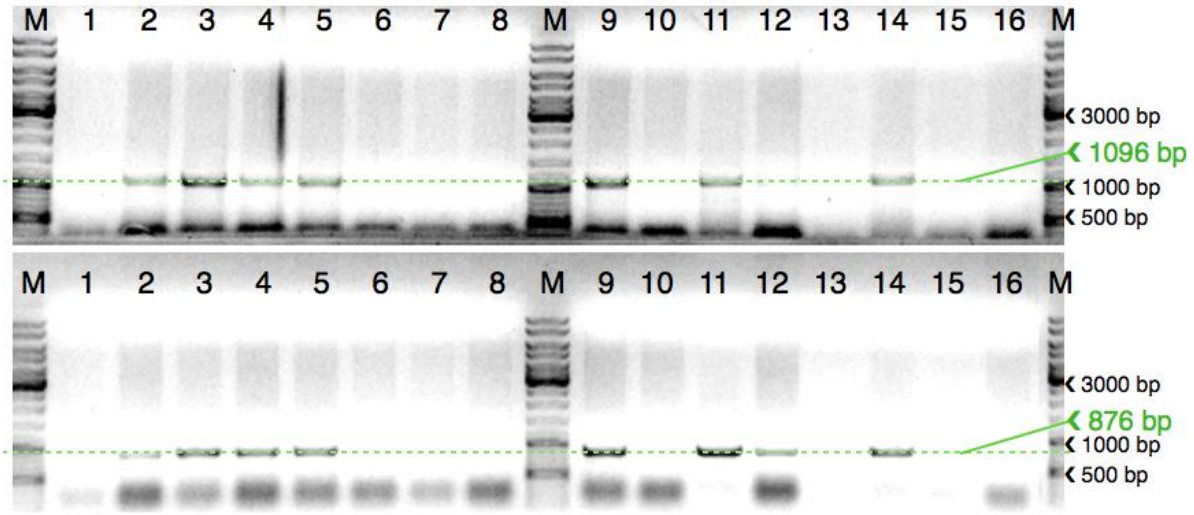


Figure 9.40: colony PCR of ligation pRMCE_SH3H6_HC with insert SH3H6_LC, row 1 - 8 were amplified with the primer-pair pMG433_FSHB_2465_as // KpnI_SH3H6_LC_as whereas row 9 - 16 were amplified with the primer-pair pCEP4_gagpol_as7 // KpnI_SH3H6_LC_s, positiv bands can be seen at clones 1, 2, 4, 6 and 7, M: 0.5µg generuler DNA ladder.

Before a midiprep was performed, this vector pRMCE_CH3H6_IgG were checked with a control digestion and by sequencing. As control digestion the restriction enzymes *BsiWI* was chosen. *BsiWI* should cut this plasmid two times (**Figure 9.41**). One restriction site is located between CH3H6_HC and poly A signal, the other one cuts in between the SH3H6_LC insert.

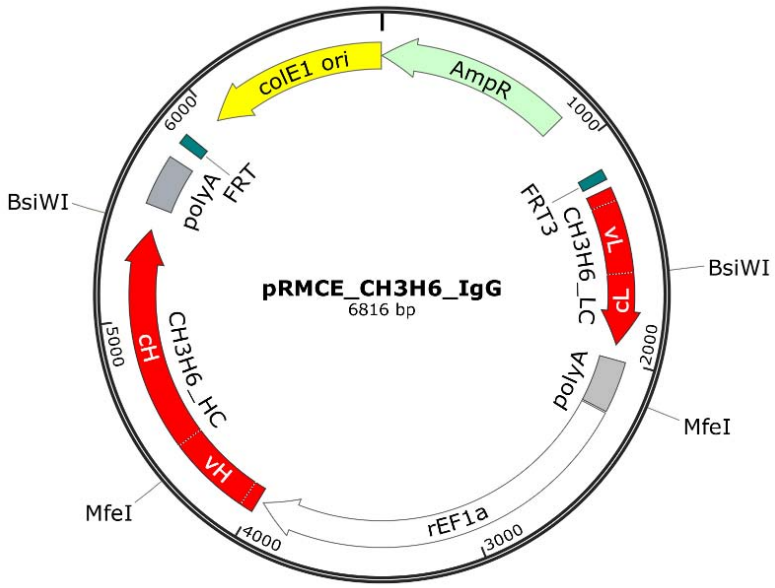


Figure 9.41: Plasmid map of pRMCE_SH3H6_IgG with highlighted restriction sites *BsiWI* and *MfeI-HF*.

Figure 9.42 shows the agarose gel electrophoresis plot of this digestion. Only one band at around 6800 bp was visible at the electrophoresis plot. Another enzyme *MfeI-HF*, which also cuts the plasmid at two sites (**Figure 9.41**), was chosen to perform a new control digestion. In **Figure 9.42** it appears that the digestion with this enzyme leads to the correct bands. Doing a sequencing of this plasmid should provide the information why enzyme *BsiWI* only cuts the plasmid one time.

Figure 9.42: left figure: 1: undigested vector pRMCE_CH3H6_IgG [500 ng] 2: digestion of pRMCE_CH3H6_IgG [500 ng] with restriction enzyme *MfeI_HF* results in 2 fragments (2210 bp and 4606 bp) 3: digestion of pRMCE_CH3H6_IgG [500 ng] with *BsiWI* results in only one visible band with 6816 bp, M: 0.5µg generuler DNA ladder; **right figure:** simulated gel electrophoresis of the same digestion with *BsiWI* (lane 1) and *MfeI-HF* (lane 2), in lane 1 there are 2 bands visible with the desired length.

9.2.2.8 Sequencing of final vector pRMCE_CH3H6_IgG

Also as for pRMCE_SH3H6_IgG five different primers were used for sequencing the vector as shown in **Figure 9.43**.

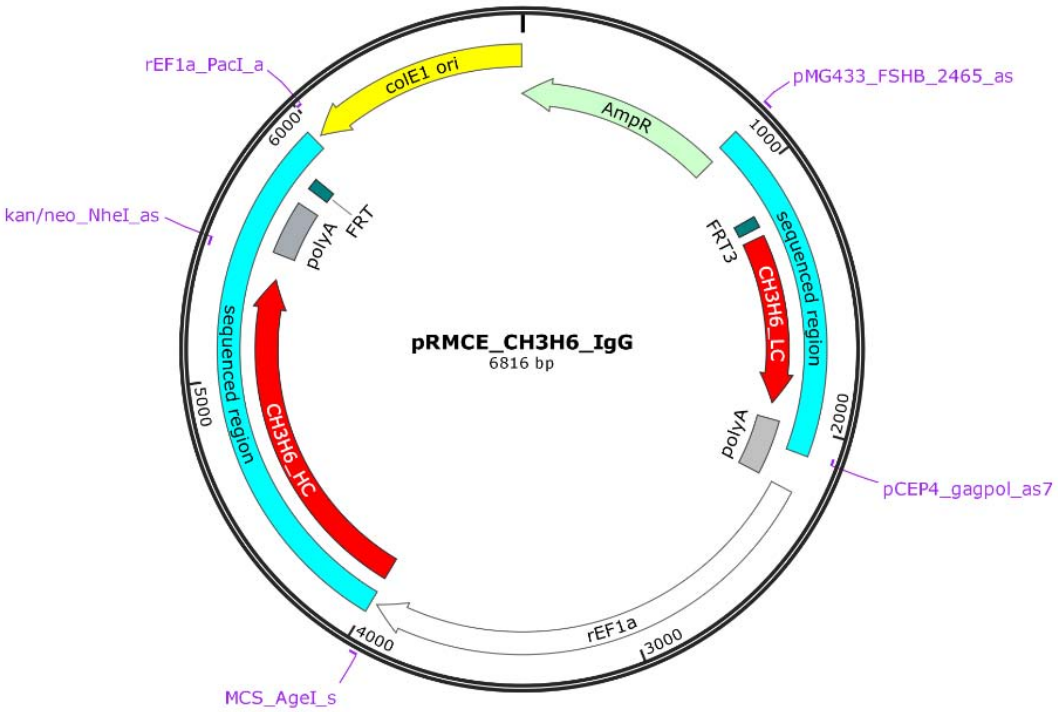


Figure 9.43: Plasmid map of pRMCE_SH3H6_IgG with primers which were used for sequencing and highlighted sequenced region.

To confirm the CH3H6_LC light chain sequence and the FRT3 signal, same two primers were used as for SH3H6_LC (**Figure 9.44**). The obtained sequences were aligned to the sequence of the plasmid map by using the program SnapGene Version 2.6.2 (free testversion). One insertion and on exchange could be identified in the none coding region upstream of the FRT sequence and between FRT3 and light chain sequence. Three exchanges in the region between light chain and poly A signal. All this mentioned disparities can be ignored because they are located in non-relevant spots. One more deletion, of three bases next to each other, was detected in the coding region of the light chain. Because of

this deletion the *BsiWI* restriction side got destroyed (**Figure 9.45**). This explains the failed digestion with *BsiWI* earlier see **Figure 9.42** where only one band occurred in the electrophoresis plot. The deletion affected the first two amino acids of the constant region (arginine and threonine). Because of the deletion of three bases no frameshift happened. The original DNA-Code which codes for arginine and threonine (**CGTACG**) was replaced by **CGG** which also codes for arginine. Generally spoken only one amino acid (threonine) was lost because of this deletion.

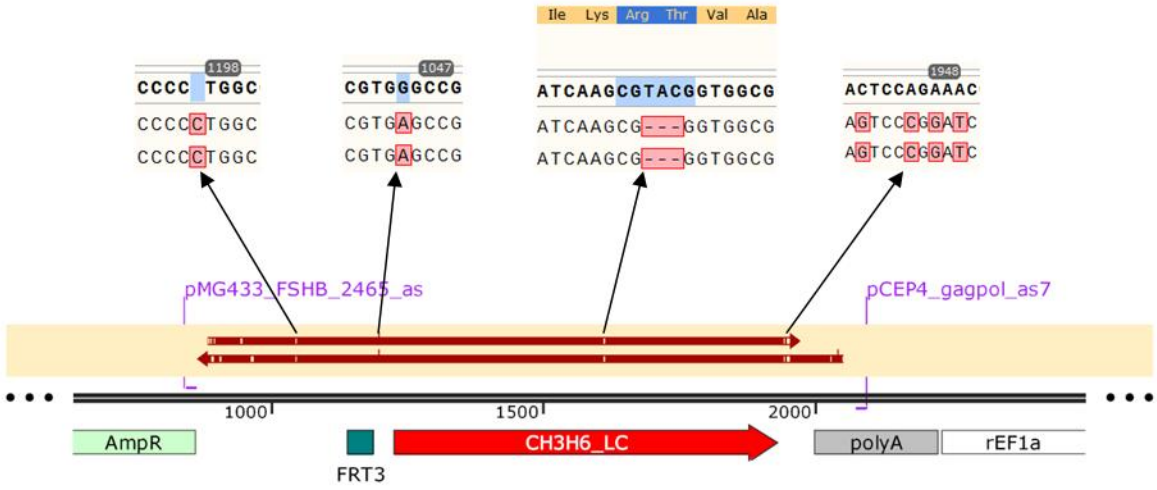


Figure 9.44: Alignment of CH3H6_LC were done with SnapGene[R], red arrows symbolizes the alignment, white spots in this red arrows are showing irregularities with the original plasmid map.

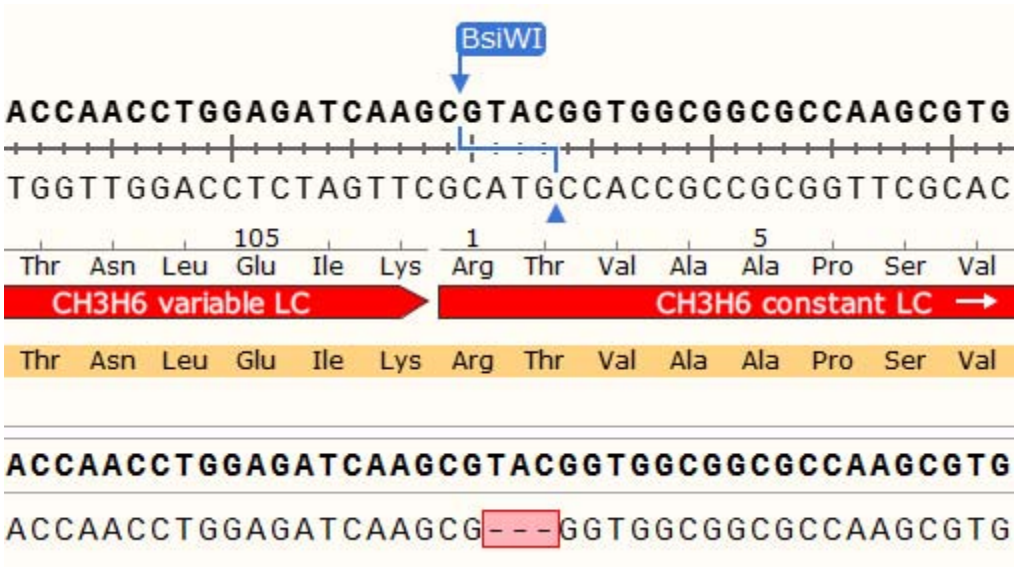


Figure 9.45: Alignment of CH3H6_LC with highlighted deletion in coding region

To check CH3H6_HC heavy chain sequence and the FRT signal three primers shown in **Figure 9.46** were used. Primer MCS_AgeI_s is located upstream the heavy chain sequence, primer kan/neo is located between poly A signal and heavy chain whereas primer rEF1a_PacI_as is located downstream of the FRT signal. No deletion or exchange of bases where observed in the coding region of heavy chain nor in the FRT signal. Still the same deletions and insertions as in vector pRMCE_SH3H6_IgG were detected in between FRT and poly A signal. As for pRMCE_SH3H6_IgG this disparities can be ignored because they are located in non-relevant spots.

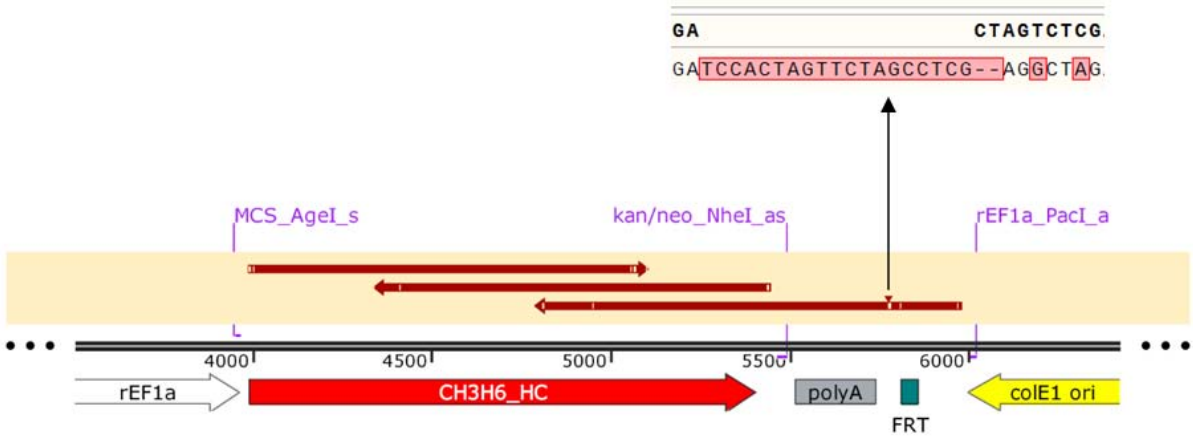


Figure 9.46: alignment of CH3H6_HC were done with SnapGene^{R}, red arrows symbolizes the alignment, white spots in this red arrows are showing irregularities with the original plasmid map.

After verification of the final plasmid a midiprep preparation was done to gain enough plasmid DNA. After this procedure the concentration of finally cleaned plasmid was measured with photometer and results in 993 ng/µl.

10 Discussion

The objective of this work was to develop two vectors (pRMCE_SH3H6_IgG and pRMCE_CH3H6_IgG), which comprises the sequence of an antibody, for transfection into RMCE competent CHO (DUKX-B11) cell line. Therefore both sequences heavy chain and light chain had to be cloned into the pRMCE_dual vector (which comprises of all relevant sequences for transfection into RMCE competent CHO cells) in a way that later, after transfection, the CHO cells are producing IgG antibodies. These two vectors should only differ in the variable domains of light chain and heavy chain. This was done to provide plasmids for further studies concerning productivity.

In constructing the pRMCE_SH3H6_IgG plasmid everything worked out as planned. All ligation steps worked out at first or second attempt. The most time-consuming steps were enzymatic digestion procedures. Some of them had to be redone because of insufficient digestion. At the beginning of this study some steps had to be repeated because of handling troubles, which after some practice hardly occurred later one. After all cloning steps the sequences of heavy chain and light chain were successfully sequenced. Over the entire length of sequences no deletions, insertions or exchanges were detected in between the coding regions. For later cell culture work around 0.5 mg plasmid DNA was produced by doing a midiprep.

Same problems occurred in constructing the pRMCE_CH3H6_IgG plasmid, whereas much more cloning steps were involved. Also after all cloning steps the heavy and light chain were sequenced. In the sequence of heavy chain no deletions, insertions or exchanges of bases were observed, whereas in the coding region of the light chain a deletion of 3 bases was detected. This involves the second, third and fourth base at the beginning of the constant domain. Mainly it was observed after digestion of pRMCE_CH3H6_IgG with the enzyme

BsiWI which should create three bands but only two were detected. After sequencing the whole light chain it reveals that exactly at the position of the restriction site of *BsiWI* 3 bases were missing. Because three bases were deleted no frame shift happened. Because of the deletion a threonine is missing at the beginning of the constant light chain peptide sequence (**Figure 10.1**).

```
                    10        20        30        40        50        60
original LC:    MALQTQVFISLLLWISGAYGETTVTQSPASLSMSIGEKVTIRCITSTDIDDDMNWYQQKP
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
sequenced LC:   MALQTQVFISLLLWISGAYGETTVTQSPASLSMSIGEKVTIRCITSTDIDDDMNWYQQKP

                    70        80        90        100       110       120
original LC:    GEPPRLLISDGNTLRPGVPSRFSSSGYGTDFVFTIENMLSEDVADYYCLQSDNLPYTFGG
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
sequenced LC:   GEPPRLLISDGNTLRPGVPSRFSSSGYGTDFVFTIENMLSEDVADYYCLQSDNLPYTFGG

                    130       140       150       160       170       180
original LC:    GTNLEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQ
                ||||||||*|||||||||||||||||||||||||||||||||||||||||||||||||||
sequenced LC:   GTNLEIKR-VAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQ

                    190       200       210       220       230
original LC:    ESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||
sequenced LC:   ESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
```

Figure 10.1: alignment of the original and the sequenced light chain sequence, the leader sequence is colored in red letters, the variable light chain sequence is colored in green letters and the constant light chain sequence is colored in blue letters.

This deletion could be because of error rate from ligation enzyme or it happened during digestion of variable light chain with *BsiWI*. It also is possible that the 5'-end of the variable light chain insert got degraded. However because no frame shift happened and only one amino acid got deleted the altered constant light chain should not cause any substantially changes in the antibody behavior. Preliminary results (not shown) suggest that the antibody function was not influenced by this deletion. By running a blast (Basic Local Alignment Search Tool) search at http://blast.ncbi.nlm.nih.gov/Blast.cgi with the altered constant light chain protein sequence no 100% match where found. Also of this vector 0.5 mg plasmid DNA was produced for later cell culture work by doing a midiprep.

11 List of tables

12 List of figures

13 References

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I declare that I have authored this thesis independently, that I have not used other than the declared sources / resources, and that I have explicitly marked all material which has been quoted either literally or by content from the used sources.

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