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Integration of retargetable FLP/FRT sites into DUKX-B11 CHO cells for Recombinase Mediated Cassette Exchange (RMCE)

Master thesis

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2 Abstract

Traditional concepts for the development of recombinant Chinese hamster ovary (CHO) producer cell lines are based on transfection methods that introduce the gene of interest into random genomic positions with unpredictable copy numbers. This strategy lacks reproducibility and requires a very time-consuming selection procedure for the most suitable cell clone. Recombinase mediated cassette exchange (RMCE), using the FLP/FRT system, provides a technique to introduce any gene of interest at the same genomic locus by the event of site specific recombination. Integration of any gene of interest into pre-characterized genomic loci with defined copy numbers would lead to predictable and more comparable expression patterns of the generated producer cell lines. In order to establish producer cell lines a RMCE-competent cell line has to be generated that can be used for the targeted recombinase mediated integration of any gene of interest for high-level recombinant protein expression.

In this study two successive rounds of RMCE were accomplished in CHO DUKX-B11 cells to generate RMCE-competent cell lines (called RMCE2 clones) with retargetable genomic loci. Exchange vectors were constructed by cloning experiments and introduced by different transfection methods. RMCE events were tracked using the green fluorescent protein (GFP) as reporter protein and analyzed by UV microscopy and flow cytometry. Suitable clones were selected based on different positive selection markers. Integrated RMCE cassettes were detected by polymerase chain reaction (PCR). Using this strategy RMCE-competent cell lines could be established in less than three months. During the two successive rounds of RMCE the disappearance and re-establishment of fluorescence due to exchange of the GFP marker was shown by UV microscopy and confirmed with flow cytometric analyses. Additionally the exchange of different cassettes integrated at each RMCE step could be verified by PCR. With the chosen strategy cell pools were generated successfully that have integrated RMCE-competent loci conferring green fluorescence and G418 resistance. These genomic loci could be used for subsequent exchange by any gene of interest.

3 Zusammenfassung

Traditionelle Techniken zur Erstellung rekombinanter Produktionszelllinien aus chinesischen Hamsterovarienzellen (CHO) basieren auf Transfektionsmethoden welche ein rekombinantes Gen in eine zufällige genomische Position und mit unvorhersagbarer Kopienzahl integrieren. Diese Vorgehensweise ist im Allgemeinen nicht reproduzierbar und basiert auf einem sehr langwierigen Selektionsverfahren für den besten Zellklon. Eine Technik namens Rekombinase mediierter Kassettenaustausch (RMCE) bietet die Möglichkeit zur gezielten Integration eines beliebigen „gene of interest“ in eine definierte genomische Position durch ortsspezifische Rekombination unter Verwendung des FRT/FLP Systems. Durch diese gezielte Integration des rekombinanten Gens in eine definierte genomische Position und mit einer bestimmten Kopienzahl könnten Zelllinien erstellt werden, die vorhersagbare und vergleichbare Expressionsprofile besitzen.

In dieser Masterarbeit wurde die Voraussetzung für eine nachfolgende RMCE geschaffen, nämlich die Etablierung der Wirtszelllinie mit einer RMCE-kompetenten Genkassette. Dazu wurden zwei aufeinanderfolgende RMCE Runden in CHO DUKX-B11 Zellen durchgeführt. Die erstellte Zelllinie (sogenannte RMCE2 Klone) trägt eine RMCE-kompetente Kassette, die nachfolgend durch verschiedene rekombinante Genkassetten ausgetauscht werden könnte. Die RMCE Austauschvektoren wurden durch molekularbiologische Klonierungsexperimente erstellt und modifiziert und mittels verschiedener Transfektionsmethoden in Säugetierzellen (CHO) eingefügt. Rekombinase mediierter Kassettenaustausch wurde mittels grün fluoreszierendem Protein (GFP) als Reportergen nachgewiesen und die erstellten Klone mit UV-Mikroskopie und Durchflusszytometrie analysiert. Mit Hilfe verschiedener positiver Selektionsmarker wurde auf geeignete Klone selektioniert. Die integrierten RMCE-Kassetten wurden mit Polymerasekettenreaktion (PCR) detektiert. Mit dieser Methode konnten innerhalb von 3 Monaten die gewünschten Zelllinien entwickelt werden. Die Erstellung und das Verschwinden der GFP-Fluoreszenz während verschiedener RMCE-Runden konnte mittels UV-Mikroskopie und Durchflusszytometrie beobachtet werden. Nach jeder RMCE-Runde konnte der Kassettenaustausch durch PCR Analysen verfolgt werden. Mit dieser Strategie konnten rekombinante Zelllinien

entwickelt werden, die einen RMCE-kompetenten genomischen Lokus besitzen, fluoreszieren und resistent gegenüber der Substanz G418 sind. Diese RMCE-kompetenten Loki könnten anschließend durch beliebige rekombinante Genkassetten ersetzt werden.

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5 Abbreviations

ampR	ampicillin resistance
bp	base pair(s)
BSA	bovine serum albumin
cDNA	complementary DNA
CHO	Chinese hamster ovary
CMV	cytomegalo virus
DAPI	4',6'-diamidino-2-pheylindole
dH2O	distilled water
dhfr	dihydrofolate reductase
$dhfr^-$	dihydrofolate reductase deficient
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphates
E.coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic
G	gravity
gDNA	genomic DNA
GFP	green fluorescent protein
HBS	HEPES buffered Saline
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HT	hypoxanthine and thymidine
kb	kilo base pairs
LB-medium	Luria-Bertani medium
L-gln	L-glutamine (4mM)
M	molar [mol/L]
mRNA	messenger RNA
MTX	methotrexate

neoR	neomycin resistance
OD	optical density
ori	origin of replication
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEI	polyethylenimine
polyA	polyadenylation
PPi	pyrophosphate
RNA	ribonucleic acid
rpm	revolutions per minute
S/MAR	scaffold/matrix attachment region
SOC medium	super optimal catabolite medium
SV40	Simian Virus 40
TAE	tris-acetate-EDTA
Tm	melting temperature
TRIS	Tris(hydroxymethyl)-aminomethan
U	unit
UV	ultraviolet
V	volt
w/v	weight/volume

6 Introduction

Chinese Hamster Ovary cells (CHO) are extensively used in the production of biopharmaceuticals with a worldwide market value of 99 billion USD in 2009 [1]. Typical products derived from this expression systems are growth factors, hormones, blood factors and recombinant therapeutic antibodies, the latter of which had a market value of $43 billion in 2010 [2].

Specific advantages of CHO cells are their safety for use in humans, capability of human-like glycosylation and posttranslational processing, as well as the availability of a useful technique for gene amplification (e.g. dhfr/MTX system), transfection and adaptation to serum/protein-free suspension culture [3, 4].

However, despite several unique advantages of CHO cells and mammalian cells in general, these expression systems depend on complex nutritional requirements and exhibit lower volumetric productivities and growth rates compared to microbial expression systems [5]. Several optimization strategies have therefore been developed to produce biopharmaceuticals in a cost effective manner that focus on the improvement of cultivation conditions or, more recently, improvements in the early steps of cell line development at the level of molecular biology and genetics.

Traditional development of high-producing CHO cell lines

Currently, the selection process for the most suitable clone starts with the transfection of the gene of interest into the host cell. Appropriate transcription regulatory elements drive expression of marker and/or selection genes that can be used for subsequent selection of stable transfectants by cultivation in media containing cytotoxic antibiotics or in the absence of metabolic precursors by using the dhfr/MTX or glutamine synthetase (GS) system respectively [4]. A gene amplification step is often performed in order to increase protein expression levels. Further optimization is done for cultivation in bioreactors and during scale-up.

The selection process for the best producing and performing clone is very time-consuming and lacks reproducibility since conventional transfection methods cause random integration into the host genome. Because of this random event, the integrated gene copy numbers and the integration locus

vary in different transfection experiments even when the same gene of interest construct is used. This leads to unpredictable expression behavior of the generated cell line. Therefore each generated cell line has to be characterized separately.

Efforts were made to develop techniques that overcome this laborious selection procedure in order to establish stable, predictable and high-producing cell lines.

Traditional concepts of generating recombinant cell lines rely on the random integration of the gene of interest into an unpredictable genomic locus. Integration of a gene of interest into a transcriptionally inactive site often leads to reduced expression levels of a cell population [6], [7]. The phenomenon that the transcription level of a gene of interest depends on its site of integration within the genomic host DNA and its surrounding chromatin structure is known as the “position effect” [8]. Beside the locus of integration, also the number of integrated gene copies is unpredictable in traditional transfection and selection procedures. Because of the fact that the integration locus and gene copy number can neither be controlled nor predicted with conventional transfection methods, it is very difficult to conduct comparative studies to investigate cause and nature of different expression levels in cell lines expressing different constructs (e.g. monoclonal antibodies).

In the last few years several methods were developed to integrate the gene of interest at pre-defined genomic positions and with a defined gene copy number which should result in the generation of cell clones with high and predictable expression patterns.

Recombinase Mediated Cassette Exchange (RMCE)

Recombinase mediated cassette exchange (RMCE), developed in 1994 by Bode et. al. [9], is a “tag and exchange” strategy [10] that can be used for introducing the gene of interest into a pre-determined chromosomal position (Figure 6.1). The host cell line is tagged with a construct carrying a reporter gene or selection marker flanked by two “heterospecific”, meaning non-compatible, recombinase targeting sites (RTs). Commonly used reporter/selection genes include the green fluorescent protein (GFP) for flow cytometric analysis, hygromycin- or neomycin phosphotransferase for positive selection, or thymidine kinase for negative selection. The reporter/selection gene can be used for pre-characterization of the genomic locus. Clones with the highest expression levels of the

marker/reporter can be selected. These clones should carry the recombinant gene at beneficial genomic loci leading to high transcription rates. These genomic loci can be targeted and exchanged by any construct carrying the same heterospecific recognition target sites. The underlying mechanism is mediated by a recombinase enzyme.

RMCE-competent clones can be used in a very flexible way. Using the FLP/FRT system, any gene of interest can be integrated into the same genomic locus by replacing the RMCE tag, leading to predictable and comparable expression levels of different cell clones each expressing a certain protein. All recombinant producer cell clones generated from the same RMCE-competent clone would have the gene of interest at the same genomic locus with the same gene copy number.

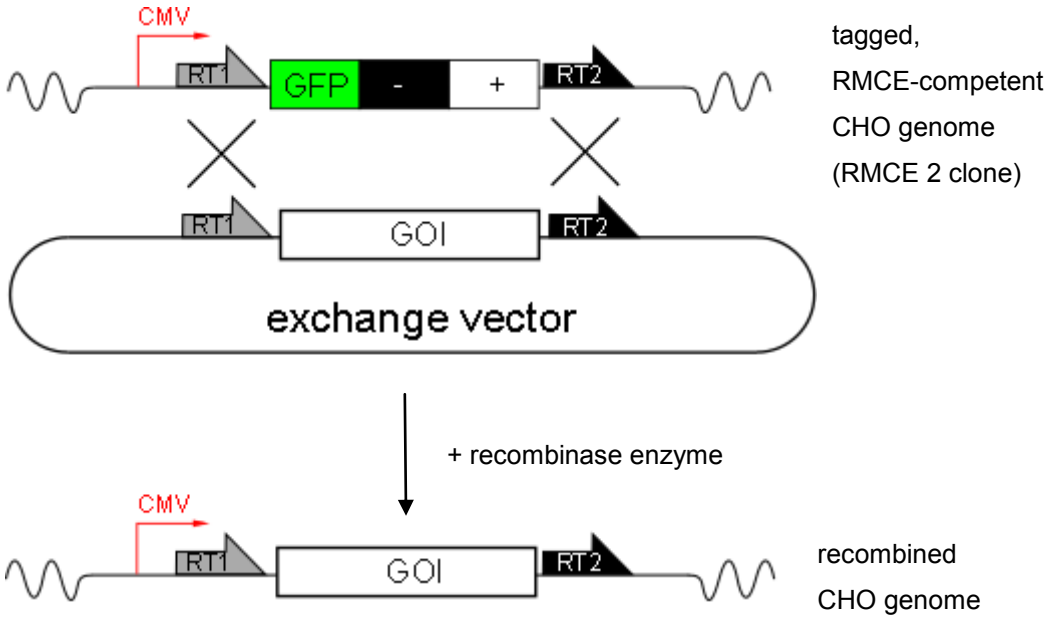

Figure 6.1: Concept of Recombinase Mediated Cassette Exchange (RMCE).

A tag in the final RMCE-competent cell line (RMCE2 clone), consisting of a marker/selection gene (GFP, green fluorescent protein; -, negative selection marker; +, positive selection marker) flanked by two heterospecific recognition target sites (RT1 and RT2), can be exchanged under the influence of a recombinase enzyme by a cassette consisting of a gene of interest flanked by the same set of heterospecific recognition target sites.

Initially the RMCE concept was developed with the yeast flippase (FLP) enzyme. Later the more active phage-derived Cre enzyme was preferentially used for RMCE techniques. Today several optimized FLP enzyme variants and different mutant forms of FLP recognition target sequences (FRTs) are available allowing flexible use of RMCE leading to comparable effectivities to the Cre mediated cassette exchange methods [11].

FLP/FRT System

The DNA site specific tyrosine recombinase FLP, derived from the autonomously replicating 2µ plasmid of *Saccharomyces cerevisiae*, was manipulated by Buchholz et. al. [12] to improve enzymatic activity at 37°C by a protein evolution strategy in *E.coli*, in order to reduce thermolability and to improve the rather moderate activity at 37°C of the wild-type FLP enzyme. Further optimization was accomplished by Raymond et. al. [13] by improving codon-usage in mammalian cells. In order to enhance translational efficiencies in mammalian cells by optimized codon usage the codons were altered according to the mouse tRNA availability without changing the amino acid sequence of the FLP enzyme. During codon optimization sequence motifs were avoided causing internal TATA-boxes, ribosomal entry sites, AT- and GC-rich regions, repeat sequences, RNA secondary structures, cryptic splice or polyadenylation sites to end up with improved mRNA stability. Finally, this thermostable, codon-optimized FLP variant (FLP optimized, FLPo) exhibits similar recombination efficiencies like the often used site-specific Cre recombinase [13].

The recombination event is initiated by co-transfection of the FLP expression plasmid and a plasmid containing the DNA sequence that should be recombined with the host chromosomal DNA. The FLP plasmid can be transiently expressed and does not have to be integrated into the host genome, whereas the stable expression of the recombined DNA sequence depends on integration into the host chromosome.

The recombination process starts at special DNA sequences called recognition target sites (RTs). The 48 bp long FLP recognition target sites (FRT) consist of a 8 bp spacer sequence flanked by two 13 bp inverted repeats and a third 13 bp repetitive sequence separated 1 bp from the first repeat at the 5’ site (Figure 6.2). The asymmetric structure of the 8 bp spacer determines the orientation of the respective FRT site. Depending on the relative orientation of one or several FRT sites on a given DNA construct, the FLP recombinase can catalyse the inversion, excision, integration or translocation of DNA sequences.

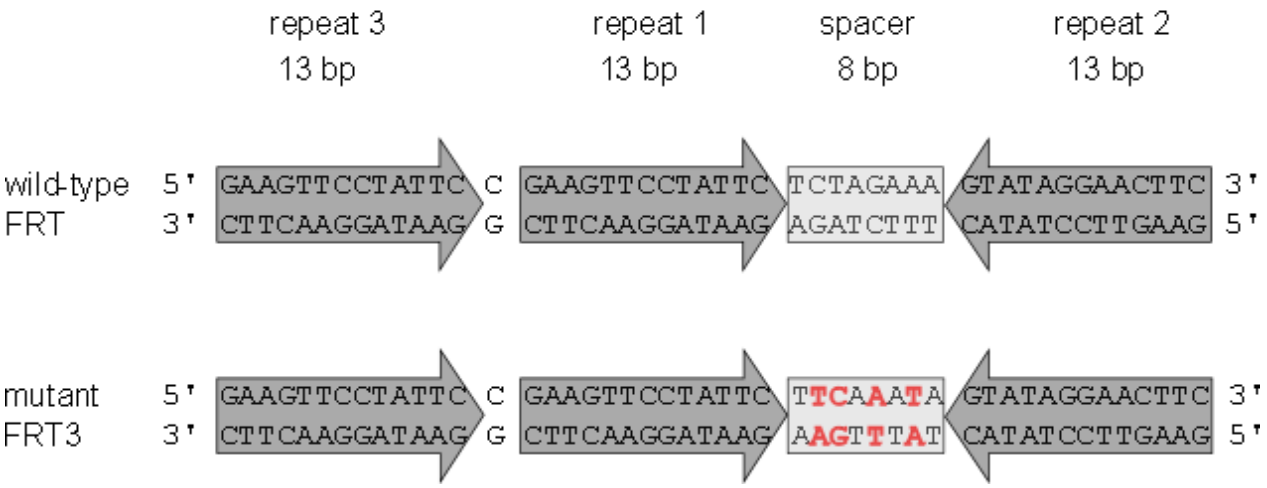

Figure 6.2: sequence structure of wild-type and mutated FLP recognition target sites (FRT).

Umlauf and Cox [14] postulated three rules of sequence properties that have to be met by a functional FRT site: Firstly, a 5' polypyrimidine tract starting within repeat 1 and extending into the spacer region should not be interrupted. Secondly, the AT content of the spacer region should be above 75% and, as a third prerequisite for a functional FRT site, the first 5' and last 3' position of the spacer sequence should be unchanged. Based on these rules several mutant FRT sites were developed that show high recombination efficiencies between two identical FRT sites but no cross-reactivity between two different FRT sites. All created mutant sites differ in their 8 bp spacer region which plays a crucial role in the recombination mechanism. To catalyse recombinase mediated cassette exchange (RMCE) two heterospecific FRTs with high self-recognition but no cross-reactivity were developed and their suitability for the intended purpose for RMCE was shown [9].

Enzymatic mechanism of FLP recombination

A general mechanism of all recombinase enzymes includes binding of protomers to their respective target sequences [11]: For the FLP/FRT system, it was shown that only two 13 bp inverted repeats, directly flanking the 8 bp spacer, are necessary for a successful recombination event. The third 13 bp repeat (repeat 3) only acts as a regulatory element (Figure 6.3 A)

The recombination mechanism starts with binding of two FLP protomers at two 13 bp repeat sequences (Figure 6.3 B). A second complex consisting of the same FRT bound to two FLP

protomers is associated with the first DNA/FLP complex in an antiparallel orientation, forming a so-called “synaptic complex”. Dimerization of the two protomers on each DNA double-strand leads to the bending of the respective spacer DNA. One of the two protomers is activated and exposes an active-site tyrosine residue (Y) at the enzyme amino acid position 343. The exposed tyrosine forms a covalent bond with the outermost nucleotide phosphate group of the spacer sequence leading to a single-strand break. The exposed free 5’-hydroxyl (OH) group acts as a nucleophile for the attack of the phosphor-tyrosine linkage of the partner DNA duplex. This so-called “OH-endonuclease action” [11] leads to the formation of a holliday junction intermediate. A second cycle of cleavage and rejoining completely reconstitutes the FRT sequence which can be used for further rounds of recombinase mediated cassette exchange (RMCE).

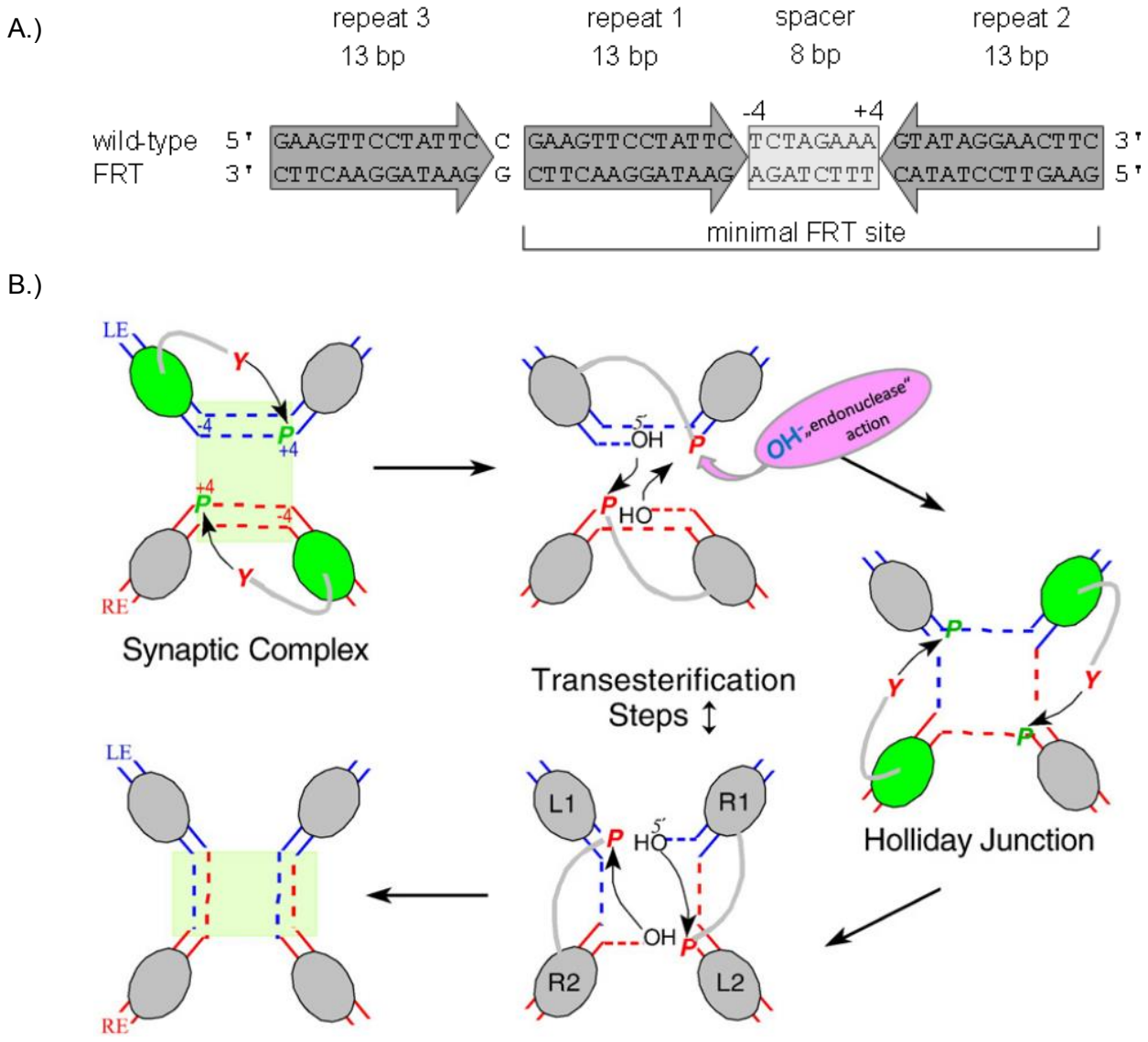

Figure 6.3: A.) sequence structure of the wild-type FLP recognition target site B) FLP mechanism taken from ref. [11].

7 Objectives

The RMCE strategy for the introduction of two heterospecific FRT sites into the CHO DUKX-B11 genome used in this study was adapted from Bode et. al. [10]. Thereby we generated a parental cassette harbouring the enhanced green fluorescent protein (d2eGFP) as reporter, flanked by two heterospecific FRT sites (Figure 7.1). The transcription of the reporter gene is controlled by a CMV promoter that is located upstream of the first 5' FRT site. This promoter-trap strategy, in which the promoter lies outside of the recombined sequence, should prevent the expression of any randomly integrated exchange gene that did not undergo recombination. Only recombinase mediated cassette exchange enables the gene of interest on the exchange vector to get under the control of the promoter and to be transcribed.

A second construct carrying the same set of heterospecific FRT sites was used for recombinase mediated cassette exchange. The d2eGFP gene is exchanged by a fusion gene of hygromycin-phosphotransferase and thymidine kinase that allows positive and negative selection, respectively. Recombination can be observed under the UV microscope by the vanishing of fluorescence since the GFP reporter gene should be replaced by the first RMCE cassette (RMCE1).

Recombined clones were selected in hygromycin B and were used for a second round of recombinase mediated cassette exchange (RMCE2) in which a triple-fusion gene of d2eGFP, thymidine kinase and neomycin phosphotransferase was introduced by recombination. These two rounds of RMCE should ensure that only clones with RMCE-competent loci can survive. Final RMCE2 clones and the previous RMCE1 clones were analysed by flow cytometry and PCR amplification.

For clarification of nomenclature of the different cell clones it should be emphasized that DUKX-B11 cells transfected only with the d2eGFP cassette were called "parental clones", clones after the first RMCE round (RMCE1), by co-transfection of "parental clones" with FLP plasmid and pF3hygTKF, were called RMCE1 clones and the final RMCE-competent clones, constructed by co-transfection of RMCE1 clones with FLP plasmid and pF3GTNF, were called RMCE2 clones (see Figure 7.1).

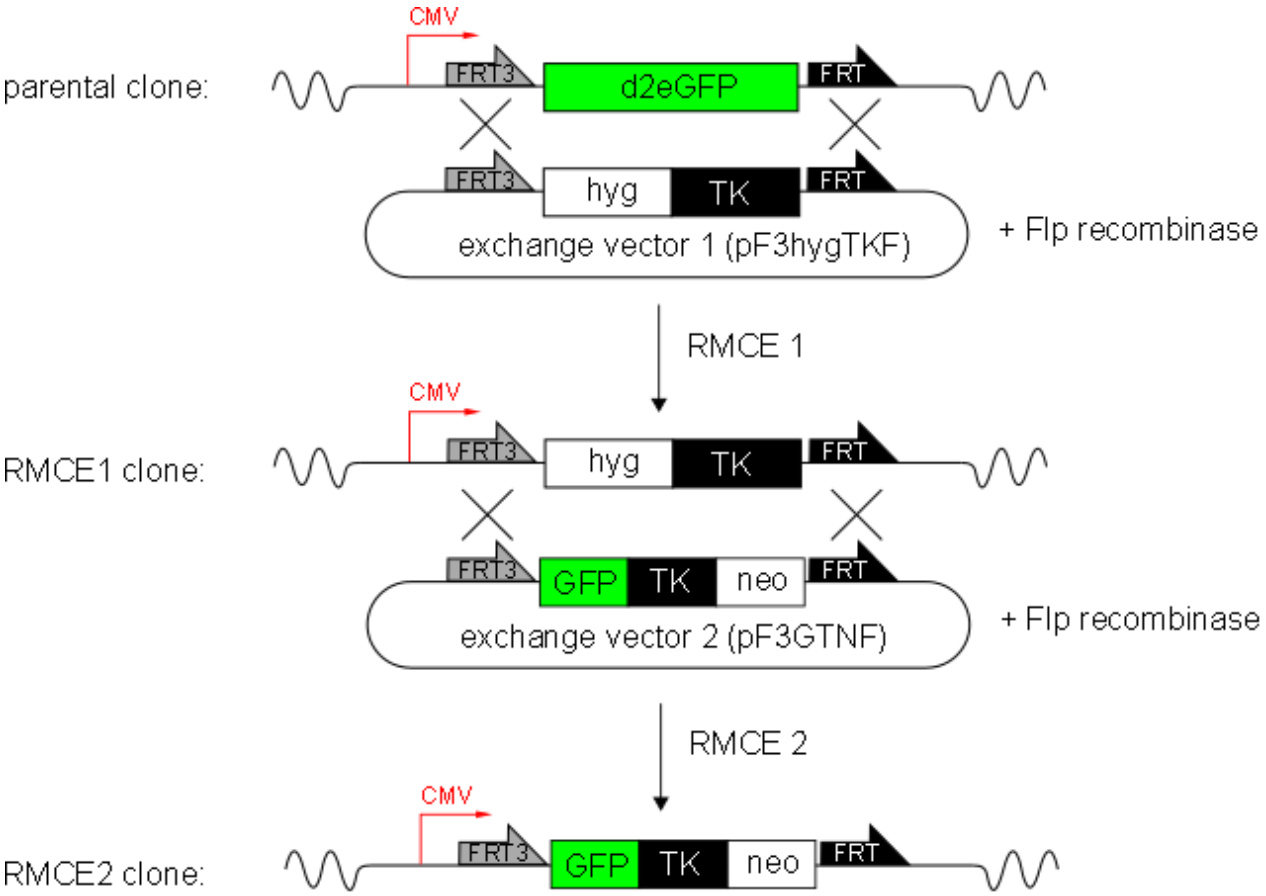

Figure 7.1: Strategy based on two rounds of RMCE for the generation of a RMCE-competent CHO cell line.

8 Materials and Methods

8.1 Equipment

Coulter Counter	Z2 Coulter Particle Count and Size Analyzer, Beckman Coulter, Item No. 6605700,
Photometer	Implen NanoPhotometer P-300
Flow cytometer	Gallios™ flow cytometer, Beckman Coulter
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 100 µL, Gislon, Cat. CN61618 Pipetman 20 µL, Gilson, Cat. CM57486 Pipetman 200 µL, Gilson, Cat. DK54448 Pipetman 10 µL, Gilson, Cat. P58933A Pipetman 1000 µL, Gilson, Cat. 51990
Hemocytometer	Neubauer 0.100 mm depth, 0.0025 mm^2, Optik Labor
CO_2 incubator	Heracell 150, Thermo scientific
Laminar flow hood	MSC-advantage, BSL-2, Thermo Scientific
pipetting controller	Pipet Help Accumax Matrix Cell Mate II Pipetboy acu, Integra Biosciences
multipipette	Gilson, 8x200 µL, Ref. F14403
centrifuge	Megafuge 16 Heraeus, Thermo scientific, Cat. 75004230 Eppendorf centrifuge 5424 Eppendorf centrifuge 5415R
autoclav	Varioklav, H+P Labortechnik GmbH
heating oven	Heraeus 50042293

vortex mixer	Vortex genie 2, scientific industries, Model. G560E
electroporator	Gene Pulser Xcell, 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 Doc XR, BioRad
gel electrophoresis chamber	BioRad
power supply for electrophoresis	Power Pac Basic, BioRad

8.2 Material

8.2.1 Chemicals and reagents

Glycerol	Merck, Cat. 1.04092.2500
Agarose	peqGOLD Universal-Agarose, peQLab, Cat. 35-1020
Agar-agar	Merck, Cat.1.01614.1000,
4',6'-diamidino-2-pheylindole (DAPI)	Sigma, Cat. D9542
Coulter isoton II diluent	Beckman Coulter, Item No: 8448011
COULTER CLENZ	Beckman Coulter, 5L (International), Item No:8448222
Trypan blue solution (0,4%)	Sigma, Cat. T8154, Lot. RNBB7800
polyethylenimine (PEI)	Polysciences Inc., Warrington, Pennsylvania, USA linear, 25 kDa, Cat. 23966
Gibco-Optimum	Gibco, Cat. 31985-062
Lipofectin	Invitrogen Cat. 18292-037
Hygromycin B	Invitrogen, Cat. 10687-010
G418 sulfate	PAA, Cat. P11-012, Lot P01210-2837
Phenol red	Sigma, Cat. P0290

HT supplement (50×)	Cat. 41065-012, Gibco, Life Technologies
Pluronic F68 (100×)	Cat. P1300, Sigma-Aldrich
Soja Peptone/UF (50×)	Polymun Scientific L0021
PF-supplement (100×)	Polymun Scientific L0018
L-glutamine (200 mM)	stable, Cat. M11-006, PAA
DMEM/HAM‘s F-12 1:1	F4815, Biochrom
T4 DNA Ligase buffer	Cat. B0202S, New England Biolabs
Thermopol buffer (10×)	Cat. B9004S, New England Biolabs
dNTPs (10mM stocks)	Cat. N0447L, New England Biolabs
BX loading buffer	Cat. R0611, Fermentas
Generuler ladder (0,5 µg/µL)	Cat. SM0331, Fermentas
BSA (100×)	Cat. B9001S, New England Biolabs
Buffer NEB1 (10×)	Cat. B7001S, New England Biolabs
Buffer NEB2 (10×)	Cat. B7002S, New England Biolabs
Buffer NEB3 (10×)	Cat. B7003S, New England Biolabs
Buffer NEB4 (10×)	Cat. B7004S, New England Biolabs

8.2.2 Consumables

Membrane filter	Millipore Express PLUS Membranfilter, Cat. SCGPU05RE
Pipette tips	Micronic Precision Tips, Cat. MP228C2
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
PP-test tubes	Cell star tubes, greiner bio-one, Cat. 227261
	Cell star tubes, greiner bio-one, Cat. 210261
Cryo-tubes	NuncTM cryotubeTM vials, Cat. 375418
Culture flasks	NuncTM NunclonTM surface T25, Cat. 163371

	NuncTM NunclonTM surface T80, Cat. 153732
	NuncTM NunclonTM surface T175, Cat. 156502
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
96 well plates	Nunclon™ Surface Nunc™ 96 well, Cat. 167008
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

Wizard Plus SV Minipreps, Promega, Cat. A1460

NucleoBond Xtra Midi EF, Cat. 740420.50

Nucleofector Kit V amaxa cell line, Lonza, Cat. VCA-1003

QIAamp DNA blood Mini Kit (50), Quiagen, Cat. 51104

8.2.4 enzymes

All enzymes were purchased from New England Biolabs.

T4 DNA Ligase	Cat. M0202S

Taq DNA Polymerase	Cat. M0267L
DNA I Polymerase Klenow	Cat. M0210S
Restriction DNA endonucleases	BspEI, Cat. R0540S
	KpnI , Cat. R0142S
	AgeI, Cat. R0552S
	NheI-HF, Cat. R3131S
	XcmI, Cat. R0533S

8.2.5 buffers and solutions

Phosphate buffered Saline (PBS) buffer

2 mM	KH_2PO_4
10 mM	$Na_2HPO_4 \cdot 2\ H_2O$
2.7 mM	KCl
140 mM	NaCl

pH 7.2-7.4

Cell lysis solution for Coulter® counter

0.1 M citric acid

2% (w/w) Triton X-100

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

1×	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

8.2.6 Oligonucleotides

Lyophilized primers purchased from Sigma (Table 8.1) were dissolved in dH_2O according to the manufacturer's instructions to yield a 100 µM stock solution. For PCR experiments and sequencing a 10 µM working stock solution was prepared by 1:10 dilution with dH_2O.

Nomenclature	Tm	sequence (5' > 3')
pIRESdhfr_CGA_578_sense	52.3°C	GCAGTACATCTACGTATTAGTC
pIRESdhfr_CGA_394_sense	63.1°C	CCATAGTAACGCCAATAGGGAC
SV40pAas	56.3°C	TGAGTTTGGACAAACCACAAC
pCIneo_5302sense	57.7°C	GGGAAACGCCTGGTATCTTT
HygTK_as	63.6°C	AATTCCCCAATGTCAAGCAC
d2eGFP_sense	66.3°C	GTGAGCAAGGGCGAGGAG
d2eGFP_as	64.0°C	TGCTCAGGTAGTGGTTGTCG
HygTK_AgeI_sense	71.2°C	ATACCGGTATGAAAAAGCCTGAACTCACC
HygTK_SpeI_as	67.9°C	GTACTAGTTCAGTTAGCCTCCCCCATCT
neo_as	62.5°C	TCAGAAGAACTCGTCAAGAAGG
hyg_sense	62°C	ATGAAAAAGCCTGAACTCACC
hyg_as	66.7°C	GGACTTCGGGGCAGTCCT
neo_sense	61.4°C	ATTGAACAAGATGGATTGCAC

Table 8.1: Oligonucleotides used for PCR and sequencing reactions

8.2.7 Plasmid DNA

pECMVF3d2eGFPFW: The 7.4 kb plasmid was obtained from Bode et. al. [10]. It consists of a S/MAR-E element followed by a CMV promoter that drives expression of the d2eGFP gene flanked by two heterospecific FRT sites. A HSV thymidine kinase polyA signal followed by a S/MAR-W element should ensure efficient transcription, RNA processing and translation of the d2eGFP protein. For bacterial amplification the ColE1 origin of replication and the ampicillin resistance marker (ampR) are present on this plasmid (Figure 9.1).

pEF3PtkhygTKFW: This plasmid was obtained from Bode et. al. [10] containing a hygromycin phosphotransferase thymidine kinase fusion protein under control of a thymidine kinase promoter flanked by two heterospecific FRT sites and the S/MAR-E and S/MAR-W sequence. The ColE1 origin of replication and the ampicillin resistance gene enable bacterial plasmid amplification.

pF3GTNF: This 5.2 kb promoter free plasmid harbours a triple fusion gene (GTN: d2eGFP, thymidine kinase and neomycin phosphotransferase) flanked by two heterospecific FRT sites. The polyA signal is derived from the HSV thymidine kinase gene. For high-copy number replication in bacterial cells the ampicillin resistance marker and the ColE1 origin of replication are present (Figure 9.3). This plasmid was obtained from Bode et. al. [10].

pPGKFLPobpA: This 5 kb mammalian expression vector was purchased from Addgene (plasmid 13793). A phosphoglycerate kinase (PGK) promoter drives expression of the FLPo enzyme. Bacterial amplification is possible by the presence of the ampicillin resistance marker and the pBR322 origin of replication (Figure 9.4).

8.2.8 Bacterial growth media for *E. coli*

Bacterial growth media were autoclaved for 20 min. at 121°C. Heat labile components (glucose, ampicillin) were sterile filtered (0.22 µm) before addition to the autoclaved medium.

Luria-Bertani (LB) ampicillin medium

Peptone, yeast extract and NaCl (Table 8.2) were dissolved in distilled water, aliquoted into 500 mL flasks, autoclaved at 121°C for 20 min. and stored at 5°C. Before usage 500 µL of sterile filtered (0.22 µm) ampicillin stock solution (1000 ×) was added to 500 ml of LB-medium to give a final ampicillin concentration of 100 µg/mL.

Concentration	component
10 mg/mL	peptone from casein
5 mg/mL	yeast extract
10 mg/mL	NaCl
100 µg/mL	ampicillin

Table 8.2: composition of LB-amp medium (Luira-Bertani ampicillin)

Luria-Bertani (LB) ampicillin agar plates

500 mL of LB medium (for composition see Table 8.2) without antibiotics were mixed with 16 g/L agar and stored at 5°C. For preparation of plates the LB agar was melted, cooled to approx. 50°C, 500 µL sterile filtered (0.22 µm) ampicillin stock solution (100 mg/mL) was added to reach a final ampicillin concentration of 100 µg/mL. Agar plates were prepared and solidified in a laminar flow hood.

Super Optimal Catabolite (SOC) medium

1 M glucose stock solution was autoclaved separately before it was added to the other autoclaved components (Table 8.3).

Concentration	component
2% (w/v)	Trypon (Bacto)
0.5% (w/v)	yeast extract
10 mM	NaCl
3 mM	KCl
20 mM	Glucose
10 mM	$MgCl_2 \cdot 6\ H_2O$
10 mM	$MgSO_4 \cdot 7\ H_2O$

Table 8.3: composition of SOC medium

8.2.9 Mammalian cell culture media

DMEM/HAM's F12 1:1 medium

Pre-mixed Dulbecco's Modified Eagle Medium with Ham's F12 in a 1:1 ratio (Biochrom) was supplemented with 4 mM L-glutamine, HT supplement (hypoxanthine 100 µM, thymidine 16 µM), 0.25% (w/v) soya peptone, 0.1% (w/v) Pluronic F68 and an in-house developed protein-free supplement.

ProCHO5 medium

ProCHO5 medium (Lonza) was supplemented with 4 mM L-glutamine, HT supplement (hypoxanthine 100 µM, thymidine 16 µM) and 11 mg/L phenol red (Sigma).

Hygromycin B selection medium

ProCHO5 medium (Lonza) was supplemented with 4 mM L-glutamine, HT supplement (hypoxanthine 100 µM, thymidine 16 µM), 11 mg/L phenol red (Sigma) and 200 µg/mL hygromycin B (Invitrogen).

G418 selection medium

ProCHO5 medium (Lonza) was supplemented with 4 mM L-glutamine, HT supplement (hypoxanthine 100 µM, thymidine 16 µM), 11 mg/L phenol red (Sigma) and 500 µg/mL or 1 mg/mL G418 (PAA).

8.2.10 Strains and cell lines

Escherichia Coli (*E.coli*) strain "Top10" with following genotype was used for cloning experiments:
F- mcrA (mrr-hsdRMS-mcrBC) 80lacZM15 lacX74 recA1 ara139 (ara-leu)7697 galU galK rpsL (Str^R) endA1 nupG>

Dihydrofolate reductase (dhfr)-deficient Chinese Hamster Ovary Cells DUKX-B11 (ATCC, CRL-9096, [15]) were used in all experiments.

8.3 Molecular biological methods

8.3.1 Restriction of plasmid DNA

Restriction of plasmids was done in an analytical or preparative scale. DNA endonuclease enzymes were purchased from New England Biolabs. Reaction conditions and composition (Table 8.4) were adapted according to the manufacturer's technical references. If possible, restriction endonucleases were inactivated through incubation at increased temperatures (65°C or 85°C) for 20 min. according to the manufacturer's instructions.

Component	amount
DNA	5 µg (preparative) or 0.5 µg (analytical)
buffer (NEB 1-4, 10x)	5 µL
endonuclease*	5 - 20 U
BSA (100x)	0.5 µL
dH_2O	ad. 50 µL

* one, two or three enzymes depending on the digestion setup

Table 8.4: composition of a typical analytical or preparative scale digestion reaction

8.3.2 PCR

A PCR master mix containing all components of Table 8.5 was prepared and 30 µL were aliquoted into PCR tubes. Standard PCR program of Table 8.6 was applied with 35 cycles (step 2 to 4). Alternatively a gradient for annealing temperatures (Ta) was used to find the optimal Ta. After amplification 6 µL of BX buffer was added and approximately 15 µL were loaded on 1% agarose gels and separated at 100V.

Component	volume/30 µL	final concentration
DNA template*	x µL	0.17-3.3 ng/µL
Sense primer (100×)	0.3 µL	100 nM
Antisense primer (100×)	0.3 µL	100 nM
Thermopolymerase buffer (10×)	3 µL	1 ×
Taq DNA polymerase (5 U/µL)	0.12 µL	20 U/mL
dNTP stock (10 mM each dNTP)	0.6 µL	200 µM each dNTP
sterile dH_2O	ad 30 µL	

* 5-100 ng template DNThe Taq DNA polymerase enzyme was added just before starting the PCR reaction.

Table 8.5: single reaction mix for PCR

Step	temperature	time	events
1	95°C	2 min.	DNA denaturation, cell breakage in colony PCR
2	94°C	30 sec.	
3	Ta	30 sec.	annealing of primers
4	72°C	1 min. /	
		1 kb product	Primer elongation
5	72°C	5 min.	additional extension step
6	4°C	forever	

Table 8.6: standard PCR program

8.3.3 Bacterial clone selection by colony PCR

A PCR master mix containing all components of Table 8.5 without any template DNA was prepared. 30 µL of the master mix were transferred into each PCR tube. For each reaction, one single colony was picked with a sterile toothpick from the agar plate, suspended in the PCR mix and subsequently streaked onto a LB-ampicillin master plate. PCR program according to Table 8.6 was applied with 35 cycles (step 2 to 5). For agarose gel electrophoresis 6 µL BX buffer were added and 6 µL were separated in a 1% agarose gels at 100V.

8.3.4 Agarose gel electrophoresis

Agarose gels were prepared by melting 1% (w/v) agarose in TAE in a microwave. The solution was cooled to approximately 50°C and supplemented with ethidium bromide to a final concentration of 200 ng/mL. Prepared gels were stored at 4°C in TAE buffer supplemented with 200 ng/mL ethidium bromide.

DNA samples were mixed with BX-buffer and separated in TAE running buffer at 100 V for 1h. Gel bands were visualized under UV illumination at 254 nm.

8.3.5 DNA isolation and purification from agarose gel

DNA fragments separated by agarose gel electrophoresis were extracted using the "Wizard SV Gel and PCR Clean-up system" (Promega) according to the manufacturer's protocol. DNA was eluted with 50 µL nuclease free water.

8.3.6 Preparation of electro-competent *E.coli* cells

For transformation experiments competent *E. coli* Top 10 cells were cultivated in 25 mL LB-media overnight (37°C, 200 rpm). A total volume of 8 mL of this starter culture were transferred into 800 mL LB-media and incubated until $OD_{600\ nm}$ reached a value between 0.6 and 0.8 (approx. 4h). The cell suspension was centrifuged (20 min., 5000 rpm, 4°C) washed three times with 500, 300 and 100 mL HEPES (15 min., 5000 rpm, 4°C) and resuspended in 20 mL Glycerine (10% v/v). After an subsequent centrifugation step (10 min., 5000 rpm, 4°C) the final cell pellet was resuspended in 1.5 mL Glycerin (10% v/v). 40 µL aliquots were prepared and stored at –70°C after flash freezing in liquid nitrogen. All washing steps were done at 4°C.

For determination of antibiotic resistance two 40 µL cell aliquots were electroporated with 3 µL dH2O, incubated in 1 mL SOC medium (1h, 200 rpm, 37°C), plated on LB-ampicillin (100 µg/µL) and LB-kanamycin agar plates (50 µg/µL) and incubated over night at 37°C/200 rpm. Absence of colonies indicated that that *E. coli* cells are free from antibiotic resistance.

For determination of transformation competency, 40 µL cell aliquots were electroporated with 3 µL of 100 pg/µL, 10 pg/µL and 1 pg/µL pUC19 plasmid solutions (Invitrogen). After incubation in 1 mL SOC medium (1h, 200 rpm, 37°C) the suspensions were plated on LB-ampicillin (100 µg/µL) and incubated over night at 37°C/200 rpm. A competency of 2.2×10^8 colonies/µg pUC19 was calculated.

8.3.7 Transformation of *E. coli* by electroporation

40 µL competent TOP10 *E. coli* aliquots were thawed on ice and gently mixed with 2-3 µL plasmid DNA solution. The mixture was transferred into a pre-cooled cuvette and an electric pulse (1800 V, 25 µF, 200 Ω) was applied. After addition of 250 µL SOC-medium transformed cells were incubated at 37°C for 1h and 200 rpm. 50 and 150 µL of this bacteria suspension was plated on LB-agar plates containing 100 µg/mL ampicillin.

8.3.8 Ligation

Ligation experiments were performed with an insert to vector DNA ratio of 1:1, 3:1 and 0:1 using 100 ng vector DNA in a reaction mixture containing insert DNA, 2 µL Ligase buffer and 400 U T4 DNA ligase filled up to a total volume of 20 µL with dH_2O. The used amount of insert DNA was calculated according to equation 8-1:

Equation 8.1:

$$mass\ of\ insert\ DNA\ (ng) = \frac{mass\ of\ vector\ DNA\ (ng)\ \times length\ of\ insert\ DNA\ (bp)\ \times excess}{length\ of\ vector\ DNA\ (bp)}$$

The mixture was incubated for 1h at room temperature and T4 DNA ligase was inactivated by incubation at 65°C for 10 min. 2 µL were used for transformation in *E.coli* and transformed cells were plated on LB-agar plates containing 100 µg/mL ampicillin. Selected colonies were screened by PCR using primer pairs where one bound specifically within the vector DNA sequence and the other one specifically within the insert DNA sequence. Positive clones containing the right plasmids were used for DNA mini-scale preparation.

8.3.9 Plasmid Preparation

Mini-scale plasmid DNA isolation:

Plasmid DNA in mini scale was accomplished by following the manufacturer's protocol (Promega, Wizard Plus SV Minipreps). For this a 10 mL over-night culture of *E.coli* was prepared by inoculating LB-ampicillin (100 µg/mL) medium with a single colony of *E.coli* carrying the respective plasmid with an ampicillin resistance gene (amp^R). After incubation over-night (12-16h) at 37°C / 200rpm, cells were harvested by centrifugation at 5000 G for 10 min. After purification the plasmid DNA was eluted with 100 µL "nuclease free water" supplied by the manufacturer.

Midi-scale plasmid DNA isolation:

For midi-scale plasmid DNA isolation the NucleoBond® Xtra Midi EF kit was used following the manufacturer's protocol (Macherey-Nagel). A starter *E.coli* culture was prepared by inoculating 5 mL LB-ampicillin (100 µg/mL ampicillin) medium with *E.coli* carrying the respective plasmid with the ampicillin resistance. After 6h of incubation at 37°C / 200 rpm, 100 µL of this starter culture suspension was added to 100 mL LB-ampicillin (100 µg/mL) medium and incubated over-night (12-16h) at 37°C / 200 rpm. After purification the plasmid DNA was eluted with 500 µL "H_2O-EF" supplied by the manufacturer.

8.3.10 Genomic DNA preparation

Genomic DNA for PCR analysis was isolated from 2×10^6 viable CHO cells and purified using the "QIAamp DNA blood Mini Kit (50)" (Quiagen) according to the manufacturer's protocol. DNA was eluted with 200 µL "ultra-purified water" and stored at 4°C.

8.3.11 DNA quantification

Concentration and purity of plasmid or genomic DNA samples were measured photometrically on a NanoPhotometer P-300 (Implen) by measuring the absorption at 260 nm and application of equation 8.2.

Equation 8.2:

DNA concentration [µg/mL] = $A_{260\ nm}$ * 50 µg/mL

The purity of DNA samples was determined by the ratio $A_{260\ nm}/A_{280\ nm}$ and lay between 1.7 and 1.9. Residual proteins exhibit an absorption maximum at 280 nm and therefore decrease the $A_{260\ nm}/A_{280\ nm}$ ratio.

8.3.12 Sequencing of PCR products and plasmids

DNA sequencing analysis of plasmid DNA was performed by the company MWG Operon. A typical reaction mixture contained 50-100 ng/µL purified plasmid DNA and 15 pmol sequencing primer in 15 µL dH_2O.

8.4 Cell culture methods

8.4.1 Cultivation of CHO DUKX-B11 host cells

CHO DUKX-B11 host cells were cultured in T25, T80, T175 and spinner flasks with Dulbeccos's Modified Eagle's Medium DMEM/Ham's F12 1:1, supplemented with 4 mM L-glutamine, 0.25% (w/v) ultrafiltrated soy peptone, 0.1% (w/v) Pluronic F68, an in-house developed protein free supplement (Polymun Scientific) and 1 × HT supplement (Biochrom AG, Germany) at a final concentration of 100 µM hypoxanthine and 16 µM thymidine. Cells were passaged after 3-4 days in a ratio between 1:2 and 1:8 with a starting total cell concentration of 2 × 10^5 cells/mL.

8.4.2 Determination of cell number and viability

Hemocytometer

500 µL cell suspension was mixed with 100 µL trypan blue (0,4%) to visualize dead cells which incorporate the blue solution because of lost cell membrane integrity. Each large square of the used hemocytometer (Neubauer counting chamber) has an area of 1 mm^2 with a depth of 0.1 mm resulting in a total volume of 0.1 µL per large square. 4 large squares per sample were counted for viable and dead cells. Cell concentrations and viability were calculated using equation 8-3 and 8-4:

Equation 8.3:

$$cell\ concentration\left(\frac{cells}{mL}\right) = \frac{number\ of\ counted\ cells\ \times 1.2\ (dilution\ factor) \times 10^4}{number\ of\ counted\ large\ squares}$$

Equation 8.4:

$$viability\ (\%) = \frac{viable\ cell\ concentration\ \times\ 100}{total\ cell\ concentration}$$

Coulter Counter

The automated particle counter Coulter Counter Z2 (Beckman Coulter) was used to minimize errors resulting from cell aggregates. 2 mL cell suspension was centrifuged at 1000 rpm for 10 min., resuspended in 1 mL triton citric acid buffer and incubated for 2 h at room temperature to disintegrate the cell membrane. 100-300 µL of this nuclei suspension was diluted with 9 mL Coulter isoton solution to get optimal number of counts between 10,000 and 20,000 particles. Only particles with a diameter larger than 2.875 µm were counted in duplicate by the Coulter Z2 Accu Comp software (Version 3.01a). Total cell concentration was calculated according to equation 8-5.

Equation 8.5:

$$total\ cell\ count\left(\frac{cells}{mL}\right) = \frac{a\ \times 2\ \times 9 + b}{b\ \times c}$$

a… average of two particle count results

b… used volume of cells in isoton solution (100-300 µL)

c… volume of harvested cell suspension (2 mL)

A multiplication factor of 2 is chosen since the measured volume in the coulter counter is 0.5 mL but the final results should be presented in cells/mL

8.4.3 Transfection of CHO DUKX-B11 cells

Electroporation

For electroporation the Amaxa Cell Line Nucleofector Kit V was used. 2×10^7 living cells were collected by centrifugation (1000 rpm, 10 min.), resuspended in 100 µL Cell Line Nucleofector Solution V (82 µl of Nucleofector solution plus 18 µl of supplement) together with 20 µg donor plasmid 1 (pECMVF3d2eGFPFW) and transferred into supplied certified cuvettes. After applying Nucleofector Program U-24, immediately 500 µL ProCHO5 + L-glutamine + HT medium were added and transferred into 1.5 mL pre-warmed ProCHO5 + L-gln + HT medium in a T25 roux flask. Transfection efficiency was evaluated under a UV microscope after cultivation for 24 h in a humidified incubator (37°C, 7% CO_2). 8 mL ProCHO5 + L-gln + HT medium was added and cell viability was determined by a hemocytometer.

PEI transfection

10^6 living cells were collected by centrifugation (1000 rpm, 10 min.), resuspended in 2 mL ProCHO5 + L-gln + HT medium and transferred into a T25 roux flask.

2 µg FLPo-plasmid (pPGKFLPobpA) was equilibrated with 6 µg pF3hygTKF or pF3GTNF and HBS buffer in a total volume of 100 µL for 10 min. at room temperature.

80 µg PEI was equilibrated with HBS buffer in a total volume of 100 µL for 10 min. at room temperature.

The two equilibrated 100 µL solutions were pooled and incubated for another 10 min. at room temperature. 200 µL of this solution were added to 2 mL pre-warmed cell suspension and incubated for 4 h in humidified air (37°C, 7% CO_2). Medium was exchanged by centrifugation (1000 rpm, 10 min.) and 2 mL of ProCHO5 + L-gln + HT medium.

The medium exchange step is very important since residual PEI induces cytotoxicity [18].

Lipofection

2 × 10^6 living cells were collected by centrifugation (1000 rpm, 10 min.), resuspended in 0.8 mL ProCHO5 + L-gln + HT medium and transferred into a T25 roux flask. 1.2 µg FLPo-plasmid (pPGKFLPobpA) was equilibrated with 3.6 µg pF3hygTKF and "Opti-Mem I" medium in a total volume of 100 µL for 15 min. at room temperature. 25 µL Lipofectin reagent (1 mg/mL) was equilibrated in 75 µL "Opti-Mem I" medium for 15 min. at room temperature.

The two equilibrated 100 µL solutions were pooled and incubated for another 15 min. at room temperature. 200 µL of this solution were added to 0.8 mL pre-warmed cell suspension. After incubation for 4 h in humidified air (37°C, 7% CO_2), 1 mL of ProCHO5 + L-gln + HT was added.

8.4.4 Selection and expansion of cell clones

24 h after co-transfection of the FLP expression plasmid and the RMCE cassette plasmid containing the selection marker, RMCE transfectants were selected with the appropriate selection medium in a limited dilution approach for a period of 3 weeks. Since successful co-transfection of the FLP expression plasmid and the RMCE cassette plasmid into the nucleus of mammalian cells followed by RMCE is considered to be a very inefficient event, a seeding density of 10^4 cells in 100 µL per well of a 96 well plate was chosen. After each week 100 µL of fresh selection media were added. ProCHO5 medium supplemented with 4 mM L-glutamine, HT supplement and hygromycin B (200 µg/mL) was used as selection medium for RMCE1 transfectants. RMCE2 clones were initially selected by using 500 µg/mL G418 for 3 weeks before selection pressure was increased to 1 mg/mL G418.

Selected clones were expanded to T25 flasks by 1:2 passages every 2-3 days.

8.4.5 Cryopreservation

5 × 10^6 cells in logarithmic growth phase were harvested by centrifugation at 1000 rpm for 10 min. The cell pellet was resuspended in 1 mL pre-cooled "CryoMaxx II" (PAA Laboratories) solution containing DMSO and was cooled to -80°C with a cooling rate of lower than 1°C/min. For long term storage the frozen cell suspension was transferred into liquid nitrogen (-196°C).

8.5 Flow cytometry

GFP expression was analysed on a Gallios™ flow cytometer (Beckman Coulter). 0.5 to 1 × 10^6 cells were washed (1300 rpm, 10 min.) with 1 mL PBS and resuspended in 200 µL PBS supplemented with 50 ng/µL 4',6'-diamidino-2-pheylindole (DAPI). CHO DUKX-B11 host cell line was used as negative control. For analysis with Kaluza® Software (Version 1.2, Beckman Coulter) and WinMDI (Version 2.9) viable cells were gated based on scatter properties (forward and side scatter) and absence of DAPI signal.

Results can be represented in diagrams by plotting GFP intensity (FL-1) signal as the abscissa vs. number of cells (in per cent of all gated cells) as the ordinate. Quantitative information of a single clone is given by the x-mean and x-mode value used for comparison of different clone populations. The x-mean value indicates the mean GFP intensity within a single clone population. The x-mode value represents the point on the abscissa containing the highest number of cells with that GFP intensity.

9 Results

9.1 Verification and modification of plasmids

9.1.1 pECMVF3d2eGFPFW

The 7.4 kb plasmid pECMVF3d2eGFPFW (Figure 9.1), used for the construction of tagged parental clones for RMCE, was initially constructed by Bode et. al. [10] comprising the 2.2 kb S/MAR-E (scaffold/matrix attachment region, [19]) followed by the cytomegalo virus (CMV) promoter and the destabilized variant of the green fluorescent protein (d2eGFP, [20]) flanked by two 48 bp heterospecific FLP recognition target (FRT) sequences followed by the HSV thymidine kinase polyA signal. The first FRT site contains the mutated 8 bp spacer sequence (FRT3: TTCAAATA), whereas the second FRT sequence contains the wild-type 8 bp spacer sequence (FRT: TCTAGAAA). The last element of this cassette consists of the 1.3 kbp S/MAR-W sequence. Additionally, on the plasmid there is a bacterial origin of replication (ColE1 ori) and the ampR (ampicillin resistance) gene for plasmid amplification and selection in *E. coli.*

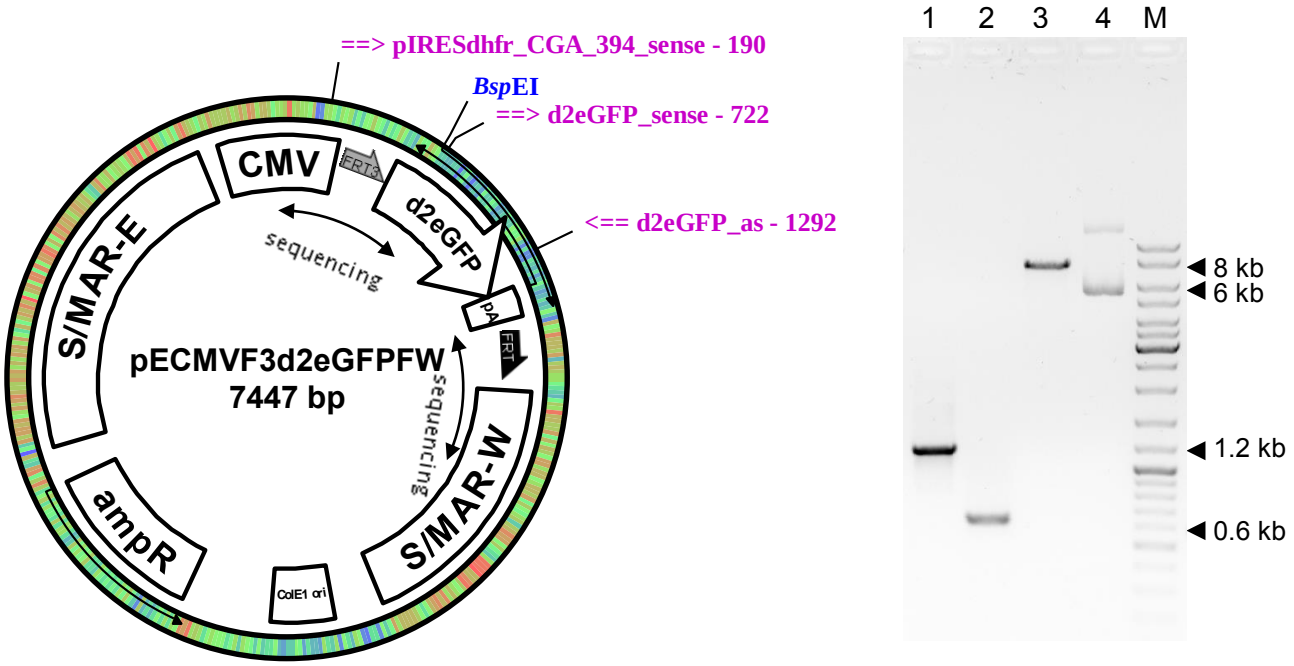

Figure 9.1: pECMVF3d2eGFPFW plasmid.

Left (plasmid map): S/MAR-E: scaffold/matrix attachment region E, CMV: cytomegalo virus promoter, d2eGFP: green fluorescent protein, FRT: wild-type FLP recognition target site, FRT3: mutant FLP recognition target site, S/MAR-W: Scaffold/Matrix attachment region W, ori: colE1 bacterial origin of replication, ampR: ampicillin resistance gene. Primers used for PCR analysis are depicted in pink with their respective binding site position in bp and orientation (right arrow= forward primer, left arrow= reverse primer). Single cutter restriction enzyme sites are indicated in blue and available sequencing results are indicated.

Right (agarose gel): lane 1: PCR analysis with primer pair pIRESdhfr_CGA_394 and d2eGFP_as which gave the expected 1.1 kb amplicon. Lane 2: PCR analysis with primer pair d2eGFP_sense and d2eGFP_as which gave the expected band at 600 bp. Lane 3: BspEI restriction analysis. The single BspEI restriction site could be verified resulting in the linearized plasmid at 7.5 kb. Lane 4: 100 ng of plasmid DNA used for transfection was applied. M: 0.5 µg of gene ruler DNA ladder. Sequence analyses were accomplished with primer pIRESdhfr_CGA 578 sense or SV40pAas.

Plasmid pECMVF3d2eGFPFW is used for the establishment of parental clones that carry two heterospecific FRT sites which can be used for subsequent rounds of recombinase mediated cassette exchange. The CMV promoter is placed 5' upstream of the first FRT site which constitutes a promoter-trap.

The d2eGFP reporter gene enables easy evaluation of transfection efficiency under UV microscope and can also be used as a first indicator for successful RMCE1 events since the d2eGFP cassette should be exchanged and therefore cells lose their fluorescence. Furthermore GFP-fluorescence can be analysed in flow cytometry without the need for fluorochrome labelled antibody dyes.

Scaffold/matrix attachment sites (S/MAR) are AT-rich sequences with a length of <3 kb that are known to attach at the nuclear membrane and establish locally independent chromatin domains that isolate the comprised DNA sequences from repressive effects of heterochromatin. Furthermore it was shown that S/MAR sequences could favour RMCE processes [6]. The 2.2 kb long S/MAR-E sequence initially was isolated from an EcoRI restriction fragment from the human interferon gene [21]. The 1.3 kb S/MAR-W was derived from the first intron of the potato leaf stem-specific protein ST-LS1 [22].

9.1.2 pF3hygTKF

The promoterless plasmid pF3hygTKF (Figure 9.2) for RMCE1 was constructed by PCR amplification of the hygromycin-thymidine kinase fusion protein with primers HygTK_SpeI_as and HygTK_AgeI_sense that contain SpeI and AgeI restriction sites, respectively. The PCR amplicon was digested with SpeI and AgeI and ligated as an insert into the compatible AgeI and NheI digested pF3GTNF (Figure 9.3) vector backbone.

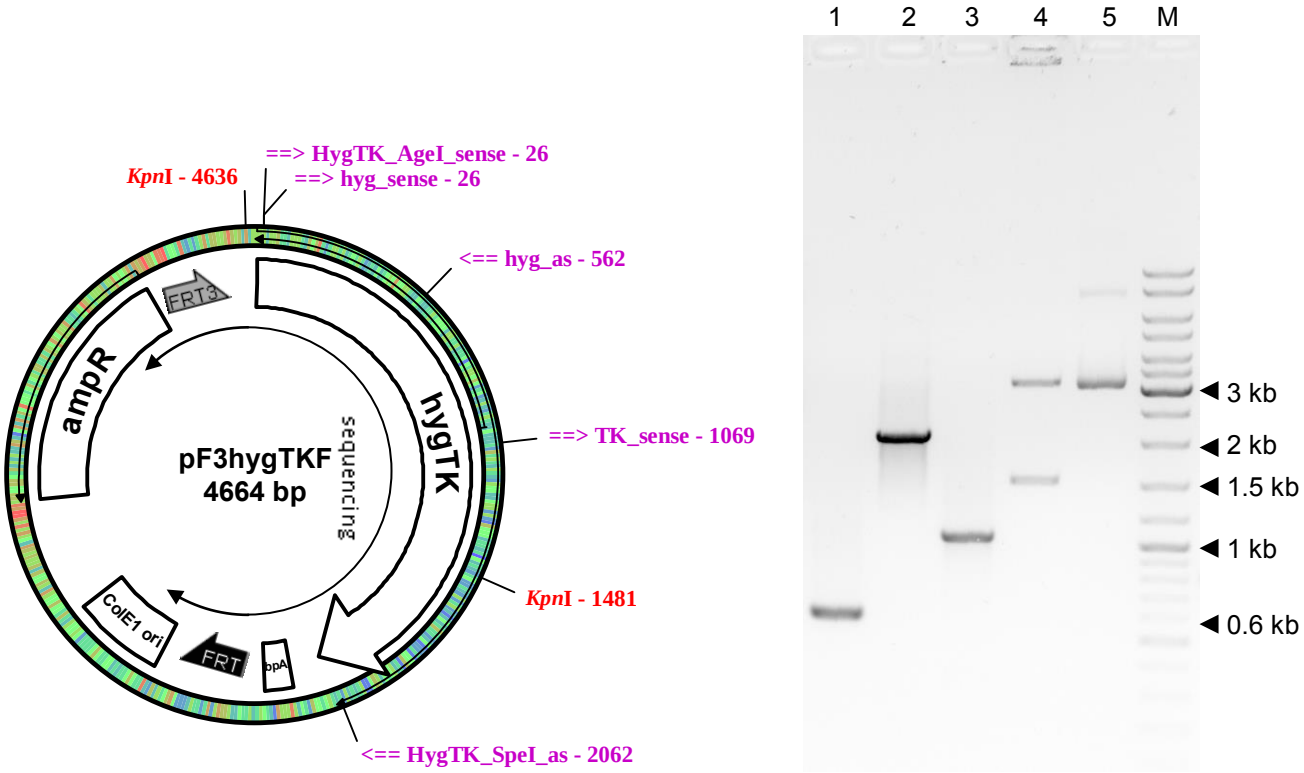

Figure 9.2: pF3hygTKF plasmid.

Left (plasmid map): FRT3: mutant FLP recognition target site, FRT: wild-type FLP recognition target site, ampR: ampicillin resistance gene, ColE1 ori: bacterial origin of replication, hygTK: hygromycin-thymidine kinase fusion protein. Primer for PCR are shown in pink (right arrow: forward primer, left primer: reverse primer). Double cutter restriction enzyme binding sites are indicated in red.

Right (agarose gel): plasmid verification and integrity; lane 1: PCR with primer pair hyg_sense and hyg_as gave the expected 576 bp amplicon. Lane 2: PCR with primer pair HygTK_AgeI_sense and HygTK_SpeI_as shows the expected 2 kb amplicon. Lane 3: PCR with primer pair TK_sense and HygTK_SpeI_as gave the expected 993 bp amplicon. Lane 4: KpnI restriction analysis showed two fragments with an expected length of 1509 bp and 3155 bp. Lane 5: 100 ng of pF3hygTKF plasmid DNA applied on gel. M: 0.5 µg of gene ruler DNA ladder. Sequence analyses were accomplished with primer HygTK_AgeI_sense, HygTK_SpeI_as, HygTK_as or pCIneo_5302sense.

9.1.3 pF3GTNF

The promoterless 5.2 kb plasmid pF3GTNF (Figure 9.3) for RMCE2 constructed by Bode et. al. [10] includes the exchange cassette comprising two heterospecific FLP recognition target sites flanking an enhanced green fluorescent protein –thymidine kinase – neomycin phosphotransferase fusion gene (“GTN”). For amplification in *E. coli* the ampicillin selection marker (ampR) and a bacterial origin of replication (ColE1 ori) is used.

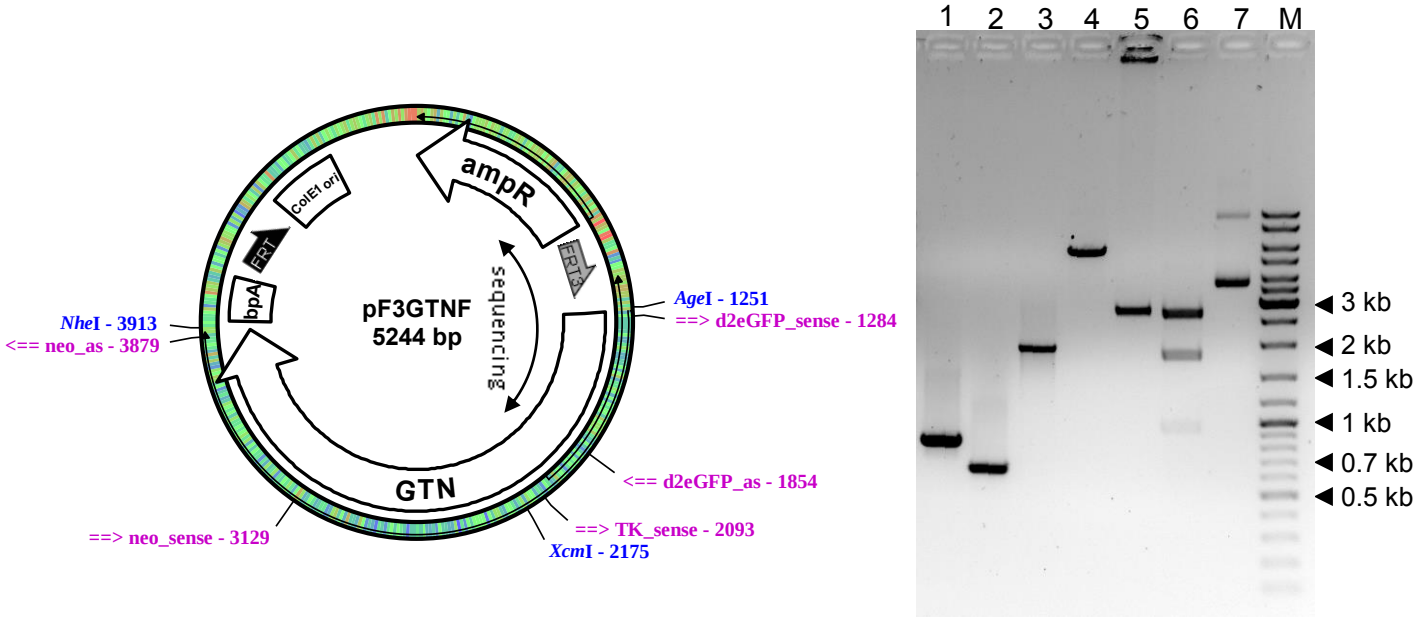

Figure 9.3: pF3GTNF plasmid.

Left (plasmid map): GTN: d2eGFP – thymidine kinase – neomycin phosphotransferase fusion gene, FRT: wild-type FLP recognition target site, FRT3: mutant FLP recognition target site, ampR: ampicillin resistance gene, ColE1 ori: bacterial origin of replication.

Right (agarose gel): lane 1: PCR with primer pair neo_sense and neo_as generated the expected 791 bp amplicon. Lane 2: PCR with primer d2eGFP_sense and d2eGFP_as showed the 607 bp amplicon. Lane 3: PCR with primer TK_sense and neo_as resulted in a single band at 1786 bp. Lane 4: Restriction analysis with AgeI alone gave the linearized vector at 5244 bp. Lane 5: AgeI and NheI double digestion resulted in a single gel band which is constituted by two fragments with approximately the same length of 2662 bp and 2582 bp. Lane 6: Triple enzyme digestion that is also used for the construction of pF3hygTKF with enzymes AgeI, NheI and XcmI devides the plasmid into three distinguishable fragments with a length of 2582 bp, 1738 bp and 924 bp. Lane 7: 100 ng of plasmid DNA. M: 0.5 µg gene ruler DNA ladder. For sequence analysis the primer d2eGFP_as was used.

9.1.4 pPGKFLPobpA

The 5 kb plasmid pPGKFLPobpA, obtained from Addgene (plasmid 13793), used for co-transfection in RMCE experiments contained the codon optimized FLPo construct [13] under control of the phosphoglycerate kinase promoter (PGK). For amplification and selection in *E. coli* an ampicillin resistance gene (ampR) and the bacterial f1 origin of replication (f1 ori) was used (Figure 9.4).

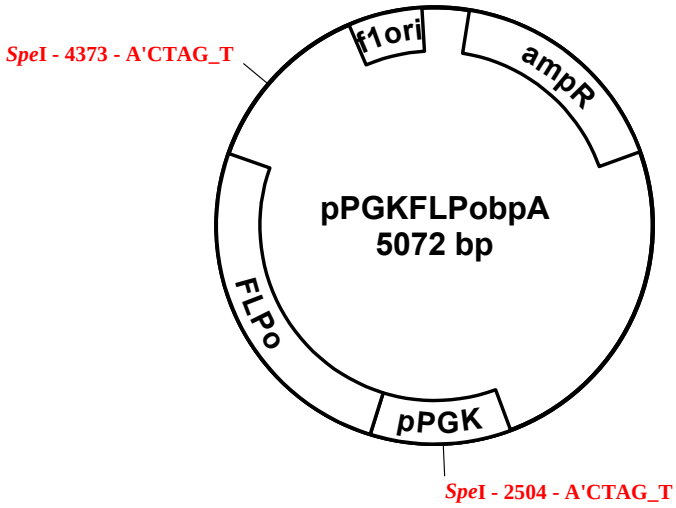

Figure 9.4: pP^{PGK}FLPobpA plasmid.

P^{PGK}: phosphoglycerate kinase promoter, FLPo: codon optimized FLP gene, f1 ori: bacterial origin of replication, ampR: ampicillin resistance gene. Double cutter restriction enzyme sites are indicated in red.

9.2 Construction of parental clones

For the construction of the final RMCE-competent clones CHO DUKX-B11 cells were initially tagged by a parental cassette (plasmid pECMVF3d2eGFPFW, Figure 9.1) to introduce the heterospecific FRT pair (Figure 9.5).

In order to determine optimal conditions for subsequent recombinase mediated cassette exchange, a pre-test with different transfection methods (PEI, electroporation, nucleofection) was done and for this pre-test the course of fluorescence was observed under the UV microscope. Table 9.1 gives an overview of different transfection experiments applied with different amounts of pECMVF3d2eGFPFW plasmid DNA. Respective UV microscopy photos are depicted in Figure 9.6.

Figure 9.5: Introduction of a recombinant RMCE-competent cassette for the construction of parental clones.

S/MAR: scaffold/matrix attachment regions, CMV: cytomegalo virus promoter sequence, FRT3: mutant FLP recognition target site, FRT: wild type FLP recognition target site, d2eGFP= enhanced green fluorescent protein sequence

Method	DNA/cell	viability	fluorescence
Electroporation Lit. [10] (360 V, 800 µF)	3 pg/cell	66% (after 24h)	-
PEI	5 pg/cell	78% (after 48h)	+
Nucleofection	3 pg/cell	67% (after 48h)	+
Nucleofection	5 pg/cell	67% (after 48h)	+
Nucleofection	2 pg/cell	58% (after 48h)	++
Nucleofection	1 pg/cell	76% (after 24h)	+++

Table 9.1: different transfection methods for the construction of fluorescent clones containing the integrated parental ECMVF3d2eGFPFW cassette.

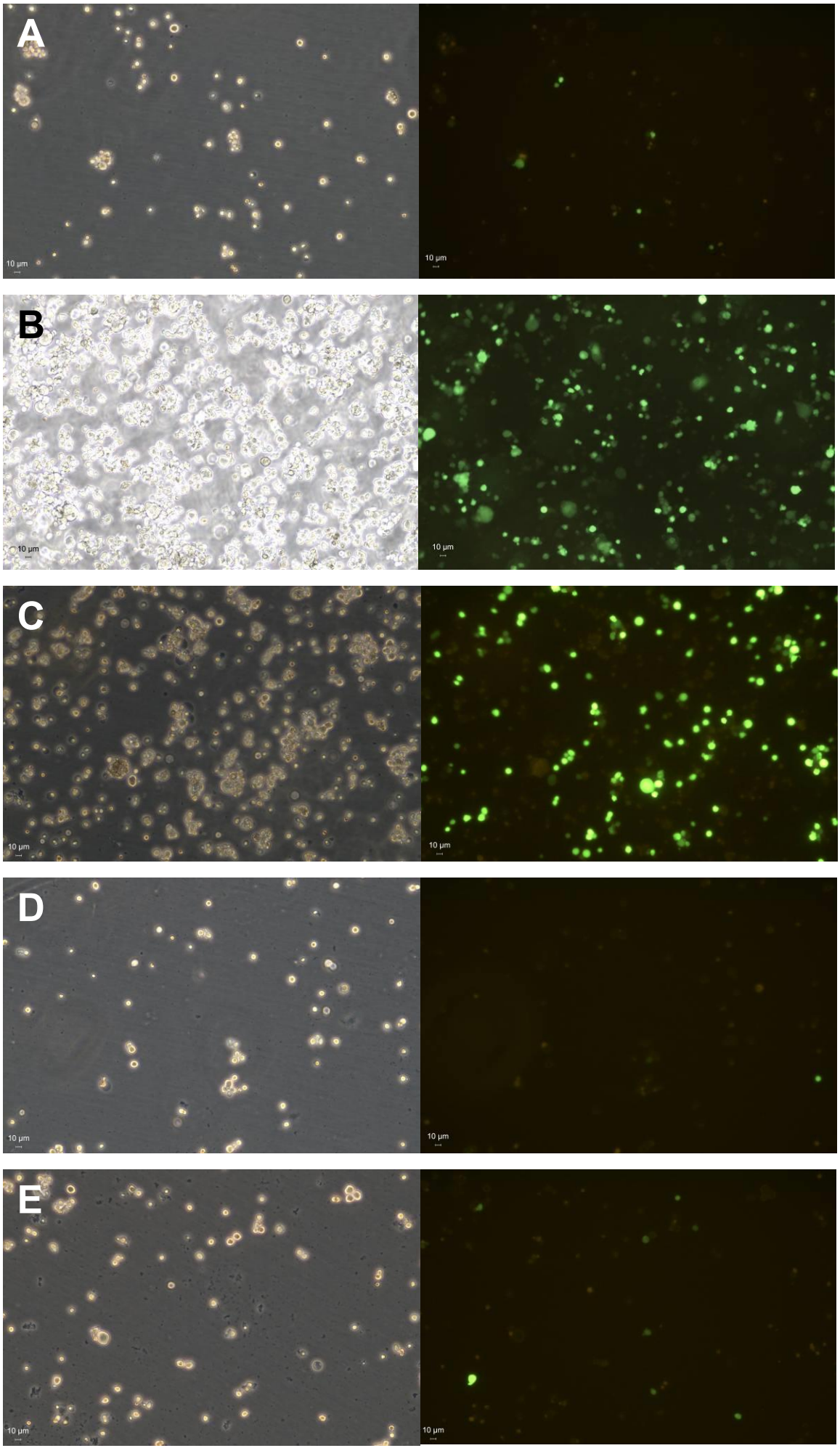
A
10 µm
10 µm
B
10 µm
10 µm
C
10 µm
10 µm
D
10 µm
10 µm
E
10 µm
10 µm

Figure 9.6: fluorescent clones containing the parental ECMVF3d2eGFPFW cassette 24 h after transfection.

A) PEI method **B)** Nucleofection 1 pg/cell **C)** Nucleofection 2 pg/cell **D)** Nucleofection 3 pg/cell **E)** Nucleofection 5 pg/cell

In the pre-tests higher amounts of fluorescent cells were obtained in the nucleofection experiments in comparison with the other transfection methods (Table 9.1). To track the disappearance of the fluorescence after nucleofection with 2 pg plasmid DNA/cell (Figure 9.6 C), transfectants were observed in course of one week. After 1 week a large proportion of cells have lost their fluorescent state (Figure 9.7). The introduction of the ECMVF3d2eGFPFW into host CHO DUKX-B11 cells is based on transient transfection experiments without application of selection pressure to select stably transfected clones. The plasmid enters the nucleus where it can be transcribed and translated into fluorescent GFP proteins after RNA processing and nuclear export no matter whether the plasmid was integrated into the host genome or was transcribed as an episomal transcription unit. For long-term recombinant protein expression, however, the recombinant gene has to be integrated into the host genome. Selection for such clones requires the use of selection pressure. In our experimental design the introduction of the first fluorescent RMCE competent cassette (ECMVF3d2eGFPFW) depends on transient protein expression without application of any selection pressure. This might be the reason why the fraction of fluorescent cells decreases within 1 week due to overgrowth of cells that have stably integrated the cassette into the genomes by cells that have lost the plasmid and lacking metabolic burden thus having a growth advantage over stable transfectants. Since our aim is to target stably integrated RMCE cassettes, further RMCE transfections have to be initiated soon after the introduction of the ECMVF3d2eGFPFW cassette to be able to target the few cells that have stably integrated this RMCE cassette.

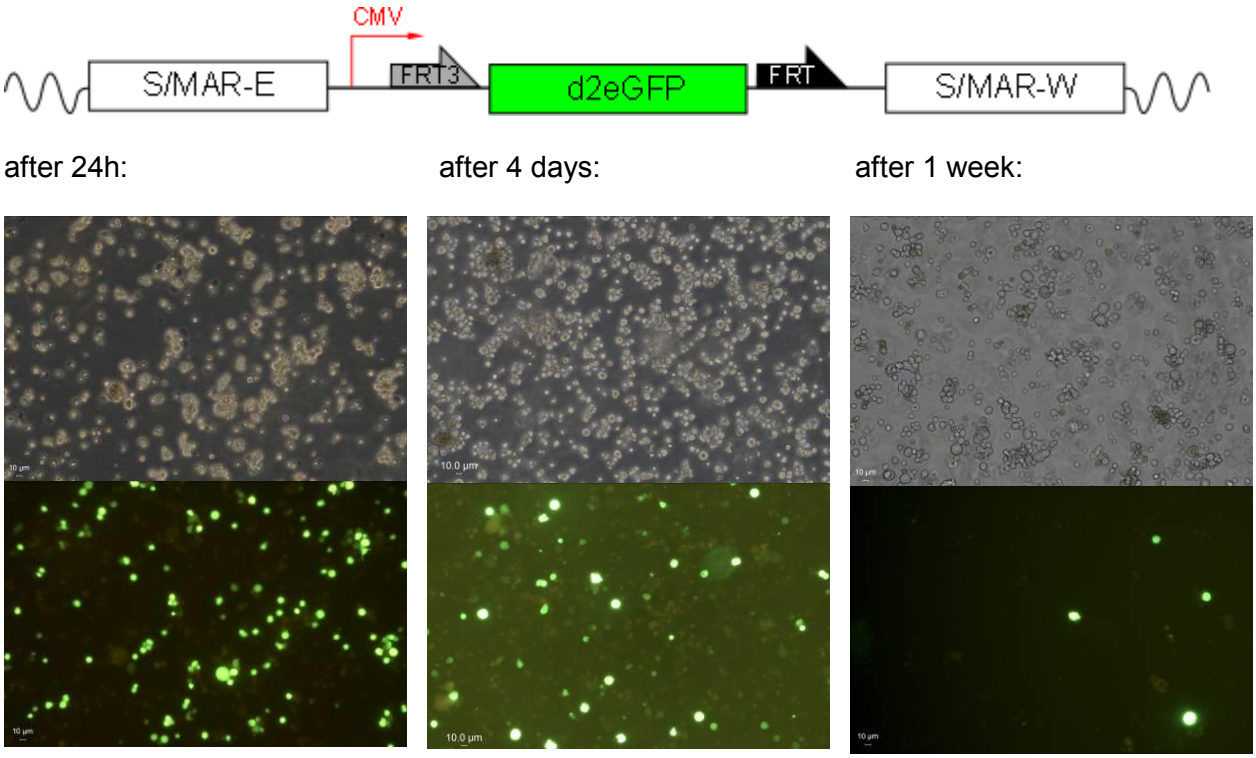

Figure 9.7: 2 pg/cell nucleofection clones observed for fluorescence after 24h, 4 days and 1 week.

The pre-tests provided two important aspects: subsequent RMCE reactions have to be initiated soon after the first transient transfection of the parental cassette and secondly, nucleofection provides large numbers of fluorescent cells. Therefore for the actual establishment of parental clones it was decided to use 2 × 10^7 living cells and transfect them by nucleofection (Amaxa cell line Nucleofector Kit V, Lonza) with 20 µg plasmid DNA (pECMVF3d2eGFPFW) which resulted in a viability of 76% after 24h and high amounts of fluorescent cells.

9.3 First recombinase mediated cassette exchange (RMCE1)

The parental clone pool, containing the d2eGFP construct, was used for the first round of recombinase mediated cassette exchange (RMCE1) in which the d2eGFP construct will be replaced by a hygTK construct (Figure 9.8) under the influence of the FLP recombinase. Co-transfection of the plasmid pF3hygTKF and the FLP expression vector pPGKFLPobpA was done 24h after the first transfection with pECMVF3d2eGFPFW for the establishment of parental clones. Co-transfection for

RMCE1 was accomplished by using the PEI and lipofection method since for efficient recombination with the FLP/FRT-system a circular and supercoiled conformation of the donor plasmid is preferred [16]. Electroporation would at least partly introduce nicks and double-strand breaks (DSB) [17] that would be detrimental.

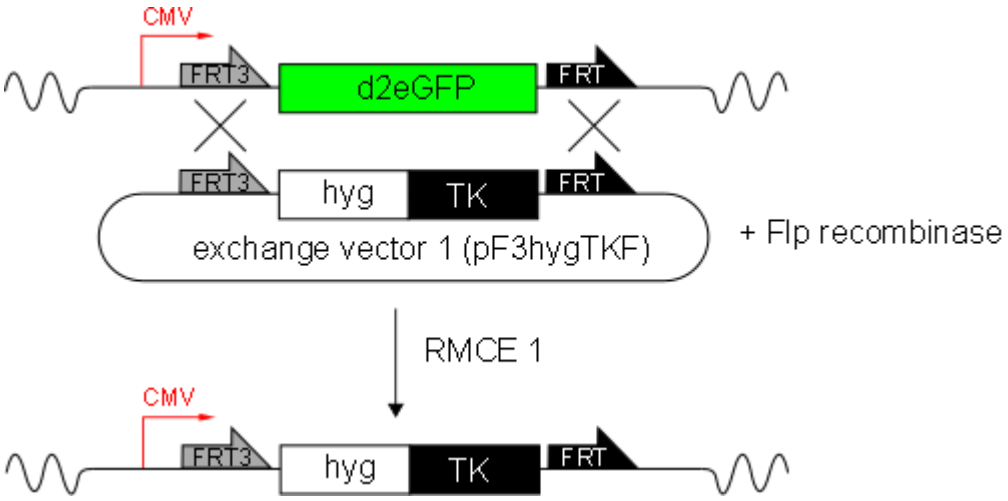

Figure 9.8: first round of recombinase mediated cassette exchange (RMCE1).

For the co-transfection with PEI reagent, 10^6 living cells were mixed with 6 µg pF3hygTKF plasmid DNA, 2 µg pPGKFLPobpA expression vector and 80 µg PEI reagent (linear, 25 kDa). The second co-transfection method was based on lipofection with 2×10^6 living cells, 3.6 µg pF3hygTKF, 1.2 µg pPGKFLPobpA and 25 µL lipofectin reagent. Clones were allowed to finish the recombinase mediated cassette exchange process and express the hygromycin thymidinkinase (hygTK) fusion protein for 24h before selection pressure with 200 µg/mL hygromycin B was applied. Cell clones were seeded with a cell density of 10^4 cells in 100 µL per well of a 96 well plate. After each week 100 µL of fresh ProCHO5 selection medium supplemented with L-glutamine, hypoxanthine, thymidine and 200 µg/mL hygromycin B were added which resulted in a final volume of 300 µL after 3 weeks of selection pressure. During that time the disappearance of fluorescence was observed under the UV-microscope as depicted in Figure 9.9 B.

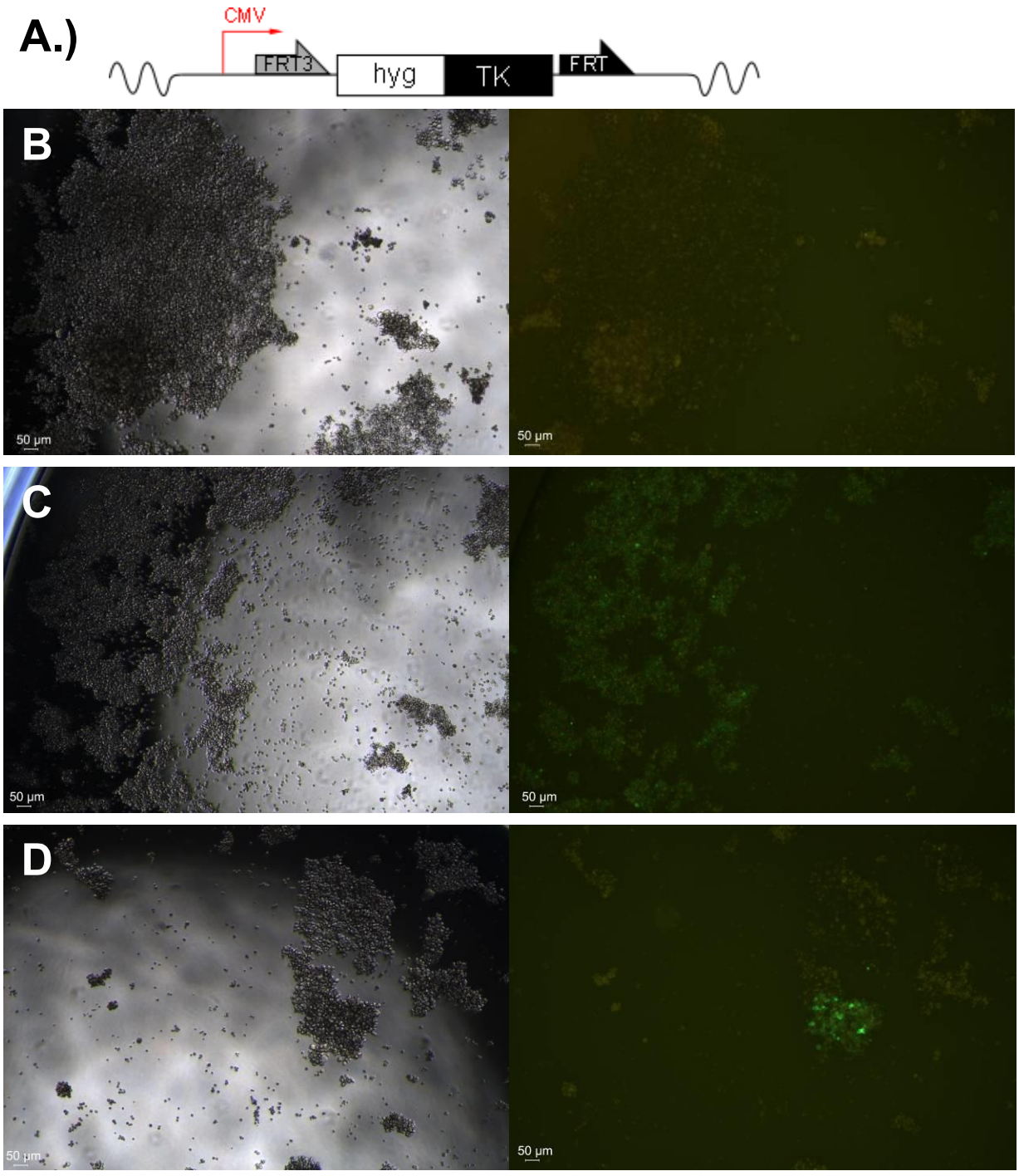

Figure 9.9: some examples of RMCE1 clones 19 days in hygromycin B selection.
A) structure of the integrated RMCE1 cassette **B)** clone PEI2 G4 **C.)** Lipo2A E10 **D.)** PEI1 C1

Colonies depicted in C) and D) of Figure 9.9 showed fluorescent and hygromycin B resistant cells. This phenotype could have been generated by multiple integration of the parental cassette into the genome of the host cell line followed by only partial recombinase mediated cassette exchange so that not all parental GFP cassettes might be exchanged completely by the hygTK cassette.

Therefore these cell clones could contain both the parental GFP cassette and the RMCE1 hygTK cassette.

Twelve clones that showed the characteristic phenotype for the first round of recombinase mediated cassette exchange, namely non-fluorescent status and hygromycin resistance (GFP -, hygR +) were selected and renamed according to Table 9.2. All 12 clones were expanded to T25 culture flasks by 1:2 dilutions every 2-3 days.

Transfection method	name of 96 well plate	well	renamed to
PEI	PEI1	D7	RMCE1-1
PEI	PEI1	F1	RMCE1-2
PEI	PEI1	H8	RMCE1-3
PEI	PEI2	G4	RMCE1-4
PEI	PEI2	G11	RMCE1-5
PEI	PEI2	H6	RMCE1-6
Lipofection	Lipo1B	D1	RMCE1-7
Lipofection	Lipo1B	D4	RMCE1-8
Lipofection	Lipo1B	E10	RMCE1-9
Lipofection	Lipo2A	H3	RMCE1-10
Lipofection	Lipo2B	C3	RMCE1-11
Lipofection	Lipo2B	G10	RMCE1-12

Table 9.2: selected and renamed RMCE1 clones

Transfection efficiency for RMCE1 clones in the 96 well plate was determined after 24 days of hygromycin B selection as the ratio of yellow wells (indicated by phenol red due to pH shift because of metabolic processes) to total well number (96 wells per plate). As indicated in Table 9.3 the PEI transfection method showed the most efficient outcome with an average transfection efficiency of 91%, whereas the lipofection method showed an average efficiency of only 37%. Therefore it was

decided that for the second round of recombinase mediated cassette exchange only the PEI transfection method was used.

Transfection method	plate name	number of grown wells	transfection efficiency
PEI	PEI1	83	86%
	PEI2	87	96%
			Mean= 91%
Lipofection	Lipo1A	39	41%
	Lipo1B	38	40%
	Lipo2A	30	31%
	Lipo2B	28	29%
			Mean= 37%

Table 9.3: Efficiency of transfection for the construction of RMCE1 clones 24 days in hygromycin B in 96 well plates

9.4 second recombinase mediated cassette exchange (RMCE2)

The second round of recombinase mediated cassette exchange should replace the hygTK cassette from the first round with the GTN exchange cassette (Figure 9.10).

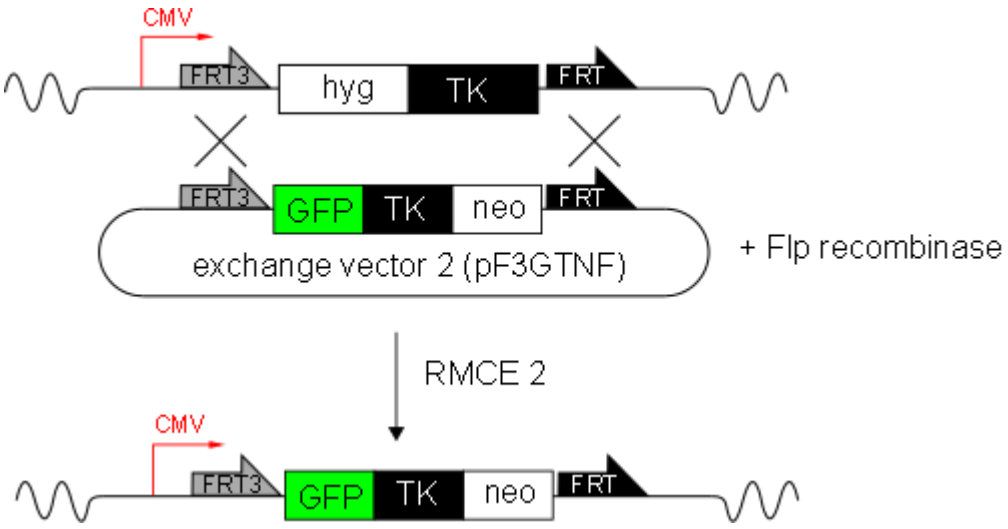

Figure 9.10: second round of recombinase mediated cassette exchange (RMCE2).

Five RMCE1 clones were chosen for RMCE2 experiments by co-transfection with pF3GTNF and the FLPo-expression plasmid, namely RMCE1-1, RMCE1-2, RMCE1-4, RMCE1-5 and RMCE1-6. The selection criterion was based on cell densities in a range of 3.7 to 7.5 × 10^5 c/mL, as determined by particle counting in a coulter counter. Especially clone RMCE1-6 showed the best growth characteristic with a cell density of 7.5 × 10^5 c/mL. All selected cell clones were observed under a UV microscope for residual fluorescence. No fluorescent cells could be observed indicating that all parental d2eGFP cassettes were exchanged by hygTK cassettes during RMCE1 (Figure 9.12 B).

Since the PEI transfection method showed the highest efficiency during RMCE1 this method was chosen as the only transfection method for RMCE2 co-transfection. 10^6 cells of selected RMCE1 clones were co-transfected with 2 µg FLPo expression plasmid and 6 µg pF3GTNF exchange vector using the PEI transfection method. 24h after transfection cells were seeded in a 96 well plate with a density of 10^4 cells/well for G418 selection with ProCHO5 selection medium containing 0.5 mg/mL G418. Two negative controls were established by transfection of RMCE1-6 cells only with the pF3GTNF exchange vector without FLPo-expression vector and vice versa.

After each week 100 µL of selection medium were added. After 20 days in 0.5 mg/mL G418 no growth could be observed for the negative control transfected only with the FLPo expression plasmid indicating that the used G418 concentration is sufficient to kill all cells that do not express the neomycin phosphotransferase enzyme encoded on the GTN fusion gene (Figure 9.11 right). Growing populations could be observed in all RMCE2 plates under the microscope which is also indicated by a metabolic pH shift to yellow depicted in Figure 9.11.

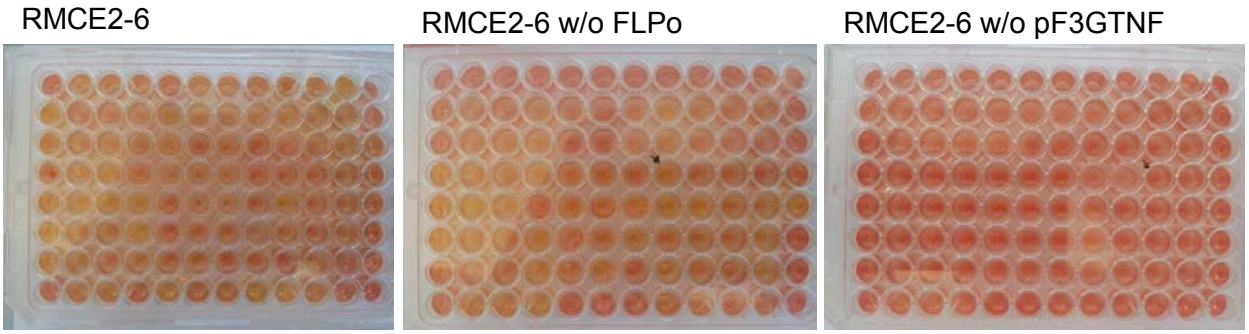

Figure 9.11: selection plates containing medium with 1 mg/mL G418.

Growth could also be observed in the RMCE2-6 negative control that was transfected only with the pF3GTNF exchange vector. Since no FLPo expression plasmid was introduced into these cells it was expected that no growth would occur since pF3GTNF should not be integrated through RMCE mechanisms catalysed by the FLPo enzyme. Also random integration should not lead to any G418 resistance since pF3GTNF lacks a promoter and therefore no transcription should be initiated. Nevertheless cell populations within this negative control were generated exhibiting sufficient resistance to the used G418 concentration for survival. This could be due to random integration downstream of endogenous promoters. Another possibility for successful integration of the F3GTNF plasmid would be initiated by the expression of the FLP enzyme present from the first (RMCE1) co-transfection experiment. If during RMCE1 co-transfection the FLP expression plasmid was introduced into the host genome the FLP enzyme would also be expressed in the RMCE2 negative control where no co-transfection of the RMCE2 cassette with the FLP expression plasmid was done but, nevertheless, leading to enough FLP activity to catalyse the recombination event of the RMCE1 with the RMCE2 cassette. The presence of integrated FLP sequences within the host genome might

be checked by PCR analysis, but this was not done in this study. Since most endogenous promoters should be less active than the used CMV promoter it was decided to increase the G418 concentration to increase the selection pressure. This was done by exchanging the medium with 100 µL of 1 mg/mL G418 selection medium. After two and six days, 100 µL fresh selection medium was added until a final volume of 300 µL per well was reached. All 96 well plates were observed under the UV microscope for fluorescence deriving from the GTN fusion protein. In all RMCE2-4 plates fluorescent cells could be detected. It has to be mentioned that the observed fluorescence intensity was much lower than that from the parental clones (Figure 9.12, A compared to C). After 9 days in 1 mg/mL G418 twelve RMCE2-4 clones were chosen for expansion to T25 culture flasks by 1:2 passages after each week (Table 9.4). Selection was based on their fluorescence properties and cell densities. For the selected clones low fluorescent cells could be observed under the UV microscope and a relatively high cell density.

RMCE1 clone	RMCE1 transfection Method	RMCE2 transfection method	well number	renamed to
RMCE1-4	PEI	PEI	D3	RMCE2-4A
RMCE1-4	PEI	PEI	D4	RMCE2-4B
RMCE1-4	PEI	PEI	D5	RMCE2-4C
RMCE1-4	PEI	PEI	D6	RMCE2-4D
RMCE1-4	PEI	PEI	B5	RMCE2-4E
RMCE1-4	PEI	PEI	F10	RMCE2-4F
RMCE1-4	PEI	PEI	H6	RMCE2-4G
RMCE1-4	PEI	PEI	C5	RMCE2-4H
RMCE1-4	PEI	PEI	C7	RMCE2-4I
RMCE1-4	PEI	PEI	C1	RMCE2-4J
RMCE1-4	PEI	PEI	C10	RMCE2-4K
RMCE1-4	PEI	PEI	F4	RMCE2-4L

Table 9.4: selected and renamed RMCE2 clones

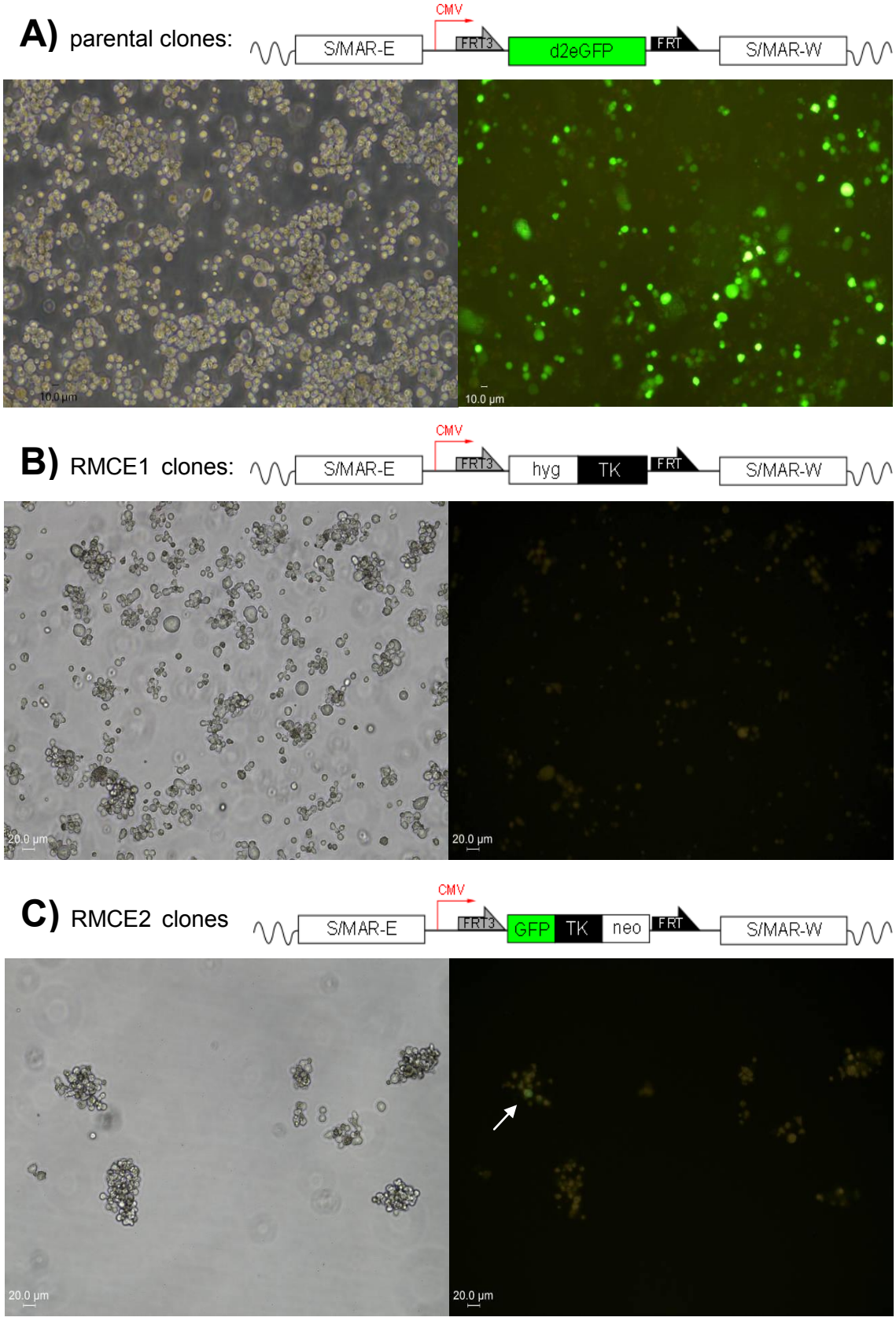

Figure 9.12: Bright -field and UV microscopy photos
A) parental clones, **B)** RMCE1-4 clones and **C)** RMCE2-4E clones.

9.5 Flow cytometric analysis of RMCE clones

Additionally to UV-Microscopy GFP signal of RMCE2-4 clones were analysed by flow cytometry. All samples were stained with DAPI. CHO DUKX-B11 cells were used as negative control. Only viable cells were selected for analysis based on forward and side scatter patterns and absence of the DAPI signal.

Overlay analysis in Figure 9.13 showed a significant difference in the fluorescence intensity of different RMCE clones. The non-fluorescent host cells were excluded by gating. This gate (#1) gave a mean and mode value of 1.34 and 1.04 in fluorescence intensity (FL-1), respectively (Table 9.5). In gate #1 only 1.38% of DUKX-B11 host cells (= negative control) were included. RMCE1 clones that should have lost fluorescence through cassette exchange gave a mean and mode value of 1.51 to 1.59 and less than 1.07, respectively. Only 15-20 % of the RMCE1-4 and RMCE1-6 cells were included in gate #1.

All RMCE2 clones showed a significant increase in the mean fluorescence intensity and mode value (= point of fluorescence intensity with the highest number of cells). Especially clone E gave the highest mean and mode value of 10.77 and 3.21, respectively. This represents a clear difference in fluorescence properties compared to their ancestor RMCE1-4 cell line. Flow cytometry analyses of CHO host and RMCE1 cells both resulted in narrow peak profiles indicating a homogeneous distribution of non-fluorescent cells. In contrast to that, flow cytometry analyses of all RMCE2 populations provided broader peaks indicating a more heterogeneous distribution of fluorescent cells within a population. Especially in the RMCE2-4H profile (Figure 9.13) two peaks could be observed indicating cells with different green fluorescence intensities.

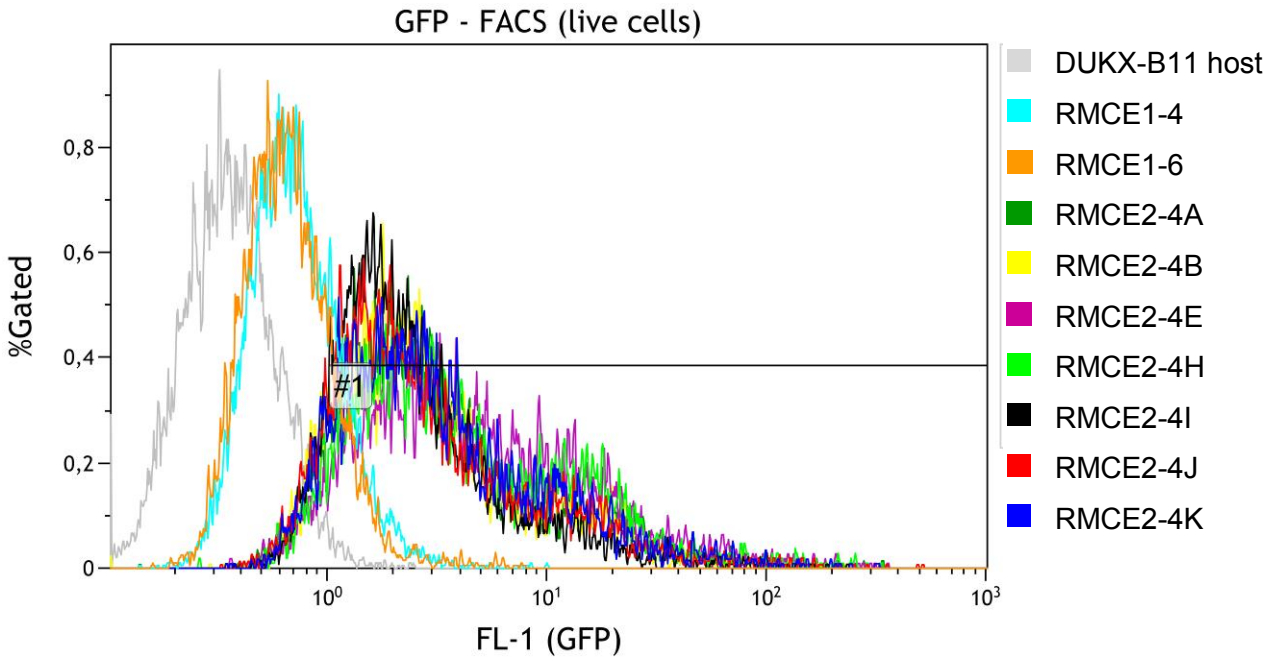

Figure 9.13: Flow cytometric analysis of the GFP fluorescence properties of viable DAPI-negative cells.

clone	gate	% gated	x-mean	x-mode
DUKXB-11 host	none	100	0.40	0.32
RMCE1-4	none	100	0.81	0.69
RMCE1-6	none	100	0.76	0.54
RMCE2-4A	none	100	6.71	1.94
RMCE2-4B	none	100	5.89	1.77
RMCE2-4E	none	100	10.03	3.21
RMCE2-4H	none	100	8.86	1.58
RMCE2-4I	none	100	4.71	1.44
RMCE2-4	none	100	6.52	1.47
RMCE2-4K	none	100	6.81	1.76
DUKXB-11 host	#1	1.38	1.34	1.04
RMCE1-4	#1	20.09	1.51	1.07
RMCE1-6	#1	15.30	1.59	1.04
RMCE2-4A	#1	89.87	7.38	1.94

RMCE2-4B	#1	88.95	6.52	1.77
RMCE2-4E	#1	92.56	10.77	3.21
RMCE2-4H	#1	92.11	9.55	1.58
RMCE2-4I	#1	88.96	5.19	1.44
RMCE2-4J	#1	88.24	7.28	1.47
RMCE2-4K	#1	89.67	7.50	1.76

Table 9.5: Flow cytometric analysis.

9.6 PCR characterization of RMCE clones

For verification of authentic RMCE events or random integration of transferred constructs, PCR methods were developed to track each RMCE step in the clone pool. Primers depicted in Figure 9.14 were designed. The target sequence, primer pair, amplicon size and PCR cycle condition are summarized in Table 9.6. All primer pairs, except for one pair, are specific for one RMCE cassette. Only primer pair pIRESdhfr_CGA394_sense and d2eGFP_as bind on both CMV-d2eGFP and CMV-GTN sequences giving amplicons with approximately the same size.

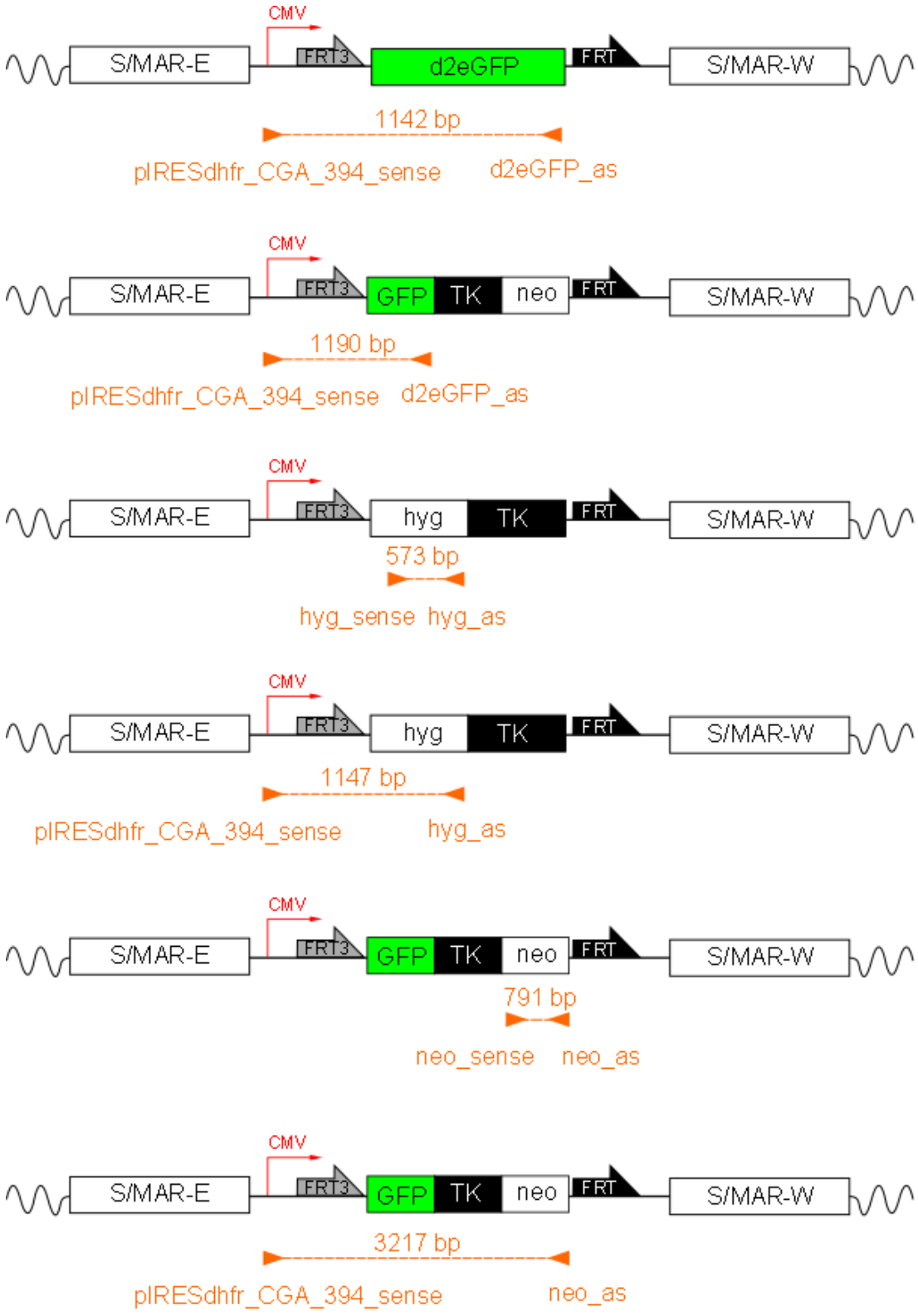

Figure 9.14: overview of primers used for PCR on gDNA of recombinant clones.

Primers are depicted as orange triangles. Right triangles indicate primers binding on the upper sense strand (= forward primer), left triangles indicate primers binding on the lower antisense strand (= reverse primer).

Target sequence	Primer 1	Primer 2	Amplicon size	PCR cycle
CMV-d2eGFP/GTN	pIRESdhfr_CGA_394_sense	d2eGFP_as	1142 bp (CMV-d2eGFP) or 1190 bp (CMV-GTN)	95°C/2', 94°C/30", 58.1°C/30", 72°C/1:30', 45 c, 72°C/5'
hygTK	Hyg_sense	Hyg_as	573 bp	95°C/2', 94°C/30", 57°C/30", 72°C/1', 45 c, 72°C/5'
CMV-hygTK	pIRESdhfr_CGA_394_sense	Hyg_as	1147 bp	95°C/2', 94°C/30", 63°C/30", 72°C/1:30', 45 c, 72°C/5'
GTN	Neo_sense	Neo_as	791 bp	95°C/2', 94°C/30", 56.4°C/30", 72°C/1', 45 c, 72°C/5'
CMV-GTN	pIRESdhfr_CGA_394_sense	Neo_as	3217 bp	95°C/2', 94°C/30", 57.5°C/30", 72°C/3:30', 45 c, 72°C/5'

Table 9.6: PCR primer pairs and conditions for the evaluation of RMCE events in gDNA of recombinant CHO cells.

Genomic DNA was isolated from 2 × 10^6 cells. The DNA concentration was measured photometrically and lay between 31-84 ng/µL using the Quiagen Blood Mini Kit. The integrity of isolated gDNA was checked by agarose gel electrophoresis (Figure 9.15). Since previous qPCR experiments in our lab indicated results with less variance for genomic DNA templates that were initially heated at 99°C for 10 min., two heat-treated gDNA samples were also run on a gel for comparison (Figure 9.15 left, lane 2 and 4). 100 ng of cooked DNA gave no visible bands at the expected length. In addition, 200 ng of cooked gDNA were applied on a 1 % agarose gel (Figure 9.15 right). A great proportion of gDNA is degraded under the used treatment condition (99°C, 10 min.) and gave fragments of with a size smaller than 1.5 kb. For PCR amplicons with sizes larger than 1 kb this heat treatment would be detrimental. Therefore it was decided not to cook the gDNA prior to conventional qualitative PCR.

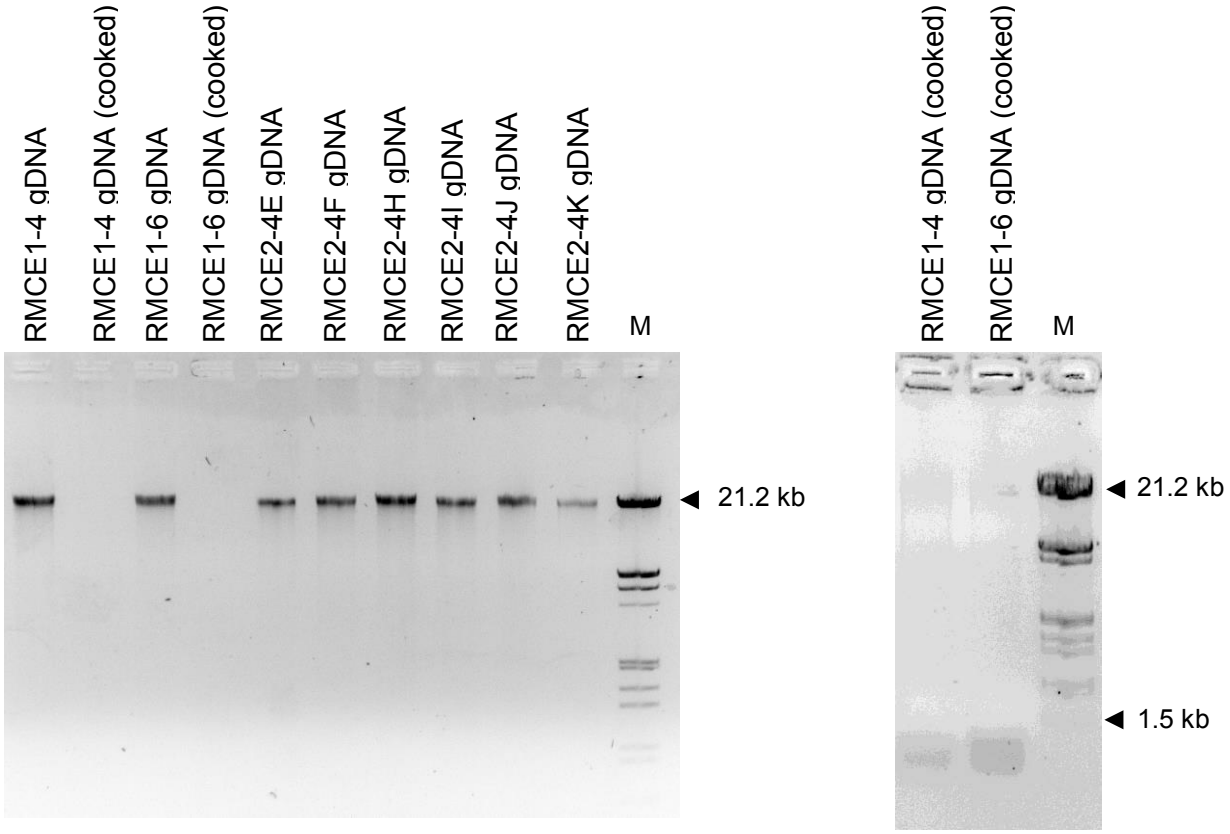

Figure 9.15: Integrity of cooked and uncooked genomic DNA. Left: integrity testing of 100 ng genomic DNA. Right: 200 ng cooked DNA were applied on each lane.

For PCR analyses 200 ng genomic DNA template were used to get a clear signal under the chosen conditions except for PCR with primer pair pIRESdhfr_CGA_394_sense and neo_as where a template amount of 400 ng was chosen to get a clear signal. One reason for the need of higher amount of template DNA for this reaction could be the fragmentation effects induced by high temperature during PCR cycles. In Figure 9.15 (right) a great proportion of template DNA is fragmented to DNA pieces shorter than 1.5 kb. These short fragments cannot serve as template DNA during PCR with the mentioned primer set that would give a 3.2 kb signal.

Screening for the d2eGFP sequence 3' downstream of the CMV promoter (CMV-d2eGFP/GTN) with the primer pair pIRESdhfr_CGA_394_sense and d2eGFP_as gave a signal with all 6 screened RMCE2-4 clones, although in RMCE2-4L only a weak signal is detectable (Figure 9.16). The used primer pair recognizes the d2eGFP sequence which is also present in the GTN fusion gene and creates a 1142 bp or 1190 bp amplicon, respectively. The two RMCE1 clones (RMCE1-4 and

RMCE1-6) did not show any amplicon. This represents a first hint that the two rounds of RMCE were successful since the d2eGFP signal is absent in RMCE1 clones and is reconstituted through the GTN-cassette recombined 3' downstream of the CMV-promoter during RMCE.

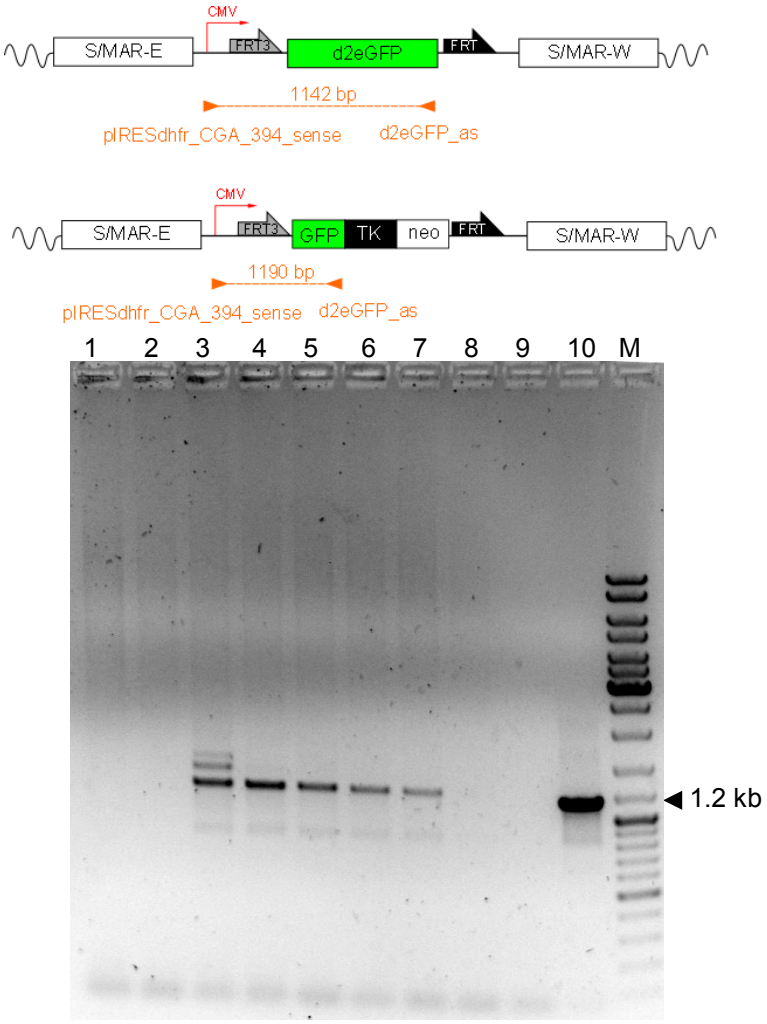

Figure 9.16: PCR screening for CMV-GFP and CMV-GTN in RMCE clones on 200 ng DNA template.

PCR cycle: 95°C/2', 94°C/30", 58.1°C/30", 72°C/1:30', 45 c, 72°C/5'. Lane 1: RMCE1-4 clone, lane 2: RMCE1-6 clone, lane 3: RMCE2-4E clone, lane 4: RMCE2-4F clone, lane 5: RMCE2-4H clone, lane 6: RMCE2-4I clone, lane 7: RMCE2-4J clone, lane 8: RMCE2-4K clone, lane 9: negative control (dH_2O), lane 10: 5 ng pECMVF3d2eGFPFW plasmid template as positive control, M: gene ruler ladder

Primer pair hyg_sense and hyg_as was designed to detect the hygTK cassette, giving a 573 bp long amplicon (Figure 9.17). For RMCE1 clones (lane 1 and 2) the hygTK cassette could be detected. Furthermore a very weak amplicon band could be detected with clone RMCE2-4H (lane 5) indicating an uncompleted RMCE event.

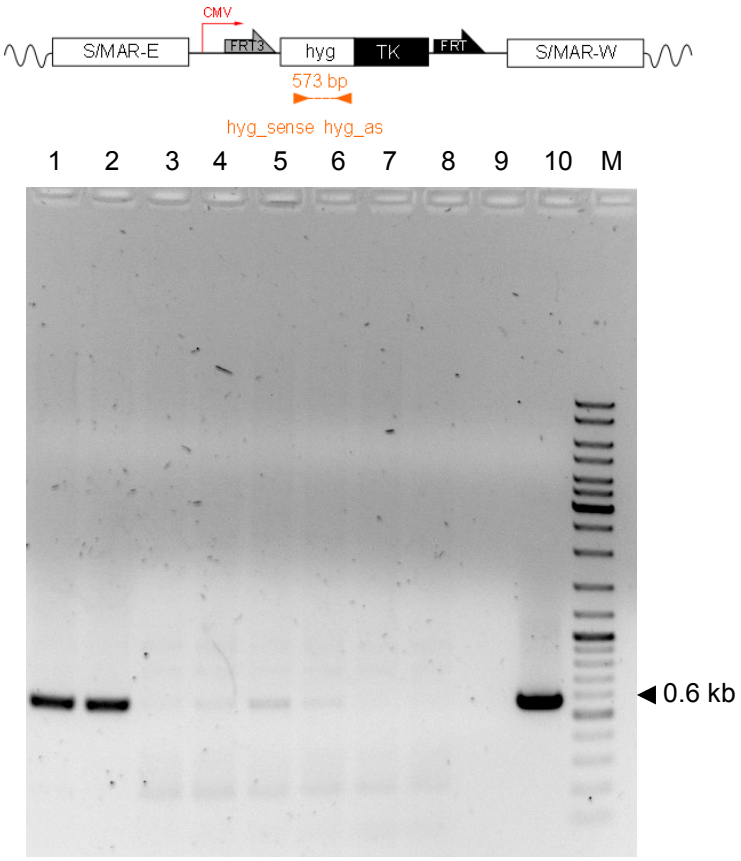

Figure 9.17: PCR screening for hygTK with primer pair hyg_sense and hyg_as on 200 ng gDNA.

Positive clones will show a 573 bp amplicon. PCR cycle: 95°C/2', 94°C/30", 57°C/30", 72°C/1', 45 c, 72°C/5'. Lane 1: RMCE1-4 clone, lane 2: RMCE1-6 clone, lane 3: RMCE2-4E clone, lane 4: RMCE2-4F clone, lane 5: RMCE2-4H clone, lane 6: RMCE2-4I clone, lane 7: RMCE2-4J clone, lane 8: RMCE2-4K clone, lane 9: negative control (dH_2O), lane 10: 5 ng pF3hygTKF plasmid template as positive control, M: gene ruler ladder

For the detection of RMCE events that introduce the hygTK-cassette 3' downstream of the CMV-promoter (CMV-hygTK) primer pair pIRESdhfr_CGA_394 and hyg_as was used that bind specifically on the sense strand of the CMV-promoter sequence and on the antisense strand of the hygTK-cassette, respectively (Figure 9.18). RMCE1-4 clones showed a clear and intense signal in lane 1. In addition a signal band can be observed in lane 5 indicating that clone RMCE2-4H also contains the hygTK-cassette 3' downstream of the CMV-promoter.

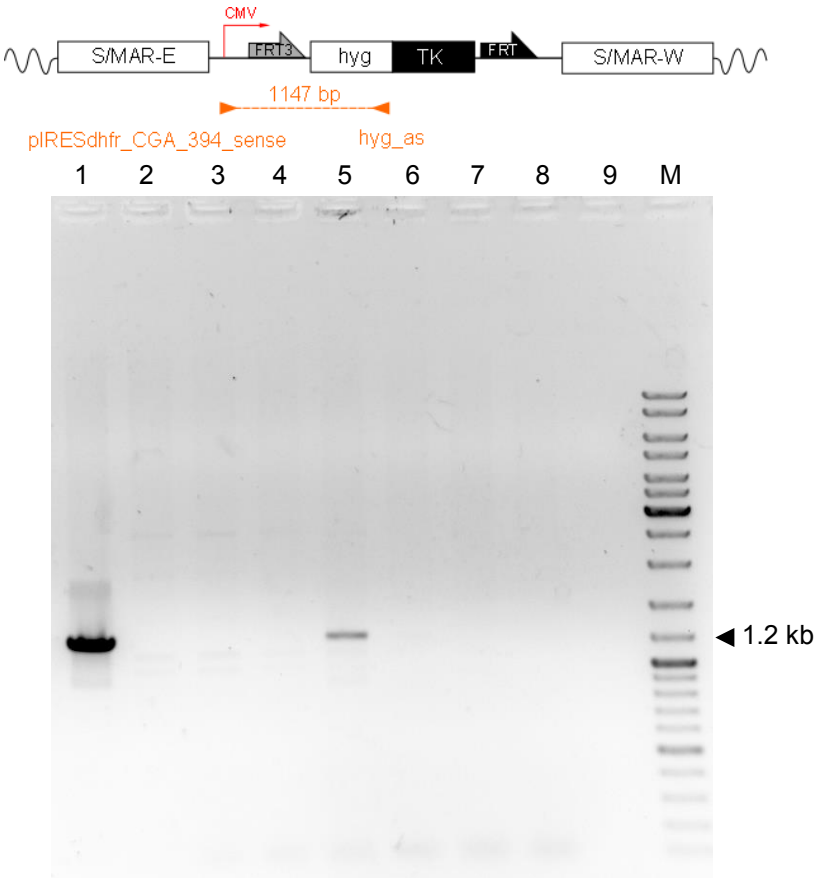

Figure 9.18: PCR screening for CMV-hygTK with primer pair pIRESdhfr_CGA_394_sense and hyg_as on 200 ng gDNA.

Positive clones will show an 1147 bp amplicon. PCR cycle: 95°C/2', 94°C/30", 63°C/30", 72°C/1:30', 45 c, 72°C/5'. Lane 1: RMCE1-4 clone, lane 2: RMCE1-6 clone, lane 3: RMCE2-4E clone, lane 4: RMCE2-4F clone, lane 5: RMCE2-4H clone, lane 6: RMCE2-4I clone, lane 7: RMCE2-4J clone, lane 8: RMCE2-4K clone, lane 9: negative control (dH_2O), M: gene ruler ladder

For detection of the GTN-cassette primer pair neo_sense and neo_as was designed that specifically binds on the neomycin phosphotransferase sequence to give a 791 bp amplicon (Figure 9.19). As expected all tested RMCE1 clones gave no signal, whereas all RMCE2 clones gave a clear band at 800 bp.

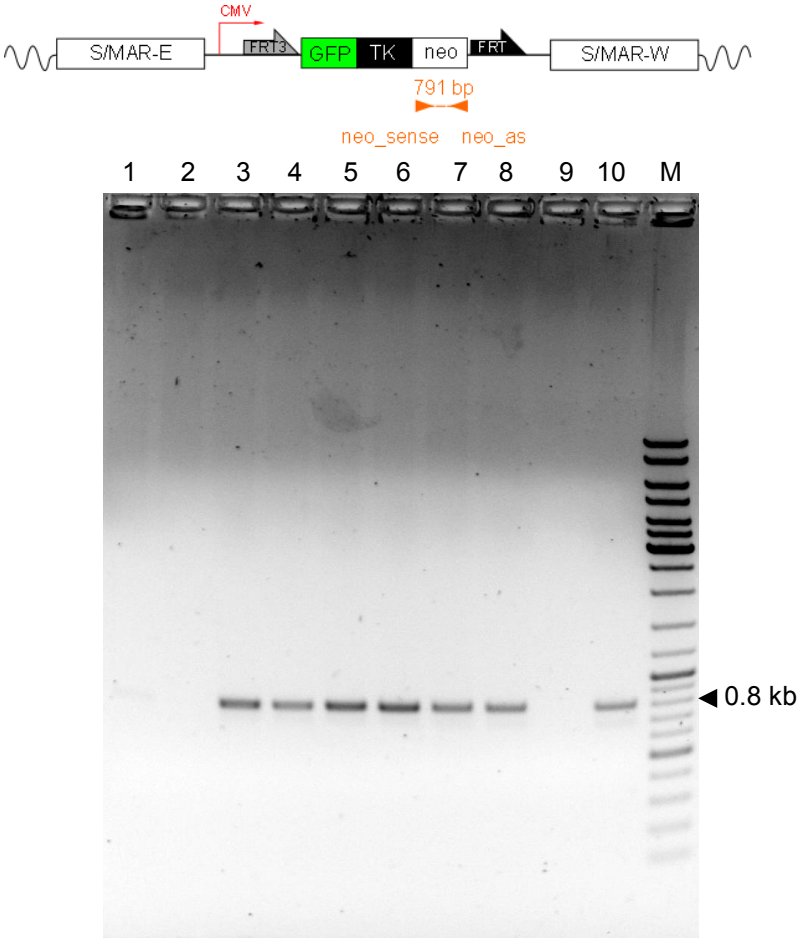

Figure 9.19: PCR screening for GTN with primer pair neo_sense and neo_as on 200 ng gDNA. Positive clones will show a 791 bp amplicon. PCR cycle: 95°C/2', 94°C/30'', 56.4°C/30'', 72°C/1', 45 c, 72°C/5'. Lane 1: RMCE1-4 clone, lane 2: RMCE1-6 clone, lane 3: RMCE2-4E clone, lane 4: RMCE2-4F clone, lane 5: RMCE2-4H clone, lane 6: RMCE2-4I clone, lane 7: RMCE2-4J clone, lane 8: RMCE2-4K clone, lane 9: negative control (dH_2O), lane 10: 5 ng pF3GTNF template for positive control M: gene ruler ladder

For evaluation of authentic RMCE that should introduce the GTN-cassette 3' downstream of the CMV-promoter, primers pIRESdhfr_CGA_394_sense and neo_as were used (Figure 9.20). In order to get a clear signal the gDNA template amount was increased to 400 ng. All RMCE2-4 clones gave a 3.2 kb amplicon indicating that RMCE events were successful in all selected RMCE2 clones.

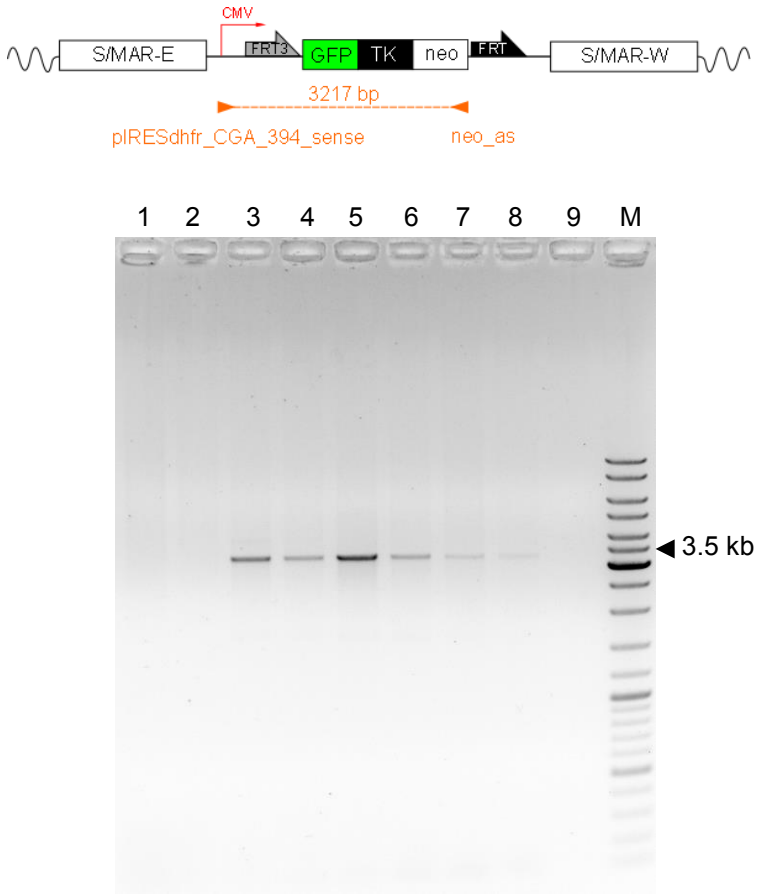

Figure 9.20: Screening for GTN recombined 3' downstream of the CMV-promoter for evaluation of authentic RMCE events.

Primer pair pIRESdhfr_CGA_394_sense and neo_as was used with 400 ng template DNA. PCR cycle: 95°C/2', 94°C/30", 57.5°C/30", 72°C/3:30', 45 c, 72°C/5'. Lane 1: RMCE1-4 clone, lane 2: RMCE1-6 clone, lane 3: RMCE2-4E clone, lane 4: RMCE2-4F clone, lane 5: RMCE2-4H clone, lane 6: RMCE2-4I clone, lane 7: RMCE2-4J clone, lane 8: RMCE2-4K clone, lane 9: negative control (dH_2O), M: gene ruler ladder.

All primer pairs used for the detection of different RMCE cassettes in RMCE clones were used to screen the gDNA isolated from the negative control pool that was transfected only with pF3GTNF without the FLP expression plasmid (Figure 9.21). A clear specific PCR band was only obtained for the primer hyg_sense and hyg_as to detect the 573 bp hygromycin amplicon in lane 2 and primer pair neo_sense and neo_as to detect the 791 bp GTN amplicon in lane 4. No integration of the GTN cassette downstream of the CMV promoter could be observed in lane 5.

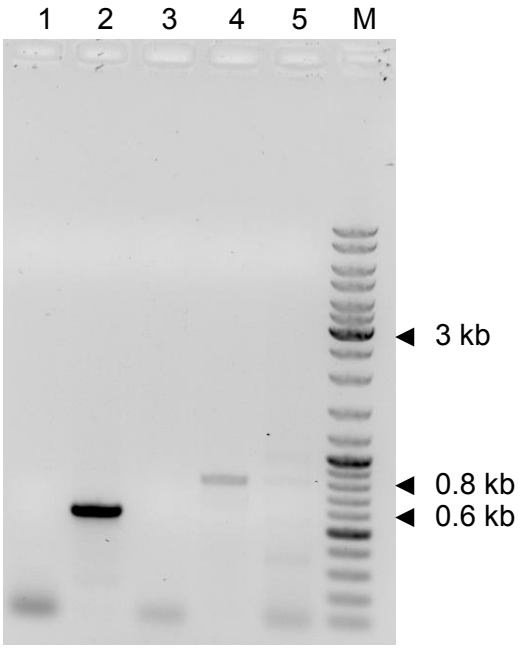

Figure 9.21: PCR results on gDNA of the RMCE2-6 negative control.

RMCE1-6 cells were only transfected with pF3GTNF. No FLPo expression plasmid was introduced and therefore no recombinase enzyme can be expressed. Lane 1: primer pair pIRESdhfr_CGA_394_sense and d2eGFP_as used to detect the CMV-d2eGFP/GTN cassette. Lane 2: primer pair hyg_sense and hyg_as to detect the hygTK cassette. Lane 3: primer p air pIRESdhfr_CGA_394_sense and hyg_as to detect CMV-hygTK sequences. Lane 4: primer pair neo_sense and neo_as to detect the GTN cassette. Lane 5: primer pair pIRESdhfr_CGA_394_sense and neo_as to detect CMV-GTN sequences. M: gene ruler ladder

10 Discussion and Outlook

The aim of this work was to develop a CHO DUKX-B11 cell line that contains the d2eGFP-thymidine kinase – neomycin phosphotransferase fusion gene (GTN) flanked by two heterospecific FRT sites under the influence of a previously introduced CMV promoter. This cell line should have following characteristics: i) the expression of d2eGFP should lead to a fluorescent state of cells (Figure 10.1) providing the possibility for easy evaluation of RMCE events under the UV-microscope or flow cytometer. This cell line can be used for the recombinase mediated cassette exchange (RMCE) of the GTN cassette by any exchange vector containing the gene of interest flanked by the same heterospecific FRT sites (FRT3 and FRT). Successful RMCE events and subsequent loss of fluorescence can be evaluated easily under the UV microscope or flow cytometer.

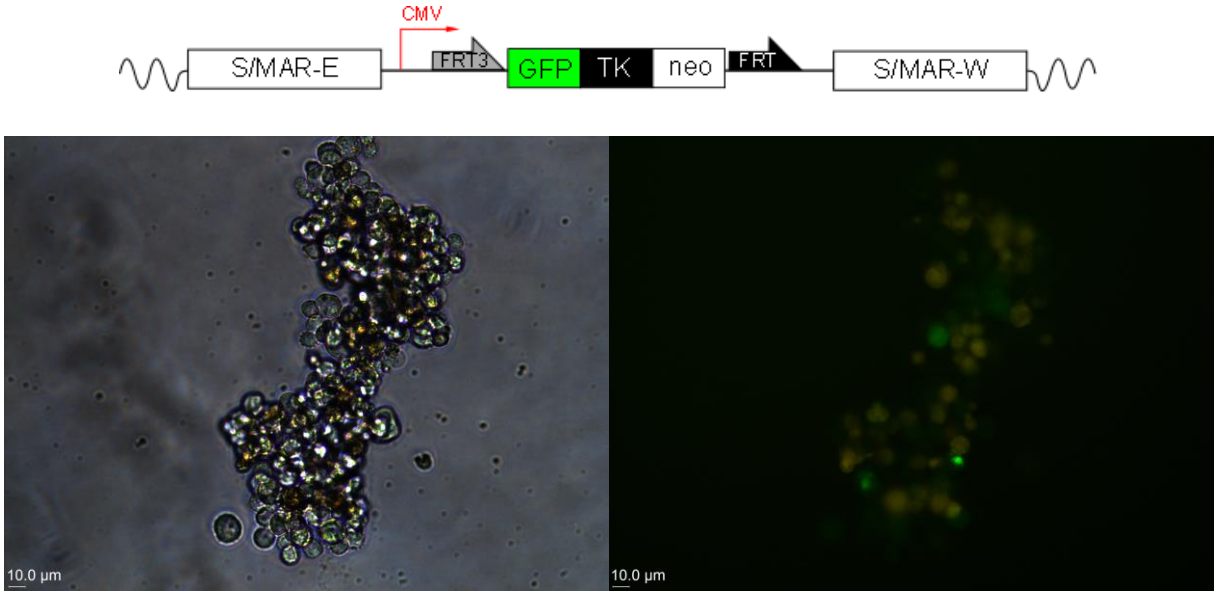

Figure 10.1: Fluorescent state of final RMCE competent DUKX-B11 cells generated by two rounds of RMCE observed under the UV-microscope.

Expression of the green fluorescent GTN fusion protein is driven by the CMV promoter and leads to green fluorescence of recombinant cells.

ii) The expression of thymidine kinase enzyme in recombinant cells provides a mechanism for negative selection with Ganciclovir. All cells expressing the GTN gene would die under Ganciclovir treatment providing another possibility to check for successful GTN integration. Cells that continue to express thymidine kinase encoded on the GTN cassette will be eliminated by Ganciclovir, a nucleoside analog which is enzymatically phosphorylated by thymidine kinase and therefore

converted to a toxic product that eliminates all cells that still contain any GTN cassette and therefore only cells containing the gene of interest at the retargetable genomic locus will be enriched. iii) Expression of neomycin phosphotransferase fusion protein leads to G418 resistance of recombinant cells providing a positive selection mechanism for stable clones. Cells could lose their GTN cassette spontaneously through genomic rearrangements or random recombination events which would lead to the loss of GTN expression. These cells can be eliminated by cultivation in G418 containing selection medium. iv) The integrated GTN cassette flanked by two heterospecific FRT sites can be used for targeted integration of any exchange cassette containing the gene of interest into the same genomic locus by replacing the GTN cassette through RMCE. This represents a method for developing cell lines with comparable and predictable expression levels since the position-effect problem can be neglected. All introduced genes of interest will be introduced at the same genomic locus via RMCE.

The application of two consecutive rounds of RMCE should ensure to generate cell lines with retargetable genomic loci (Figure 7.1). It is known that CHO cells possess a rather unstable karyotype [26]. Random recombination and chromosomal rearrangements could result in silencing of RMCE cassettes. Introduced FRT sequences could spontaneously be converted into heterochromatin and therefore be inaccessible for the recombinase enzyme which would decrease subsequent RMCE efficiencies dramatically. In order to ensure stable and accessible FRT sites, which can be reused with any exchange construct, two rounds of RMCE were chosen.

Parental clones were created by introducing a d2eGFP gene under control of a CMV promoter (Figure 9.5). In the first RMCE round this GFP cassette was exchanged by a hygTK cassette (Figure 9.8).

Flow cytometric overlay analysis of RMCE1-4 and RMCE1-6 showed that most of the analyzed cells are comparable to the DUKX-B11 host negative control in terms of their green fluorescent properties (Figure 9.13). A gate that excludes 98.62% of viable CHO DUKX-B11 cells were set up based on the GFP (FL-1) signal. Only 20% and 15% of RMCE1-4 and RMCE1-6 cells could be found within this gate, respectively, indicating that most RMCE1 cells have lost their fluorescence. Also, within

this gate, the GFP intensity with the highest number of gated cells (x-mode) is comparable to the negative control which lies between 1.04 and 1.07 (Table 9.5). This result gives a first hint that RMCE1 clones have lost their fluorescence by exchanging their d2eGFP cassettes by hygTK during recombinase mediated cassette exchange and no residual fluorescence remained. Nevertheless it was also observed that parental clones lose their fluorescence spontaneously which could be observed under the UV microscope in course of one week (Figure 9.7). The introduction of the parental cassette is based on a transient transfection strategy without application of any selection pressure. As is often the case in transfection experiments, mixed populations are established in which non-transfectants can overgrow recombinant cells since a recombinant gene may constitute a metabolic burden to recombinant cells. After the first RMCE step hygromycin selection pressure was applied. Therefore only cells should survive that have introduced the parental cassette via transient transfection and subsequently exchanged this construct by the RMCE1 cassette.

The method of PCR analysis on genomic DNA was chosen to track RMCE events and to detect specifically exchanged cassettes within the CHO genome (Table 10.1). The primer pair designed to bind specifically within the CMV promoter sequence and the GFP sequence, which is also present in the GTN fusion gene, showed that no residual GFP sequences were present in the two screened RMCE1 clones (Figure 9.16). The finding that no residual GFP cassettes are present in the RMCE1 clones is consistent with the observed GFP pattern in flow cytometry (Figure 9.13). The hygTK sequence, which confers resistance to selection media containing 200 µg/mL hygB, could be found in all tested RMCE1 clones (Figure 9.17). In order to detect authentic RMCE events, primers were used in PCR experiments that bind specifically within the CMV promoter sequence and within the hygTK sequence. Only in RMCE1-4 genomic DNA a specific PCR amplicon was detected, indicating that RMCE1-4 cells have successfully integrated the hygTK cassette via the mechanism of recombinase mediated cassette exchange (Figure 9.18). In contrast to that, RMCE1-6 clones probably have integrated the hygTK cassette at random positions within the genome and have lost their d2eGFP spontaneously. In most cases this random integration of the hygTK fusion gene should not lead to any hygromycin resistance since the used exchange vector did not contain any promoter sequence. Hygromycin phosphotransferase – thymidine kinase fusion protein expression

could be initiated after random integration of the hygTK fusion gene 3' downstream of an endogenous promoter that is sufficiently active to cause hygromycin resistance to 200 µg/mL hygB.

	CMV-GFP/GTN	CMV-hygTK	hygTK	CMV-GTN	GTN
RMCE1-4	No	Yes	Yes	No	No
RMCE1-6	No	No	Yes	No	No
RMCE2-6 w/o FLPo	No	No	Yes	No	Yes
RMCE2-4E	Yes	No	No	Yes	Yes
RMCE2-4F	Yes	No	No	Yes	Yes
RMCE2-4H	Yes	Yes	Yes	Yes	Yes
RMCE2-4I	Yes	No	No	Yes	Yes
RMCE2-4J	Yes	No	No	Yes	Yes
RMCE2-4K	Yes	No	No	Yes	Yes

Table 10.1: Summary of PCR on gDNA to evaluate RMCE events

The second round of RMCE should replace the hygTK cassette by the GTN cassette under the influence of the FLPo recombinase enzyme (Figure 9.10). It was observed that even the RMCE2-6 negative control that was only transfected with pF3GTNF but not with the FLPo expression plasmid showed some G418 resistant clones (Figure 9.11 middle). For this negative control it was expected that no cells will develop any G418 resistance since the GTN cassette cannot be recombined into the tagged locus without FLP recombinase enzyme. In addition the pF3GTNF plasmid (RMCE2-6 w/o FLPo) did not contain any promoter sequence. Therefore it was also expected that any possible random integration into the CHO genome will not lead to any transcription initiation. PCR analysis of this cell pool (RMCE2-6 w/o FLPo) indicated that the GTN cassette was introduced randomly followed by transcription initiation mediated by an endogenous promoter, leading to G418 resistant cell clones (Figure 9.21). Another indication for the presence of an active endogenous promoter that drives GTN expression was that no fluorescence could be observed under the UV microscope (not shown), indicating that a weak promoter drove expression of the GTN fusion protein that was active

enough to confer neomycin resistance but did not lead to visible GFP fluorescence. In contrast to an endogenous promoter the CMV promoter is known to be a very strong and highly active constitutive promoter. Furthermore, this negative control cell pool also contained the hygTK cassette, as detected by PCR analysis (Figure 9.21 lane 2), indicating that GTN did not replace all hygTK cassettes. This detrimental event of random integration behind endogenous promoter lead to cell clones that have not integrated the exchange plasmid through recombinase mediated cassette exchange.

To increase the probability of selecting RMCE-competent cell lines other gene-trapping strategies such as the ATG-trap could be used. The ATG trap strategy uses an ATG signal placed 5' before the first FRT side, similarly to the promoter trap strategy. Only the first parental construct introduces the ATG signal into the CHO genome. Subsequent exchange cassettes lack the ATG signal and therefore translation of randomly integrated exchange cassettes should be prevented. The ATG-trap approach could be demonstrated successfully in baby hamster kidney (BHK) cells [24]. Random integration and subsequent expression without the event of recombination could be prevented by using an exchange cassette containing a selectable marker protein without the ATG start codon. Only with the event of recombinase mediated cassette exchange the marker gene receives the in-frame ATG signal of a previously integrated target cassette leading to the initiation of translation and expression of the selection marker.

In all screened RMCE2 clones, that were co-transfected with pF3GTNF and FLPo expression plasmid, the GTN cassette could be detected by PCR (Figure 9.19). Furthermore in all selected RMCE2 clones GTN cassettes could be detected that were integrated 3' downstream of the CMV promoter indicating that two rounds of consecutive RMCE events were accomplished successfully (Figure 9.20). Only in one RMCE2 clone (RMCE2-4H) a residual hygTK cassette could be observed that was not replaced by GTN (Figure 9.17). RMCE2-4H clones contain both the hygTK (Figure 9.18) and GTN cassettes (Figure 9.20) placed 3' downstream of the CMV-promoter. With conventional qualitative PCR it was not possible to determine the number of integrated parental CMV-d2eGFP cassettes in the host genome. Multiple integration events of these cassettes could lead to multiple RMCE events which would result in genomes containing more than one RMCE-

competent locus. Genomic DNA that contain multiple integrated CMV-hygTK cassettes could be subjected to RMCE2 leading to incomplete exchange of all hygTK cassettes. Exchange of only some RMCE-competent loci would finally lead to genomes containing both hygTK and GTN cassettes (Figure 10.2).

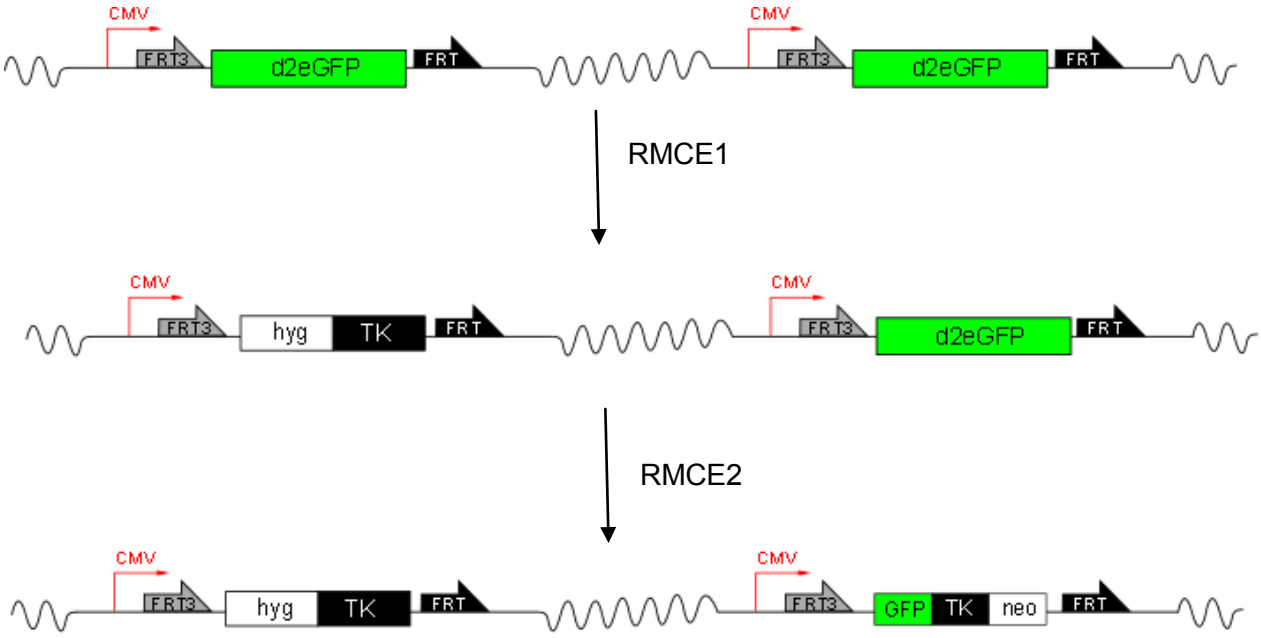

Figure 10.2: Schematic picture of how a cell line containing both RMCE cassettes could be created.

The constructed parental clone contains more than one CMV-GFP cassette which is fully replaced by hygTK cassette during RMCE1. During RMCE2 there only partial exchange leads to a cell line containing both RMCE cassettes. In this situation PCR reaction will detect the CMV-hygTK as well as the CMV-GTN cassette.

Viable RMCE2 clones were analyzed by flow cytometry (Figure 9.13). Gate #1 includes 88.2% to 92.6% of all viable RMCE2 clones with a mean GFP fluorescence intensity of 5.19 to 10.77 (Table 9.5). The point of GFP fluorescence intensity with the highest number of gated cells (x-mode) ranges from 1.44 to 3.21 which is significantly higher than the CHO DUKX-B11 host (x-mode of 1.04) and the RMCE1 clones (1.04 and 1.07). This indicated that all tested RMCE2 clones showed increased fluorescence properties upon RMCE transfection with pF3GTNF and the FLP expression plasmid. Histogram plots of all RMCE2 clones showed a very heterogeneous distribution of their fluorescence intensity (Figure 9.13). Especially for RMCE2-4H two peaks were visible indicating cell populations with lower and higher fluorescence intensities. PCR analysis revealed that in the RMCE2-4H cell pool both RMCE cassettes, namely hygTK and GTN, were present (Figure 9.17 and

Figure 9.19) indicating that not all cells have replaced their hygTK by GTN during RMCE2 as depicted in Figure 10.2. However, it is possible that some cells within this clone pool have replaced all hygTK cassettes and only harbor the GTN cassettes which would lead to higher GFP expression patterns. For further characterization high and low GFP producers should be separated by subcloning with suitable methods such as limited dilution or fluorescence activated cell sorting (FACS) followed by characterization by flow cytometry, qPCR or southern blot analysis to determine the number of integrated GTN or hygTK cassettes. This approach should lead to a more homogenous distribution of fluorescence properties within a cell pool.

The strategy used in this project is based on the selection of clones with high intracellular GFP expression. For industrial protein production a high product titer in the supernatant is required. At this point it has to be mentioned that selected clones with high intracellular reporter protein (GFP) might not necessarily lead to clones with high specific productivities after RMCE exchange with protein product vector. Beside the intracellular transcription and translation rates also other factors like glycosylation and secretion rates play an important role concerning specific productivities. The level of intracellular expression does not always correlate with the level of secretion [23]. Despite this fact the generated cell clones could be used for further evaluation during production of different protein constructs. Several genes of interest could be integrated into the same genomic locus via RMCE mechanism. Comparative studies for the expression of different gene of interests with the use of RMCE-competent cell lines will eliminate the problems arising from the position effect. All genes of interest would be introduced in the same genomic loci and should lead to similar expression levels due to the same genomic environment.

Despite the fact that high intracellular GFP expression does not mean high secretion of antibodies, after RMCE, the green fluorescent protein as a reporter gene provides many advantages. First, it enables tracking the RMCE process quickly under the UV microscope. Furthermore, it can be used for more quantitative evaluation of RMCE processes via flow cytometry. Another possibility is to use the fluorescent signal to exclude or sort for GFP positive or negative cells by fluorescent activated cell sorting (FACS). This approach would enhance the chance to select RMCE-competent cell

clones with high productivities and was demonstrated by Bode et. al [10]. For example, RMCE1 clones could be sorted for their non-fluorescent state by FACS. This would exclude RMCE1 clones that have integrated multiple RMCE competent sequences consisting of both d2eGFP and hygTK cassettes as indicated in Figure 10.2.

The flexibility of this RMCE-competent cell line for different gene of interests is another big advantage. All in all, the establishment of this cell line was accomplished in less than three months. Further evaluation for determination of the number of integrated RMCE-competent cassettes will take another 1-2 months. In total it is estimated that the generation and characterization of a RMCE-competent cell line will take 6 months. This cell line can be used for RMCE with different gene of interest exchange vectors generating clones with similar and predictable expression levels which would facilitate the screening process for high-producers. With traditional methods for developing cell lines expressing different genes of interest, each generated cell line had to be characterized separately and the final expression level could not be estimated since the integration of recombinant genes depended on the random integration that could not be targeted into a pre-determined locus.

11 List of Figures

12 References

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