universität wien

MASTERARBEIT/MASTER'S THESIS

Titel der Masterarbeit / Title of the Master's Thesis

Reporter System for splicing detection of long non-coding telomeric repeat-containing RNA

verfasst von / submitted by

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Angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Master of Science (MSc)

Wien, 2018/Vienna 2018

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	066 834
Studienrichtung lt. Studienblatt / degree programme as it appears on the student record sheet:	Masterstudium Molekulare Biologie UG2002
Betreut von / Supervisor:	Ao.Univ.-Prof. Dipl.-Ing. Dr. Klaus Holzmann

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Acknowledgements

Firstly, I would like to thank Prof. Dr. Klaus Holzmann for giving me the opportunity in his lab to perform my master project. He encouraged and supported me throughout the project and I greatly benefited from his invaluable advices, valuable discussions, and feedbacks on my work and always being available for my questions. Big thank to lab members; Medina Korkut, Theresa Kreilmeier and Elizabeth Naderlinger for their expertise and patience and for their help in numerous ways. In particular, I would like to thank Doris Mejri for her assistance and practical guidance for my project. They all provided a friendly and cooperative atmosphere and also useful comments on my work. Finally, I would like to express my gratitude to my friends for their support and continuous encouragement throughout my study and writing this thesis.

Abstract

Introduction: Telomeres are the end structures of the chromosomes and function as safeguards to protect them from degradation and potential damage. Telomeric heterochromatin contains long non-coding RNAs (lncRNAs) that have roles in telomere maintenance. These lncRNAs are telomeric repeat-containing RNAs (TERRAs) and are transcribed from half of chromosomal ends by RNA pol II with 7% found polyadenylated. Transcripts made by RNA pol II are often pre-mRNAs from coding genes, which are polyadenylated and processed by splicing to remove intronic parts. It is still remained unclear whether TERRA transcripts undergo splicing as post-transcriptional processing that may result in transcripts with new structures and functions. This work will focus on human 2p,10q and Xq/Yq chromosome ends representative for all TERRA loci with emphasis on the endogenous subtelomeric parts of 2p,10q and Xq/Yq-TERRA transcripts. The exogenous telomeric part of TERRAs cannot by analyzed due to repetitive sequences but will be studied by a minigen reporter system for RNA processing of 800 nts long telomeric part of TERRA.

Aim: The aim of this study is to provide tools and work on evidences for processing of TERRA transcripts as performed in previous work. I try to answer to the question whether there is a process described in literature for non-coding RNAs and TERRA, and whether subtelomeric and/or telomeric part of TERRA is processed by splicing. In meantime, we will search specific primers for highly repetitive sequences such as telomeres.

Material & Methods: The TERRA transcripts were analyzed using Plasmid DNA of Adenoviral Vectors (AVs) and Lentiviral vector, HEK293 cells, gel electrophoresis, transformation and transfection and PCR methods.

Results: These results confirmed that the recombinant Adenovirus pAD/CMV5-DEST and pAd/PL/DEST were infected in HEK293 cells and were caharacterized as a correct adenoviral plasmid. pAD/CMV/V5-Terra-sense and pAD/PL-Terra-sense were characterized. The exogenous TERRA (exoTerra1-5 and, exoTerrafwd-2rev, exoTerra 2-3) and total TERRRA (endogenous and exogenous) was detected (totTerra-tel1+2b). And lentiviral vectors by restriction analysis by EcoRI showed that pLenti6/UbC sense and pLenti6/UbC antisense were correctly constructed. Splicing of TERRA transcripts analysis in silico showed that subtelomeric region of Chr 2p, 10q Chr and Xq/Yq multiple potential splicing sites.

Conclusion: These adenoviral vectors and lenti-viral vectors could provide a useful model system for endogenous and exogenous TERRA expression. In silico analysis showed multiple potential splicing sites for TERRA transcripts.

Zusammenfassung

Einleitung: Telomere sind die Endstrukturen der Chromosomen und dienen als Schutzmechanismen, um sie vor Abbau und möglicher Beschädigung zu schützen. Telomeres Heterochromatin enthält lange, nicht kodierende RNAs (lncRNAs), die eine Rolle bei der Erhaltung der Telomere spielen. Diese lncRNAs sind telomerische Repeat-enthaltende RNAs (TERRAs) und werden von der Hälfte der chromosomalen Enden durch RNA pol II transkribiert, wobei 7% polyadenyliert gefunden wurden. Durch RNA pol II erzeugte Transkripte sind oft prä-mRNAs von kodierenden Genen, die polyadenyliert und durch Spleißen verarbeitet werden, um intronische Teile zu entfernen. Es ist immer noch unklar, ob TERRA-Transkripte als posttranskriptionelle Verarbeitung Splicing durchlaufen und was zu Transkripten mit neuen Strukturen und Funktionen führen kann. Diese Arbeit konzentriert sich auf menschliche 2p-, 10q- und Xq/Yq-Chromosomenenden, die für alle TERRA-Loci repräsentativ sind. Dabei liegt der Schwerpunkt auf den endogenen subtelomerischen Teilen der 2p-, 10q- und Xq/Yq-TERRA-Transkripte. Der exogene telomere Teil von TERRAs kann aufgrund von sich wiederholenden Sequenzen nicht analysiert werden, sondern wird von einem Minigen-Reportersystem für die RNA-Verarbeitung eines 800 nts langen telomerischen Teils von TERRA untersucht.

Ziel: Ziel dieser Studie ist es, Werkzeuge und Nachweise für die Verarbeitung von TERRA-Transkripten bereitzustellen, wie sie in früheren Arbeiten durchgeführt wurden. Ich versuche, die Fragen zu beantworten, ob es ein in der Literatur beschriebenes Verfahren für nicht kodierende RNAs und TERRA gibt und ob der subtelomerische und/oder der telomere Teil von TERRA durch Spleißen verarbeitet wird. In der Zwischenzeit werden wir spezifische Primer für stark repetitive Sequenzen wie Telomere suchen.

Material & Methoden: Die TERRA-Transkripte wurden mit Plasmid-DNA von Adenoviral Vectors (AVs) und Lentiviral-Vektor, HEK293-Zellen, Gelelektrophorese, Transformations- und Transfektions- und PCR-Methoden analysiert.

Ergebnisse: Die Ergebnisse bestätigten, dass die rekombinanten Adenovirusp Ad/CMV5-DEST und pAd/PL/DEST positiv in HEK293-Zellen infiziert waren und als korrekte adenovirale Plasmide caharakterisiert wurden. pAD/CMV/V5-Terra-sense und pAD/PL-Terra-sense wurden positiv charakterisiert. Das exogene TERRA (exoTerra1-5 und exoTerrafwd-2rev, exoTerra 2-3) und das gesamte TERRRA (endogen und exogen) wurden (totTerra-tel1 + 2b) fesgestelt. Die lentiviralenVektoren durch Restriktionsanalyse durch EcoRI zeigten, dass pLenti6/UbC sense und pLenti6/UbC antisense korrekt validiert wurden. Das Splicing von TERRA-Transkripten analyse in silico zeigte, dass die subtelomerische Region von Chr 2p, 10q Chr und Xq / Yq mehrere potenzielle Splicing-Stellen aufweist.

Schlussfolgerung: Diese adenoviralen Vektoren und lentiviralen Vektoren könnten ein nützliches Modellsystem für die endogene und exogene TERRA-Expression bereitstellen. In der silico-Analyse wurden mehrere potenzielle Spleißstellen für TERRA bereitgestellt.

Abbreviations

ALT: Alternative lengthening of telomeres

AVs: Adenoviral Vectors

CMV: Cytomegalovirus

DDR: DNA damage response

DNMTs: DNA-methyltransferases

DSB: Double strand breaks

ES: Embryonic stem cells

HP1: Heterochromatin protein 1

HSF: Human Splicing Finder

lncRNAs: long non-coding RNAs

ncRNAs: noncoding RNAs

NGS: Next-generation sequencing

POT1: Protection of telomeres protein 1

RAP1: Ras-related protein 1

RT-PCR: Polymerase Chain Reaction

RT: Reverse transcriptase

TERC: Telomerase RNA component

TERRAs: Telomeric repeat-containing RNAs

TERT: Telomerase reverse transcriptase

TIN2: TERF1 interacting nuclear factor 2

TPP1: Tripeptidyl-peptidase 1

TRF1: Telomeric Repeat Factor 1

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CHAPTER 1. INTRODUCTION

1.1. Telomeres

Early pioneering studies exhibited that functional structures are required to maintain genomic stability in cells and organisms to ensure an accurate separation of genetic material into the daughter cells **(Muller 1938 and McClintock 1941).** Following years, it has been found that Eukaryotic chromosomes harbors distinctive sequences called telomeres, which provides stability and integrity of genetic material **(Blackburn EH 1978).**

Telomeres are fundamental structures of chromosome ends that protect chromosomes from degradation, improper fusion, and recombination through a mechanism in which chromosome ends are prevented being recognized as sites of DNA damage **(Doksani and de Lange, 2014).** Telomeres are dynamic DNA/protein structures and supplemented with complementary proteins and heterochromatins, which provides appropriate organization to maintain genomic stability.

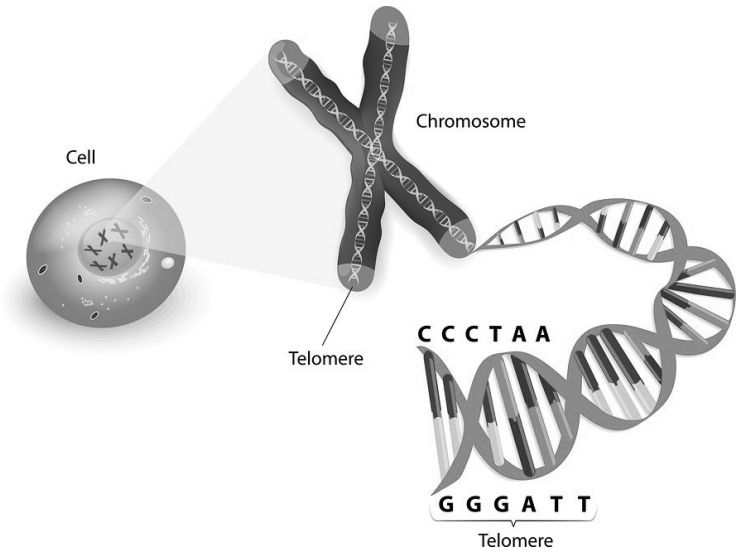


Figure 1.1. The overall structure of Telomere. Telomeres are functional Nucleo-protein structures found in the end of the chromosomes consisting of repetitive sequences to protect chromosomes **(A Bianchi et al., 1997).**

Telomeres are also essential for the replication capacity of DNA. Eukaryotic chromosomes become shortened with each cell divisions over the time because of restrictions arisen from semi-conservative DNA replication machinery that fails to fully replicate the linear DNA, which is a mechanism called "end replication problem" **(Hug and Lingner, 2006).** As the telomere length leads to senescence and genome instability over the time with each cell division and therefore it might be vitally important as a biological clock to estimate the lifetime of a cell and an organism.

1.2. Structure and function of Telomeres

Genomic stability and maintenance of cell viability is ensured by capping chromosomes in eukaryotes by telomeres. Uncapped chromosomes could be recognized as double strand breaks (DSB) which leading to the activation of DNA damage response (DDR), rearrangements of chromosomes and chromosomal end-fusions resulting with the death of cells. **(Cesare AJ et al., 2008).**

Telomeric binding proteins associate with the telomeres and create a protective structure called shelterin complex **(Palm W, 2008).** Sheltering proteins include TRF1, TRF2, TIN2, RAP1, POT1 and TPP1 (PTOP) and play an import role in the protection of telomeres from DNA damage checkpoints and regulate the telomerase activity to ensure a proper length of telomeric DNA **(de Lange T, 2010).**

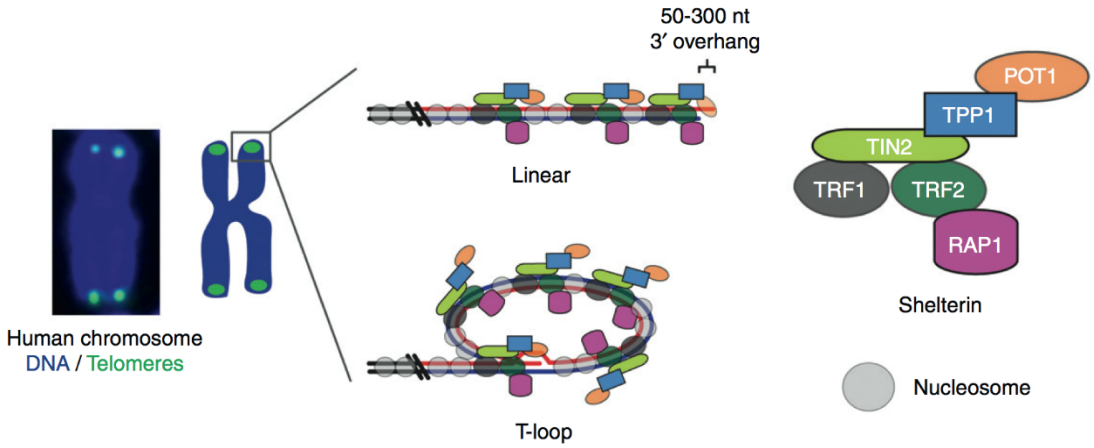


Figure 1.2. Schematic illustration of human telomeres in linear and t-loop conformation. A micrograph of a metaphase chromosome stained with blue and the telomeres stained in green (left) and the shelterin proteins (right). Mammalian telomeres are associated with shelterin proteins which are TRF1, TRF2, POT1, Rap1, TIN2 and TPP1. The binding and interaction of shelterins with each other is shown on the very right side. Telomeres also form loop structures (t-loop and d-loop). T-loop forms when telomere folds back on itself and D-loop forms when The 3' G-strand overhang folds on dsDNA of 5'-end strand tleomeric repeat sequence. The gray circles are nucleosomoes **(Anthony J. Cesare and Jan Karlseder, 2013).**

As telomeres get shortened, the ability to form this protective sheltering decreases and chromosome ends are exposed to DNA damage stimulation. **Figure 1.2** shows the entire shelterin complex and their interactions and nucleosomes. Nearly 80 % of genomic DNA is associated with histone proteins to form nucleosomes, the basic units of chromatin and In eukaryotes, nucleosomes play an important role in promoting DNA by wrapping DNA around the histones **(Pisano S et al., 2008).** TRF1 and TRF2 interact with each other and bind at the double strand DNA telomeric repeats to create bridging along with other shelterin components **(Liu D et al., 2004).** TRF1 has been shown to play an important role in the bending and remodeling of DNA which is important for the appropriate function of telomeres in mammals and TRF1 served as a DNA-binding dimerized protein through its cognate telomeric sequences in the yeast (**A Bianchi et al., 1997).** It has been reported that shelterin component TRF1 provided an efficient replication of telomeric repeats; TTAGGG and solved one of the telomere replication problem; fork stalling **(Sfeir A et al., 2009).** Telomere length is controlled by RAP1 and it binds to the telomeric DNA indirectly by TRF2 and plays an important role in the distribution of telomere length **(Bibo Li and**

Titia de Lange, 2003). TIN has been found to have implications on regulation of telomere length and protection. Researchers identified that TIN2 was associated with TRF2/RAP1 complex and simultaneously bound to TRF1 and TRF2 to create a bridge between these two proteins to stabilize TRF2 on the telomeric regions **(Ye JZ et al.2004).** TPP1 makes a link between TIN2 and POT1. TPP1 and POT1 interact with each other to establish a complex with telomeric DNA and this formation increases activity of human telomerase core enzyme during the telomere extension (**Wang F et al.,2007).**

1.3. Telomere mantainance mechanisms (TMMs) – TA and ALT

Telomerase was characzerized over the two decades by Elizabeth H. Blackburn, Carol W. Greider and Jack W. Szostak (2009 Nobel Prize) and majority of eukaryotic cells, single cell organisms and germ cells use telomerase enzyme to maintain their telomeres **(Greider CW, Blackburn EH (1985).** Telomerase is a ribonucleoprotein contain two components reverse transcriptase TERT protein and lncRNA TERC. The Telomerase reverse transcriptase (TERT) gene provides instructions for making one component of an enzyme called telomerase which maintains structures of telomeres. The telomerase enzyme consists of two major components that work together. The component produced from the TERT is known as hTERT. The other component is Telomerase RNA component (TERC), is a non-coding RNA and this component of telomerase serves as a template for telomere replication. **(https://ghr.nlm.nih.gov/gene/TERC).**

Most cancer cells, Telomerase activity leads to unlimited replicative capacity and cells continue to divide which is required for the growth of human tumors **(Shay JW et al., 1996).** Liu Y et al disrupted the telomerase-specific motif and the three transcriptase motifs of TERT gene by gene targeting in ES cell clones and mice. The mice did not show any detectable mTERT transcript. Loss of TERT caused to the

loss of telomere DNA, aging-related phenotypes, telomeric fusion and in the long term in mice and genomic instability was observed in TERT deficient embryonic stem (ES) cells **(Liu Y et al.,2000).**

Cancer cells have an unlimited proliferation capacity through protection and maintain their telomeres by a telomere maintenance mechanism **(Reddel RR 2014).** Since the majority of human cancer cells express telomerase, telomerase activity might be a novel diagnostic approach for the cancer detection. Hahn WC et al has reported that expression of a mutant human telomerase resulted with overall inhibition of telomerase activity and decreased telomere length (**Hahn WC et al., 1999).** All somatic normal human cells have telomere shortening with the cell divisions over the time. Normal human cells have telomerase activity at chromosome ends which shortening to limit the lifespan but when cells upregulate telomerase together with oncogenic changes, they become fully malignant **(Meyerson M, 2000 and Shay JW, 2016).** Telomerase inhibitors remain valuable approach to kill cancer cells because of the specific activity in tumor. For example, targeting telomere uncapping compounds would be an attractive way to target telomerase-expressing cancer cells **(Mender I et al., 2015).** In this way, telomerase-silent normal cells would not be affected but cancer cells become shrinking. Additionally, blocking of telomerase activity led to the death of tumor cells in vitro and decreased tumor growth in vivo **(Qi Z and Mi R 2016).** These observations exhibited that blocking the telomerase activity might limit the cellular proliferation and growth and further supporting human telomerase might be an important target for the anti-cancer therapy**.**

Majority of cancer cells rely on the activation of telomerase to promote unlimited proliferation and some immortalized human cell lines have no detectable telomerase activity **(Reddel RR et al 1997).** These Telomerase-negative immortalized human cells maintain their telomere length by a mechanism called alternative lengthening of

telomeres (ALT) **(Henson JD et al., 2002).** Although the molecular mechanism of ALT is largely unknown, recent reports have indicated that nearly 10% of cancers use ALT to prevent telomere shortening and recombinational mechanisms may be involved in this process **(Christopher MH et al., 2011).** The specific characteristics of ALT in telomerase negative cancer cells include rapid changes in telomere length, presence of ALT-associated PML bodies containing telomeric DNA, mmmproteins involved in telomere binding and telomere length heterogeneity **(Wei-Qin J et al., 2009). Figure 1.3** shows the model proposed for ALT mechanism which is based on Homologous recombination-dependent DNA replication. Telomeric DNA associates with chromatid recombination-mediated replication to add telomeric DNA at telomeres. To determine how the telomeric DNAs are maintained in ALT cells, the physical structures of Telomeric DNAs in ALT cells were also examined. They found abundant single-stranded regions in both G and C strands of T-DNAs and ALT-specific T-DNAs are produced by telomere metabolism specific to ALT, namely, homologous recombination **(Nabetani A and Ishikawa F, 2009).** It is suggested that ALT depends on the recombination between telomere repetitive DNAs. A recombination-mediated DNA replication process in which telomeres use other telomeric DNA as a copy template. It has been found evidence for a remarkably high level of telomeric exchange in ALT cells that was not seen in cells that do not use the ALT mechanism. **(Londoño-Vallejo JA et al., 2004).** Recently this ALT model was updated in 2016 with publications that demonstrate the induction of ALT in TA cells via inhibition of telomerase, down-regulation of the histone-chaperon ATRX and induction of a DNA damage **(Naderlinger and Holzmann, 2017 et al.)**

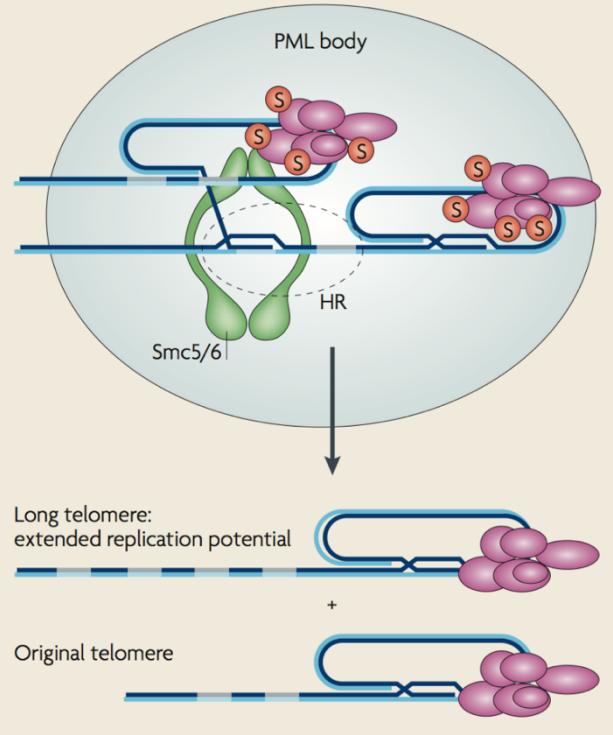


Figure 1.3. Mechanism of alternative lengthening of telomeres (ALT). Homologous recombination maintains the telomere length through the alternative lengthening of telomeres in ALT (-) cells. In these cells, telomeres associate with promyelocytic leukaemia (PML) bodies al known known as ALT-associated PML bodies (APBs). Pairs of structural maintenance of chromosome 5/6 (Smc5/6) is required for chromosomal organization and homologous recombination repair. Smc5/6 complex and Homologous recombination (HR) proteins interact with PML bodies in G2 cell cycle stage because siste chromatids are visible and functional at this phase. And sumoylation (post-translational modification) of shelterin proteins maintains telomeres at PML bodies and promotes telomere **(Murray JM et al., 2008).**

1.4. Coding and non-coding RNAs

The nature of RNA polymerization is similar in all types of organisms but in human and eukaryotes, mRNA are undergo a processing mechanism during transcription **(Figure 1.4).**

A primary transcript is the single-stranded ribonucleic acid (RNA) product synthesized by transcription of DNA and later processed to the other RNA types such

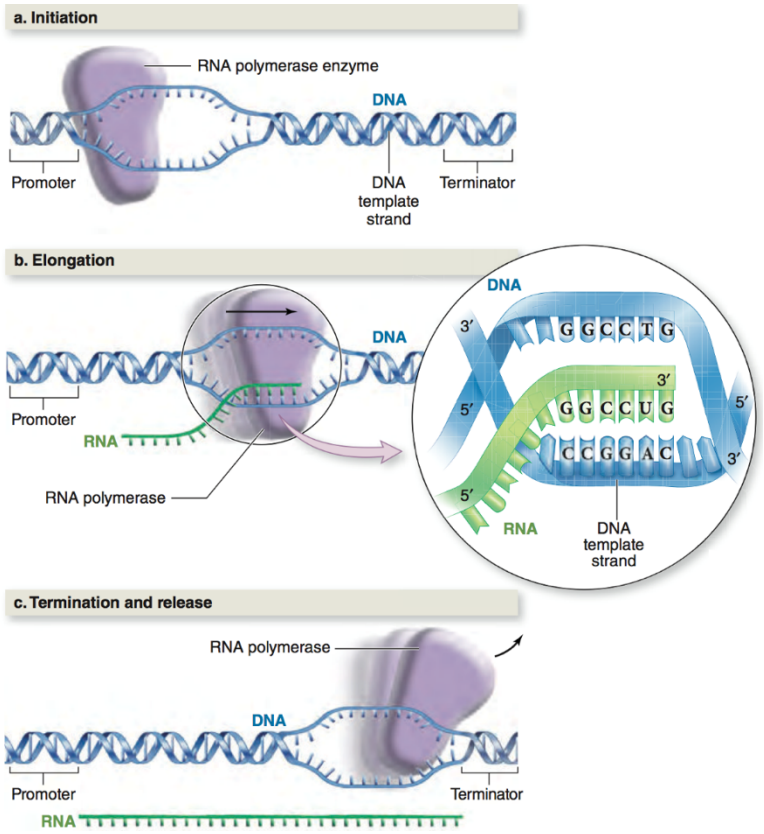


Figure 1.4. General Transcription of all RNA. a) Binding of RNA to the DNA **b)** Elongation **c)**Termination **(Hoefnagels M, 2014).**

as mRNAs, tRNAs, and rRNAs. For example pre-mRNA is a kind of primary transcript that processed into a messenger RNA (mRNA) with the general mechanism for RNA shown in **Figure 1.4**. In contrast to the bacteria, all eukaryotic mRNAs contain a cap at the 5′ end and are polyadenylated at the 3′ end. Eukaryotic mRNAs processing takes place simultaneously during transcription, followed by splicing ´and polyadenylation to terminate the activity of RNA polymerase II **(Lodish H et al., 2000).**

Transcriptional process starts with Capping of RNA polymerase II and followed by elongation process. Elongation refers to length of first transcript to be synthesized. One strand of the DNA is used as a template strand for RNA synthesis. RNA

polymerase uses a DNA template strand to synthesize primary RNA from 3' to 5' the coding end.

After the formation of pre-mRNA, first RNA polymerase is stopped and then 5′UTR starts with 5′ terminal cap structure whereas 3′ UTR ends with polyadenylation poly(A) mRNA which is a procedure of addition adenosines to the 3′ ends by RNA mpolymerase called poly(A) polymerase **(Bard J et al., 2000).** Both cap structure and polyadenylation is coordinated with splicing of introns that discrete the exons. in order to produce translatable mRNA **(Nick JP, 2016).** Nearly all eukaryotic mRNAs are polyadenylated to protect mRNA molecule from degradation in the cytosol and export mRNA from the nucleus during the transcription.

RNAs regulate multiple biological processes as protein-encoding or non-coding RNAs. A non-coding RNA is an RNA molecule which is not translated into a protein and a gene. Main non-coding RNAs that are known to be transfer RNAs (tRNAs) and ribosomal RNAs (rRNAs), microRNAs, siRNAs and are involved in cellular and developmental processes such as catalyzing the translation of nucleotide sequences to protein **(Mattick JS et al., 2006).** Long non-coding RNAs (long ncRNAs, lncRNA) are not translated into protein and they have more than 200 nucleotides which do not code any protein-coding genes **(Lei Nie, 2012).** lncRNAs are 10-fold lower than mRNAs in cells and 78% of lncRNAs are known as tissue specific **(Cabili MN et al., 2011).** Long non-coding RNAs functions in the regulation of transcriptional factors and post-transcriptional regulations in the cells such as mRNA processing and splicing **(Beltran M et al., 2008).** NcRNA are also involved in the translation to act as regulatory pressure in the neurons **(Tiedge H et al., 1993).**

1.5. RNA processing by splicing and back-splicing

RNA splicing is a process to utilize and edit pre-mRNA transcripts into the mRNA. RNA splicing is a post-transcriptional course, which takes place in the nucleus throughout transcription as described in the chapter before. After series of reactions, exons (encodes gene) are cut from pre-mRNAs and exons are joined together to produce a mRNA molecule, which later will be translated into a functional protein. On the other hand, there is produced in most cases no single mRNA product but also diverse mRNA transcripts by a process called alternative splicing, in which exons can bind to introns in a vairety of combinations . The increasing evidences has shown that alternative splicing is mainly observed in multicellular eukaryotes **(Alberto RK et al., 2012).** Alternative splicing is very important to increase different and diverse transcriptomes. With the help of next-generation-sequencing (NGS) methods, overall exon and intron mapping showed that around 92-94% of human genes are result of alternative splicing **(Wang ET et al., 2008).**

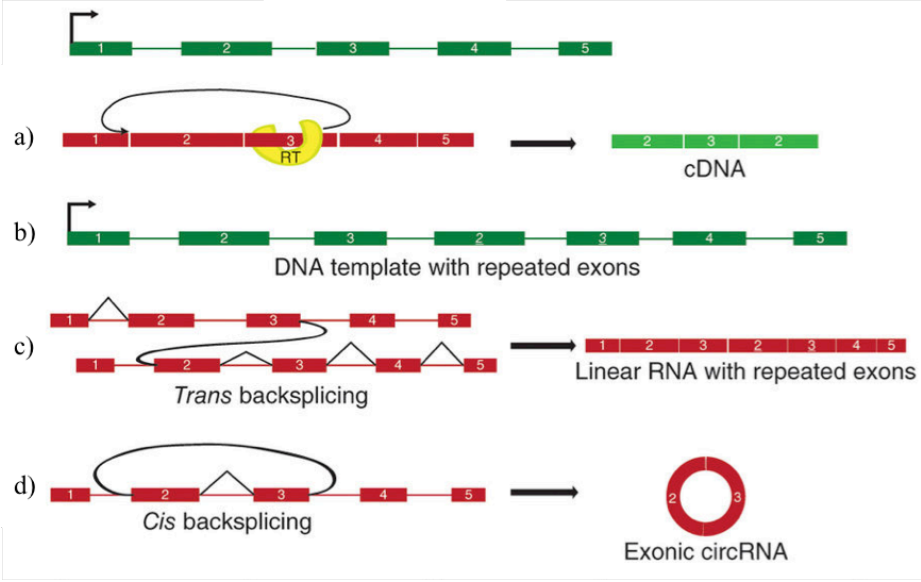


Figure 1.5. Different Types of Alternative Splicing and back-splicing. **a)** Reverse transcriptase (RT) template switching. **b)** Tandem duplications resulting in repeated exons. **c)**Trans-backsplicing. **d)** circRNAs by cis-backsplicing. DNA (green) and RNA (red). **(William R. Jeck et al., 2015).**

Figure 1.5 shows different Types of Alternative Splicing and back-splicing. Reverse transcriptase (RT) template switching produce a back-splice via transcription of a copy of upstream exon (**Figure 1.5a**). DNA region in a genome with repeated exons can produce back-splicing and transcribed mRNA displays an apparent back-splice product (**Figure 1.5b**). Two different molecules can join together to create splicing a process called trans-splicing. This splicing generally arises from two RNA molecules from same gene (**Fig. 1.5c**). Exonic circRNAs are formed by cis-backsplicing a process which exons within the same RNA are spliced together to create circular RNA (**Figure. 1.5d**). **(William R. Jeck et al., 2015).** Circular RNAs are known to be highly regulated product of splicing and have promising functions. Synthesis of Circular RNAs are generated by back-splicing which starts from 5′ exon splice site which is ligated to the 3′ upstream exon splice site.

Apart from splicing products, recently discovered long non-coding RNAs (lncRNAs) are subject of alternatively splicing from microRNAs to small nucleolar RNAs (snoRNAs) **(Yin QF et al., 2012).** Spliced introns can escape from degradation and later are processed to create noncoding RNAs (ncRNAs) suggesting that ncRNAs can be spliced out from mRNA precursors to generate diverse types ncRNAs with different functions **(Yang L 2015).**

1.4. Long non-coding telomeric repeat containing RNAs or TERRAs

Telomeres are transcribed into telomeric repeat containing RNAs (TERRAs), long non-coding RNAs (ncRNAs) and known to have functions in the formation of telomeric heterochromatin **(Montero JJ et al., 2018).** TERRAs contain 5'-UUAGGG-3' repeat sequences complementary to the telomerase RNA sequence and chromosome end specific sequences. **Redon et al.** showed that TERRA is bound to telomerase and TERRA associates with the telomerase reverse transcriptase (TERT)

(Redon, S et al., 2010). This ligand binding acts as an inhibitor of human telomerase activity**.**

TERRA has been reported in many tissues and transcribed from multiple subtelomeric ends **(Azzalin CM et al., 2007).** TERRA expression (transcripts from chromosome 2p and 18p specific for TERRA) has also been associated with good prognosis of astrocytic patients as a therapeutic value **(Sampl S et al., 2012).** Remarkably, TERRA expression reduced the colony growth and telomerase activity in human cell lines **(Kreilmeier T et al., 2016).** TERRAs have been identified in a variety of organisms, including yeast, human and mouse **(Emilio Cusanelli 2015).** It has been evidenced to be an active key player in the mechanism of telomere regulation and homeostasis to maintain integrity **(Luke B et al., 2008; Schoeftner S and Blasco MA, 2008).** TERRA molecule are expressed in most tissues and a have heterogeneous diversity in length and are transcribed from multiple subtelomeric ends **(Azzalin CM et al., 2007).** RNA polymerase II (RNAPII) transcribes TERRA from telomere ends and increasing evidences indicate that a RNAPII has a key function in TERRA transcription in human, mouse and yeast cells **(Luke B et al., 2008). Figure 1.6** illustrates the associations of TERRA with telomeric subunits and RNAP II.

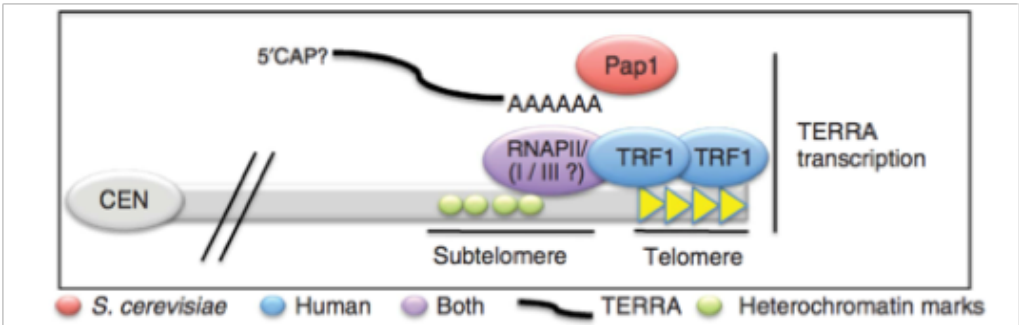


Figure 1.6. The general transcription of TERRA and binding patterns to telomere. RNAPII initiates TERRA transcription from the subtelomeric sequences into the telomeric tract. TRF1 interacts with RNAPII to promote TERRA transcription through the telomere tract. A part of TERRA is polyadenylated by poly(A) polymerase (Pap1) **(Adapted from Brian Luke and Joachim Lingner, 2009).**

TERRA transcripts display heterogeneity in lengths due to the presence of different types of promoters from half of chromosome ends in humans **(Nergadze SG et al., 2009).** Furthermore, the origin of TERRA can also be intra chromosomal due to telomeric sequences found within chromosomes, such as identified for chromosome 2. Several TERRA promoter regions have been identified at CpG islands in a human telomere subset in proximity to their telomeric repeats. In a study, subtelomeric promoters CpG dinucleotides have been shown to be methylated in vivo and CpG methylation negatively regulated TERRA expression **(Nergadze SG et al., 2009).**

TERRA contains UUAGGG in the telomeric DNA and TERRA promoters maintain expression of reporter genes in the subtelomeric region and these promoter regions are located in the upstream of TERRA transcription start sites (less than 1kb telomeric sequence) and bound by RNAPII enzyme **(Nergadze SG et al., 2009).**

Porro A et al conducted a complete analysis of molecular structure of TERRA and they identified that TERRA complexes contain 7-methylguanosine cap structures and poly(A) tail which increase stability of TERRA transcripts **(Porro A et al.,2010).** This Poly (A) is associated with chromatin which confirms its role on telomere ribonucleoprotein complexes. The study has also revealed that length of telomeric repeat of TERRA (UUAGGG) was approximately 200 nucleotides suggesting that most of the TERRA is transcribed from subtelomeric region of individual chromosomes **(Porro A et al.,2010).**

Having known that ncRNAs could modify epigenetic changes by recruiting chromatin-remodelling complexes, TERRA has also been implicated to function as a recruiter for chromatin-modifying at telomere regions to strength and stabilize the heterochromatin state **(Caiqin Wang et al., 2015).** Heterochromatin protein 1 (HP1) and histone H3 trimethyl K9 (H3 K9me3) have been shown to interact with TERRA

and knock-down of TERRA led to the increased in telomere dysfunction and a loss of histone H3 K9me3 **(Zhong D et al., 2009).** Some reports showed that TERRA promoters are heavily methylated by DNA-methyltransferases (DNMTs). Knock down of DNMT1 and DNMT3b in human colorectal cancer cells caused hypomethylation of TERRA promoter and accompanied with upregulation of TERRA levels **(Rhee I et al., 2002).** Mass spectrometry and gel electrophoresis showed that human TERRA could form parallel G-quadruplex structures to protect telomere from enzymatic digestion **(Xu Y et al., 2008).**

Correlations of TERRA transcripts (total and specific levels from chromosome 2p and 18p) and TMM in tumor tissues from 46 astrocytoma patients with grades II to IV showed that total TERRA was correlated with good diagnosis and prognosis and signifying the role of TERRA as a prognostic and therapeutic marker in astrocytic tumors **(Sampl S et al., 2012).** In a further study, the role of TERRA on cell viability and telomere length has been investigated. Human cell lines with telomerase or ALT mechanism were infected with Adeno and lentivirus constructs to express recombinant TERRA transcripts from the telomere but not the subtelomere part. Overall, ALT cells were not affected by TERRA expression but telomerase activity and colony growth of cells with telomerase was significantly decreased **(Kreilmeier T et al., 2016).**

1.7. Detection and identification methods for RNA splice products.

Splicing might lead to the different biological functions and therefore precise quantification of splicing products is fundamental to understand the role of alternative splicing in molecular processes nature of diseases. Reverse Transcription-Polymerase Chain Reaction (RT-PCR) is one of the most common tool for studying RNA transcripts **(Carleton KL, 2011).** This method is based on isolation of RNA

followed by quantitative PCR (qPCR) and combined with electrophoresis to visualize and compare of splice products.

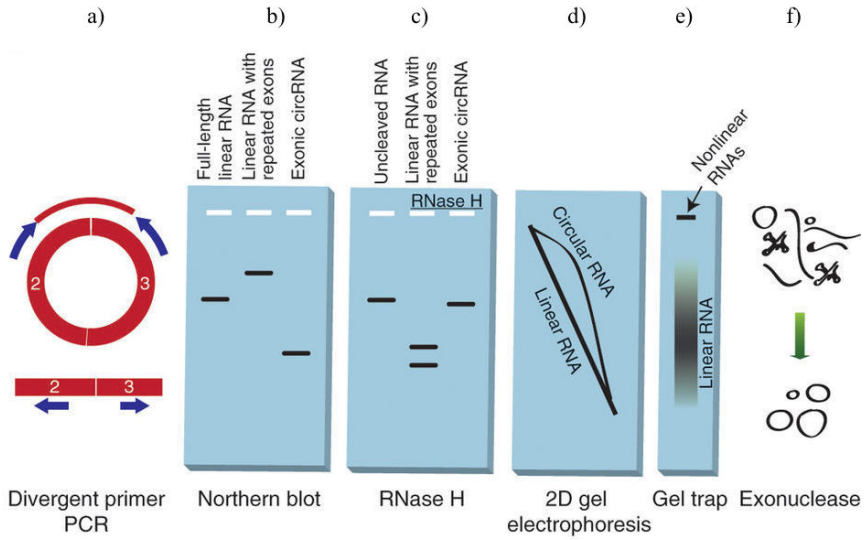


Figure 1.7. Methods to determine splice products, circRNA and illustration of expected results. a) Divergent primers are used to amplify the specific amplicon when a back-splicing occur. **b)** Agarose gel for RNA products, trans-splicing, tandem duplication or circRNA. **c)** Migration of RNA after RNAse H treatment. **d)** 2D gel electrophoresis. **e)** Gel-trapping. **f)** Exonuclease activity for linear RNAs to create a pool of circRNA (**William R. Jeck et al., 2015).**

Figure 1.7 shows some methods for splice products and circRNA with expected results **(William R. Jeck et al., 2015).** When a backsplicing connects outside sequences, divergent primers are used to amplify DNA from the connection side to distant region **(Figure 1.7a).** Denaturated agarose gel can be used to determine whether there is splicing and back-splicing as well as cirRNA. The expected RNA products migrate in a distant fashion which could have resulted from trans-splicing or tandem duplication. Because the length of exonic circRNAs will be less than total RNA and there must be trans-spliced or tandem-duplicated transcripts in the reaction environment. circRNAm will migrate faster in agarose gel. Alternatively, northern blot probing can be employed for the presence of back-splice products in the RNA pool **(Figure 1.7b).** Another approach is RNAse H treatment. RNA products

can be treated with RNAse H treatment and leading to the degradation. circRNA will be in linear form giving a single product and can be visualized on agarose gel **(Figure 1.7c).** Some other methods could provide splicing and back-splicing determination suc as two-dimensional (2D) gel electrophoresis and gel trap electro-phoresis **(Figure 1.7d,e).** In 2D gel electrophoresis, linear RNA migrate thoroughly than circRNAs in highly cross-linked gels and in gel trap electrophoresis, circRNA is combined with agarose and can not migrate through the gel and it becomes trapped.

Alternatively, Semi-quantitative PCR can be used to analyze splicing products based on designed primers for 5' and 3' splice sites. The PCR products can be visualise on an agarose gel. The seperation will be clear in spliced products based on their size making them migrate slower or faster.

Next-generation sequencing allowed supplemented developed computational techniques and bioinformatic tools us to identify and quantify of mRNA splicing (**Bryant DW et al., 2012**). Saccharomyces cerevisiae genes comprising exons and introns were characterize for the alternative splicing using a systematic analysis of mRNA sequencing data. Data revealed alternative splice sites, novel introns boand exon-intron boundaries **(Schreiber K et al., 2015).** The next-generation sequencing (NGS) technology has also provided novel spliced products in cancer. e.g., apoptotic gene BCL2L12 gene has been implicated in many human cancer and computational analysis revealed new alternative splicing patterns. PCR validation identified 50 novel BCL2L12 splicing products which may have clinical applications **(Adamopoulos PG et al., 2016).** The specific determination of splicing signals has great clinical inputs for patients because of the effect of mutations on splicing. *In silico* tools that predict effect of a genetic variant on splicing may be useful for diagnosis. A large number of prediction tools are currently available. One of the common tools is Human Splicing Finder (HSF) method which enables us to either

predict the impact of mutations on 5′ and 3′ splicing regions or define novel splicing motifs of the human genome **(François-OD et al., 2009).**

1.8. Aims of the study

This study aims to continue on the previous work performed by Nejc Kupper ("Splicing and Back-splicing of TERRA", approved 2015 at Medical University of Vienna) with the focus on the interpretation of existing data and the development of tools for expression of recombinant TERRA transcripts.

I. Answer to the question whether there is a process described in literature for non-coding RNAs and TERRA, similar to splicing in coding genes

II. Answer to the question whether subtelomeric and/or telomeric part of TERRA is processed by splicing

III. Search for gene locus specific primers for highly repetitive sequences such as telomeres.

Workflow to reach the aims;

Aim I: Literature search in public database with presentation and discussion of examples (theoretical part)

Aim II: Study of exogenous TERRA expression vector validation

RNA splicing of telomeric parts of TERRA transcripts will be evaluated using Minigene reporter System. Such systems are recombinant plasmid DNA vectors to express telomere sequences that have been already constructed by molecular cloning **(Kreilmeier et al. 2016**) or were constructed by this work. All vector plasmid constructs will be characterized by restriction analyses.

Plasmid DNA of Adenoviral Vectors (AVs) will be transfered to the recipient bacteria cells (E.coli) for large scale plasmid DNA amplification and transfer to HEK 293 cells for virus amplification. Methods that will be applied include:

- transformation of E.coli bacteria with plasmid DNA of recombinant AVs
- selection of plasmids with antibiotics such as Ampicillin
- low and large-scale isolation of plasmid DNA, restriction of DNA with restriction enzymes

- determination of the restriction fragment sizes of plasmids by gel electrophoreses and comparision with their AV plasmid maps
- release of recombinant AV from plasmid DNA via restriction with PacI and transfection of HEK 293 cells to produce AVs

For lentiviral vectors;

- validation whether recombinant TERRA transcripts are expressed in HEK 293 cells and how much compared to a reference gene
- Obtaining the vector DNA with Wizard mini preps from competent E.coli cells.
- Association of the Terra gene fragment with the vector DNA (Transformation with LR-Reactions from pENTR to Lenti vectors).
- Selection (with LB-Medium and Ampicillin)
- Isolation the Recombinant DNA vector with STETL-mini preps
- Restriction digest with EcoRV and SacI enzymes
- Electrophorese Gel Analysis

Aim III: Study of endogenous TERRA expression ;

- To figure out whether there is splicing of non-coding transcripts information.
- Using in silico analysis, to work out on subtelomeric parts of 2p,10q for TERRA

CHAPTER 2

2.Materials and Methods

2.1. Cell Lines

Materials

DMEM
MEM
McCoy's 5A
RPMI-1640
10% FBS
Penicilum/streptomycin mix

Procedure

All cell lines that I used in this study with certain characteristics are summarized in the table 1. Human embryonic kidney 293 (HEK293) cell lines was originally isolated from the embryonic kidney cells and I used this cell line to produce high titer of lentiviral vectors in DMEM medium supplemented with 10% FBS.

Table 2.1. Cell lines with some specific features used in this study.

Cell lines	ATCC no.	Tissue	cell type	disease	TMM
HEK 293	CRL-1573	Kidney	epithelial	H. embryonic kidney	TA
Wi38	CCL-75	Lung	fibroblast	None	None
SW480	CCL-228	colon	epithelial	colorectal Adenocarcinoma	TA
SW480 TL	CCL-228	colon	epithelial	colorectal Adenocarcinoma	TA&ALT
U2OS	HTB-96	bone	epithelial	osteocarsoma	ALT

Human tumor cell line SW480 was originally derived from colorectal adenocarcinoma, the SW480 LT cell line is a sub clone of SW480 posing a new phenotype and was established at the Medical University of Vienna. Both SW480 and SW480 LT cells were grown in RPMI-1640 medium supplemented with 10 % FBS and 1 % penicilum/streptomycin mixture. U2OS is an epithelial cell line derived from bone tissue of an osteosarcoma and U2OS cells were grown in modified McCoy's 5A Medium. Wi38 were originally isolated from lung and they were

sustained in minimal essential medium (MEM) supplemented with 10 % FBS. All cells were maintained in an incubator at 37 °C and 5% CO_2

2.2. Gateway cloning assay

Materials

Gateway LR clonase II enzyme mix (Invitrogen, Life technologies)
(pENTR-delta-hH1-sense, pENTR-delta-hH1-antisense, pENTR-hH1-Tel-sense, pENTR-hH1-Tel-antisense)
(pLenti4/V5/Dest (CMV), pLenti6/Ubc/V5/Dest, pLenti6/BLOCK-iT-DEST)
Gateway™ LR Clonase™ II enzyme
pENTR™-gus
Proteinase K

Procedure

Gateway LR clonase II enzyme mix (Invitrogen, Life technologies) was used to recombine TERRA from entry plasmids (pENTR-delta-hH1-sense, pENTR-delta-hH1-antisense, pENTR-hH1-Tel-sense, pENTR-hH1-Tel-antisense) into the final destination vectors (pLenti4/V5/Dest (CMV), pLenti6/Ubc/V5/Dest, pLenti6/BLOCK-iT-DEST) and plasmid DNA were isolated with STETL and Wizard® Plus SV Mini-Preps. Gateway™ LR Clonase™ II enzyme mix catalyzed in vitro recombination between an entry clone (*att*L-TERRA) and an *att*R-containing destination vector to create an *att*B-TERRA expression vector **(Figure 2.1).** Firstly, LR Clonase™ II enzyme mix was diluted into 1x as a final concentration. 2 µL of pENTR™-gus was used as a positive control. 1-7 µL entry clone was added into 1 µL destination vector up to 8 µL with TE buffer. This reaction was combined with 2 µL of LR Clonase™ II enzyme mix and vortexed and centrifuged briefly. The reactions was incubated at 25°C for 1 hour and terminated by adding 1 µL of the Proteinase K followed with vortexing and incubation at 37°C for 10 minutes.

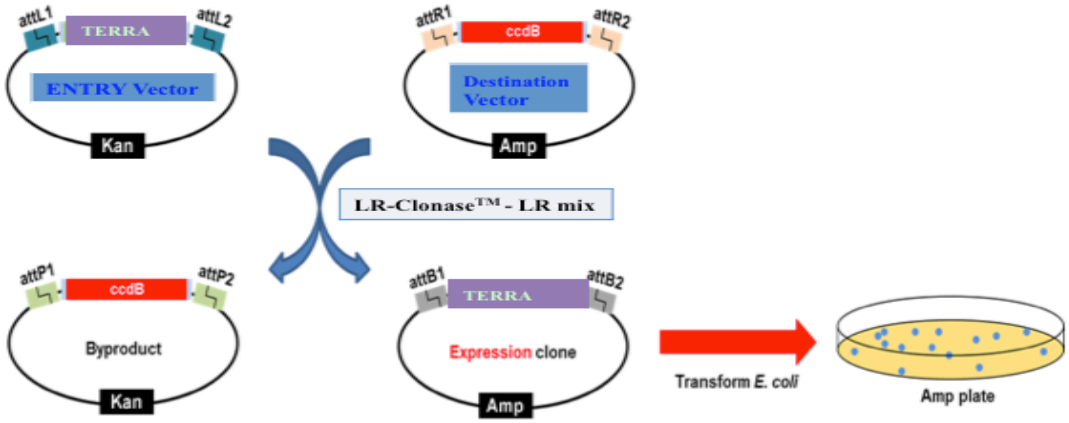


Figure 2.1. Gateway entry cloning. Diagram shows the representative illustration of transferring DNA elements (TERRA gene Fragment) from a Gateway Entry clone to an expression vector. *ccd*B (the encoded protein is toxic for the standard *E. coli* strains). Kan (kanamycin resistance) and Amp (ampicillin resistant).

2.3. Bacterial transformation

Materials

SOB-medium:

- *20g Trypton/Pepton out of casein (Roth, #8952.1)*
- *5g Select yeast extract (Sigma-Aldrich, #Y0500)*
- *0.5g NaCl*
- *10ml 250mM KCl*
- *ddH2O to 1L*
- *with NaOH to pH 7*
- *autoclave*

SOC-medium

- *1/100 volume 1M MgCL2*
- *1/100 volume 2M glucose*

Procedure

Production of competent Stbl3 E.coli bacteria for transformation with plasmid DNA. For the purpose of my project, I transformed One ShotTM Stbl3TM chemically competent E. coli bacteria (purchased from Heidi Huber). The lentvirus plasmids used for transformation were pLenti6-Blockit-dest, pLenti4-CMV-dest, pLenti6-UbC-dest prepared by LR-Reactions - LR Clonase II Mix (#11791020, Fisher Scientific, 20rct (40µl). Transformants were grown on agar plates containing amphicilin (100 µl) and a single colony was picked up to produce in large amount in LB medium.

Adenovirus constructs (AV) were consisted for expression telomere hexanucleotide repeats under control of cytomegalovirus (CMV) and human RNase P RNA H1 (hH1) promoters. Telomere was constructed using recombinant AV. Gateway cloning system (Invitrogen, Lofer, Austria) for the expression of the repertoire, and the polymerase II (CMV) and III (hH1) promoters and the respective termination signals were used. These plasmids were transformed in Stbl3 Chemically Competent Escherichia coli (Invitrogen) and procedure is following.

5ml SOB + 125µl 1M MgCl2 + 500µl 1M MgSO4 + 500µl 2M Glucose were combined to prepare SOC medium. The samples were incubated for 30 minutes. Exposed to Heat-shock in a water bath (Edith) at 42°C /45 sec and Incubated on ice for 2 minutes. 400µl liquid chinese SOC medium was added. Samples were incubated on a heat block at 37 °C for 45 minutes shaking at 180 rpm. At the same time SOC plates containing Antibiotic were preheated at 37°C for 1 hour. puc-Trafo-control also 1/100 (= 5µl= 10pg) was plated out (Amp100R). Samples were centrifuged at 1000g for 3min at RT and supernatant was discarded. Pellet was resuspended in 100µl and plated on LB-Plate (+antibiotics). Plates were incubated at 37°C overnight in a bacterial incubator.

2.4. Plasmid DNA Purification: Maxi-preps

Materials

Competent E. coli bacteria (Stbl3)
Qiagen Plasmid DNA
AdCMV-Terra-sense (35391bps)
AdPL-Terra-sens(34260bps)
70% ethanol

Procedure

The plasmids from competent E. coli bacteria which contains the desired gene fragment was isolated using Qiagen Plasmid DNA Purification Maxi-preps. I have used the competent E.Coli (Stbl3) with the following Adenoviral Plasmid DNA to isolate plasmid DNA. A single colony was picked from the agar plate and inoculated 5 ml LB medium containing amphicilin for overnight at 37°C with shaking. Next day bacterial cells were centrifuged at 6000xg for 15 min at 4°C. The bacterial pellet was re-suspended in 10 ml Buffer P1. 10 ml Buffer P2 was mixed and incubated at room temperature for 5 min. 10 ml Buffer P3 was added to the lysate and mixed. The lysate was placed into the barrel of QIAfilter Cartridge and incubate at room temperature for 10 min. The plunger was inserted into the QIAfilter Maxi Cartridge and the lysate was filtered into a 50 ml tube. 2.5 ml Buffer ER was added to the filtered lysate and mixed followed by incubation on ice for 30 min. QIAGEN-tip 500 was equilibrated in 10 ml Buffer QBT and allowed the column to empty by flowing. The filtered lysate was poured off to the QIAGEN-tip and let to remove by flowing. QIAGEN-tips were washed with 2x30 ml Buffer QC. DNA was eluted with 15 ml Buffer QN. DNA was precipitated by adding 10,5 ml isopropanol and mixed and centrifuged at ≥15,000xg for 30 min at 4°C. Supernatant was aspirated. DNA was washed with 5 ml 70% ethanol and centrifuge at ≥15,000 x g for 10 min. Supernatant was aspirated. The pellet was allowed to dry for 10 minutes at room temperature and the DNA was dissolved in 50 µl Buffer TE.

2.5. STETL miniprep Plasmid Purification Systems

Materials

STET-buffer

- *8% Saccharose (Sigma-Aldrich, #84097)*
- *0.5% Triton X-100 (Sigma-Aldrich, #T8787)*
- *50mM Tris pH8 (Sigma-Aldrich, #T87602)*
- *50mM EDTA (Sigma-Aldrich, #431788)*
- *ddH2O to 1l and autoclaved*
- *stored at +4°C*

Lysozyme: 50mg/ml stock (stored at -20°C, Invitrogen, #GIC207)
RNase A: 100mg/ml stock (in 10mM Tris-HCL pH 7.5 and 15mm NaCl heat to 100°C and cool it to room temperature, stored -20°C)
Isopropanol
EtOH 75%

Procedure

I isolated Plasmid DNA from E.Coli bacteria's, which were grown overnight with respective antibiotics. The STETL method applied is described under standard molecular biology protocols in the group of principle investigator (Reference: A rapid boiling method for the preparation of bacterial plasmids

David S. Holmes. Quigley.1981). Heat block thermocycler was adjusted to 95 °C and centrifuge cooled down to 4 °C prior to procedure. 1.5 ml overnight cultures of bacteria was centrifuged at 10,000g and supernatant was discarded. This step was repeated several times and bactarial pellet was resuspended in 100 µL lysozyme and 1 ml STETL buffer. The mixture was incubated at 95 °C on thermocycler until DANN dissolved. The samples were centrifuged at 10,000xg for 10 minutes at 4 °C. The debris was removed with a sterile toothpick dipped in RNAse A and discarded. The solution was mixed with 110 µL of isopropanol to precipitate DNA. The samples were centrifuged at 10,000xg for 10 minutes at 4 °C. Supernatant was discarded and pellet was rinsed in cold 70% ETOH 2-3 times followed by 10 minutes centrifugation. The supernatants were aspirated and pellet was dried for 10 minutes. The pellets were resuspended in 30 µl 1xTE buffer and samples were stored at -20 ° C.

2.6. Wizard® Plus-Minipreps DNA Purification Systems

Materials

Wizard® Plus kit
Nuclease-Free water
Vacuum Adapter
Ethanol

Procedure

I additionally isolated Plasmid DNA from bacteria, which were grown overnight using a kit (Promega, #L1460) from Promega. In brief, 1,5 ml bacteria culture were used

per isolation. After production of lysate, binding of plasmid DNA to column and elution with 100µl from column was performed to obtain around 10 pg to 100 ng plasmid DNA. 1–10ml of overnight culture was pelleted for 5 minutes. The pellet was re-suspended in 250µl of Cell Re-suspension Solution. 250µl of Cell Lysis Solution was added to each sample and inverted 4 times to mix. 10µl of Alkaline Protease Solution was added and inverted 4 times to mix followed by incubation for 5 minutes at room temperature. 350µl of Neutralization Solution was added, mixed and centrifuged at top speed for 10 minutes at room temperature. The vacuum Adapter was attached to manifold port and Spin Column was inserted into Adapter. The lysate was decanted into column and vacuum was applied to pull liquid through column until all liquid has passed through column. 750µl of wash solution containing ethanol was applied to pull solution through column. The vacuum was turned off and repeated with 250µl of wash Solution. The pellet was dried by applying a vacuum for 10 minutes and transferred into the column in 2ml Collection Tube and centrifuged at top speed for 2 minutes. The columns were transferred into a sterile 1.5ml micro centrifuge tubes. 100µl of Nuclease-Free water was added to the column and centrifuged at top speed for 1 minute at room temperature. Eluted DNA was stored at -20°C.

2.7. Restriction fragment analysis of plasmid DNA

Materials

FD-Buffer
Restriction Enzymes (EcoRV/EcoRI, Sigma-Aldrich Chemie GmbH #cat. 11040197001 and NdeI, Sigma-Aldrich Chemie GmbH #A1161)
Distilled H_2O
Adenoviral Plasmid DNA (pAD/CMV/V5-Terrasense(35391bps), pAD/PL-Terra sense (34260bps))
Lentiviral Plasmid DNA (pLenti4/V5/(CMV)_sense/antisense/Gus,pLenti 6/Ubc/V5_sense/antisen/Gus, pLenti6/BLOCK-iT_sense/antisense/Gus

Procedure

In order to produce one compatible end-to-end clone, characterization of Plasmid DNA for cloning was performed. The DNA to be cloned was prepared with PCR (eg. TERRA gene) processing of the pre-cloned or transferred gene fragment from a bacterial clone or population that has to be removed from genomic DNA extracted from a vector to another. (Source: Prof. Klaus Holzmann, Doris Mejri). The necessary steps for this have been taken as below.

Master mix was prepared as follow

Digestion	**Indigestion**
1µl FD-Buffer	2µl (0,5µg) DNA
0,3µl Enzyme	8ul H_2O
6,7µl H_2Odd.	**10µl Total**
2µl (0,5µg) DNA	
10µl Total	

The digestion and indigestion DNA was shaken for 1h at 37°C.

2.8. Agarose gel electrophoresis

I used the Agarose gel electrophoresis to separate the size of bands of interest and to confirm the expected size from restriction analysis. TERRA fragments were analyzed and compared with those identified by clone manager.

Materials

1g Agarose (Sigma-Aldrich)
100ml distilled H2O
2ml 50xTAE-Buffer (Tris-acetate-EDTA)

Procedure

a) Preparation of Gel

1 g. agarose was measured and slowly boiled in the microwave until became clear. The ends of the casting tray were sealed with the tapes and comb was placed in the gel-casting tray. The melted agarose solution was poured off into the casting tray and allowed to solidify approximately 1 hour. The combs were removed and the casting tray was placed in the gel chamber and run in the running buffer (1xTAE buffer).

b) Sample loading

The 10µl batches (digested and undigested DNA) were mixed with 2µl 6x Loading dye and gently applied to the slots of the solidified gel including marker (Lambda marker III).

c) Electrophoresis:

The power was applied to run with 90 Volt for 1 hour until the dye approaches the end of the gel.

d) Gel Staining and visualization

The gel was allowed to stain (1:10,000 dilution) for at least 10 minutes on shaking. The gel was rinsed with water to remove residual staining solution. And Geldoc imaging system was used to visualize DNA fragments in agarose gel.

2.9. RNA isolation with TRizol

Materials

TRIzol®Reagent(Invitrogen,#15596026)
Chloroform
Isopropanol
Ethanol 75 %
RNasin Plus RNase Inhibitor (Promega, 40u/µl, #N2611) 1x TE as solvent

Procedure

HEK 293 cells were homogenized and the mix was incubated at room temperature for 5 minutes to permit complete dissociation of the nucleoprotein complex. 200 µl chloroform per 1 mL of TRIzol Reagent was added to the homogenized mixture and tubes were gently shaken for several times. The samples were incubated for 2–3 minutes at room temperature and then centrifuged at 12,000xg for 15 minutes at 4°C. The mixture was separated into different layers and upper layer containing total RNA was transferred into the new tubes. RNA was precipitated by adding 500 µl of 100% isopropanol to the collected upper phase per 1 ml of TRIzol Reagent used for homogenization and incubated at room temperature for 10 minutes. The samples

were centrifuge at 12,000xg for 10 minutes at 4°C. RNA pellet was washed with 1 ml of 75% ethanol and vortexed briefly and then centrifuged at 7500 × *g* for 5 minutes at 4°C. Supernatant was aspirated and RNA samples were dried up for 5–10 minutes. RNA was dissolved in 30-50 µl of RNase free water and followed by an additional incubation at 55 °C on a heat block for 15 minutes to allow the complete dissolve.

2.10. Determination of DNA and RNA concentrations

Materials

Spectrophotometer NanoDropTM 1000 (ND-1000, Thermo Scientific)
RNase free water

Procedure

DNA and RNA yield were assessed by absorbance (optical density) method using a spectrophotometer (Spectrophotometer NanoDropTM 1000 (ND-1000) from Thermo Scientific). DNA, RNA quantity and quality were determined by reading the whole absorption spectrum with absorbance ratio at 260 and 280 nm. The machine was calibrated with 1 µl RNase free water and cleaned according to the manufacturer's instructions and 1 µl volume of samples was applied for measurement. Absorbance readings were carried out at 260nm and 280nm absorbance and purity of DNA was considered as 2 at ratios of 260/280 and purity of RNA was estimated as 1,8 at 260/280 ratios.

2.11. cDNA synthesism

Materials

RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific)
5x RT-buffer
dNTP mix (10nM)
Revers Transcription (RT) primer
RNase Inhibitor (40U/µl)
Maxima enzyme mix (Thermo Fisher Scientific, K1671)

Procedure

After the isolation of RNA from HEK 293 cells with TERRA inserted vectors, cDNA was prepared using (RevertAid First Strand cDNA Synthesis Kit from Fermentas). In the first place, the following components were combined and incubated at room temperature for 30 minutes.

1 µg RNA
1µl Random Hexamer Primer
Completed with RNase free water up to 12 µl

The mixture was mixed briefly and incubated at 65°C for 5 minutes. And following 4 µl master mix was added.

2µl 5xReaction Buffer
0,25µl Ribonuclease inhibitor 40U/µl (Kit)
0,25µl H_2O + Inhibitor
1µl dNTPs 10mM mix
0,5 µl Revert Aid Premium Reverse Transcriptase

All mixture was mixed and collected by a gentle centrifugation followed by incubation steps as below;

5 minutes at 28°C incubation
30 minutes at 55°C incubation
Termination of reaction at 85 °C for 5 minutes and storing samples at -20.

2.12. PCR and qPCR Analysis

Materials

GoTaq® qPCR Master Mix (Promega, #A6001)
SYBR Green Reference Dye
For recombinant TERRA expression the following primers were used
- *exoTERRAfwd_1 Box/270-273*
- *exoTERRArev_1*
- *exoTERRAfwd_2*
- *exoTERRArev_2*
- *Tel1b*
- *Tel2b*
- *36B4d*
- *36B4u*
- *eGFP_fwd/rev*

7500 Fast Real Time PCR System (Applied Biosystem)

Procedure

Endogenous TERRA expressions were determined by quantitative PCR (qPCR). 1 µg of RNA was converted with random hexanucleotide primers into cDNA. Total TERRA levels were amplified with telomere-specific (T) primers. The exogenous TERRA primers are exoTerra1-5 and, exoTerrafwd-2rev, exoTerra 2- and total TERRA (endogenous and exogenous) primer is totTerra-tel1+2b. Recombinant TERRA 5`-1 are used to amplified for hH1 promoter/mcs and Recombinant TERRA 3`-1 are used to amplified for term promoter/mcs. The Primers Tel 1 and 2b were used for Telomere length. 36B4 is reference gene was used for normalization. GFP primers were used for GFP-av contructs of GFP cells

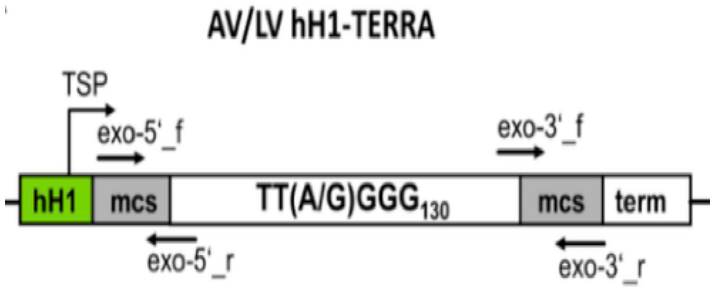


Figure 2.2. Recombinant TERRA Expression of Lenti and Adenivirus constructs with specific primer provide from Theresa Kreilmeier **(Theresa K et al., 2016).**

Table 2.2. Primer for TERRA transcripts to test in qPCR.

Chromosomes	**Primer name**	**Sequence (5´-3´)**
Chr 2p	1_Chr2p_Pos1_fwd 1_Chr2p_Pos303_rev	CGTATCCCACACACCACA GGGGTTCGGGTTAGGGTAA
	2_Chr2p_Pos 255_fwd 2_Chr2p_Pos736_rev	AACCCTAACCCTAACCGTAA AAAGGCGAAGCAGCATTCTC
	3_Chr2p_Pos 722_fwd 3_Chr2p_Pos 1370_rev	TGCTGCTTCGCCTTTACG TGCGACTGTGCGCTTATG
	4_Chr2p_Pos1351_fwd 4_Chr2p_Pos1783_rev	TGCATAAGCGCACAGTCG AGCTGCCACTACACTTGGAG
	1_Chr10q_Pos44_fwd	CTGCGTAGTCCCCATCT

Chr 10q	1_Chr10q_Pos166_rev	GGCCCTCTTCCTCACAT
	2_Chr10q_Pos133_fwd 2_Chr10q_Pos393_rev	GGTGTGCTGCCACTATAATG TCTGAAAAGCCCGTTTCG
	3_Chr10q_Pos330_fwd 3_Chr10q_Pos1284_rev	GTGGGCAAGCTGGTCCTGTAGTG GAAGAACTCTGCTCCGCCTTCGC
	4_Chr10q_Pos1174_fwd 4_Chr10q_Pos1705_rev	GGGCGGAGTTGCGTTCTCTTTAG C CGAACCCGAACCTGAACCCTAAC C
Chr Xq/Yq	1_ChrXq_Y q_Pos8_fwd 1_ChrXq_Y q_Pos455_rev	TCCCTATAATCCGCCACTAC TGTGTTGCAGGAGCAAAG
	2_ChrXq_Y q_Pos434_fwd 2_ChrXq_Yq_Pos1147_rev	GCGACTTTGCTCCTGCAACAC TCCGCACTGAACCGCTCTAAC
	3_ChrXq_Y q_Pos1041_fwd 3_ChrXq_Y q_Pos1503_rev	ACTGGCTTTGGGACAACTC CCCTAACCCTAACCCTTAACC
	4_ChrXq_Y q_Pos1390_fwd 4_ChrXq_Y q_Pos2235_rev	TAGGGTTGGGGTAGGGGTA CCCACACACCACACCCTAA

Table 2.3. Exogenous TERRA expressions primers

qPCR assay	**Oligonucleotide**	**Sequenze; 5'-3'**
Recombinant TERRA 5´	R. TERRA 5´ fwd R. TERRA 5´ rev	CGATCCCCGGGTACCGAG CCCCAACCCCAACCGGAAT
Recombinant TERRA 3´	R. TERRA 3´ rev R. TERRA 3´ fwd	TTAGGGTTAGGGTTCGGAAT AAGTGGAATTGTCGATCTGATA
Total TERRA	Total TERRA fwd Total TERRA rev	CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGT GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT
Reference primer	36B4 fwd 36B4 fwd	CAGCAAGTGGGAAGGTGTAATCC CCCATTCTATCATCAACGGGTACAA
GFP Primer	eGFP-av-fwd eGFP-av-rev	TGACCCTGAAGTTCATCTGCACCA TCTTGTAGTTGCCGTCGTCCTTGA

Primer mix was prepared as follow

4ul Forward + 4ul Reverse + 32ul H_2O

5 difference Master Mix were prepared

a) Fwd 1 + Rev 1(Recombinant TERRA)

b) Fwd 2 + Rev 2 (Recombinant TERRA)

c) Tel1b + Tel2b (Total Terra)

d) Fwd 1 + Rev 2

e) eGFP as control

Master mix (GoTaq® qPCR Master Mix);

1 reaction	18 total reaction
0,16 ul Primer Mix	2,88 ul Primer mix
4 ul Master mix	72 ul Master mix

for eGFP (0,8 ul primer mix and 20 ul Master mix)
for control (4 ul Mix and 4 ul H_2O)

4µl master mix was pippeted into 96-well plate and then 4µl cDNA sample was added. The following PCR steps were used;

95°C, 5" initial denaturation o
95°C, 30"" denaturation
60°C, 1" annealing/extension
60°C, 5" final extension
Melt curve
40 cycles

2.13. Delta-delta Ct method

The delta-delta Ct (2–ΔΔCt) method was used to calculate the relative fold gene expression of samples from qPCR results. Cycles threshold (Ct) of samples from reaction by qPCR were employed to calculate gene expression was calculated using ΔΔCt equation (ΔCt = Ct (gene of interest) – Ct (housekeeping gene). Basically, Ct values for genes of interest were normalized housekeeping gene 36B4. The results produced were taken as relative gene expression values.

CHAPTER 3

3. RESULTS

3.1. Limited information is available about splicing of non-coding transcripts including TERRA

A systematic literature search by Pubmed was performed on September 2017 to identify publications about splicing of non-coding transcripts **(Table 3.1).** Key words related to the subject and logical operators were used. Numbers of identified publications for original articles and review articles are listed. Search for "splicing (transcript or RNA)" in pubmed resulted in 42246 articles and 4102 reviews, that indicate the expected high number of research since several decades. Berget and Chow for the first time identified mRNA splicing they showed genes are present in different segments and splits in higher organisms **(Chow LT and Berget SM, 1977).** A number of publications were found associated with the keyword "coding" (5224 articles and 553 reviews) in contrast to "non-coding" (1007 articles and 244 reviews). From the latter publications a significant amount was associated with the keyword "long non-coding" (400 articles and 92 reviews) but not with the keyword "telomeric repeat-containing" (only one review was chosen). Furthermore, 242 articles and 90 reviews were found from lncRNA biogenesis search in pubmed and 1 article and 1 review were carefully read and evaluated if RNA transcripts were indeed reported as directly spliced and were described in more detail in the discussion section.

Table 3.1. Systematic literature search by Pubmed on Dec. 10th, 2018

		Number of hits		Selected	
Search	**keyword's**	**Articles**	**Reviews**	**Articles**	**Reviews**
1	splicing (transcript or RNA)	42246	4102	n.d.	n.d.
2	search #1 and (coding)	5224	553	n.d.	n.d.
3	search #1 and (non-coding)	1007	244	n.d.	n.d.
4	search #1 and (long non-coding)	400	92	n.d.	n.d.
5	search #1 and (telomeric repeat)	32	3	n.d.	1
6	lncRNA biogenesis	242	90	1	1

n.d. not determined

3.2. Characterisation of Adenoviral plasmids for exogeneous TERRA expression by restriction fragment analyses and transfection of human HEK293 cell lines

In my study, to determine the expression pattern of TERRA, I used the generated and available expression vectors containing the coding sequence of the recombinant TERRA **(Kreilmeier T et al., 2016)**. Adeno- and lentivirus constructs (AV and LV) were established for transient and stable expression of approximately 130 units of telomere hexanucleotide repeats under control of cytomegalovirus (CMV) and human RNase P RNA H1 (hH1) promoters with and without polyadenylation, respectively **(Figure 3.1).**

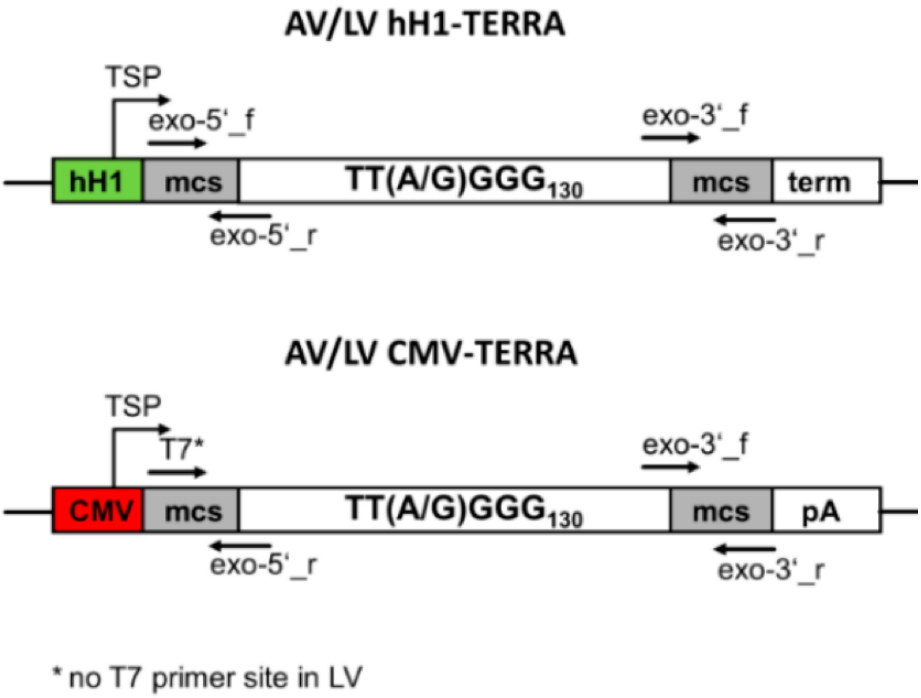


Figure 3.1. Schema of recombinant TERRA expression of AV and LV constructs in human cancer cells (from Kreilmeier T et al., 2016). The constructs (AV and LV) with specific exo-5 and exo-3 primer pairs and H1 RNA polymerase III promoter (hH1), human cytomegalovirus RNA polymerase II promote (CMV). Transcription start point (TSP) and orientation is indicated at hH1 and CMV transcription start point (TSP), polymerase III termination site (term), Polymerase III termination site (term), polyadenylation signal (pA) and multi-cloning-site (mcs) are indicated.

These Adeno- and lentivirus constructs (AV and LV) were recently published **(Kreilmeier T et al., 2016)**. However, beside these published recombinant sense transcript expression constructs, also constructs for anti-sense expression were generated in part by Christian Stern (Diplomarbeit "TERRA transcript expression in tumor cell lines and establishment of constructs for alteration of this expression", Christian Stern, approved at University of Vienna 2011) with limited information about characterisation of all these plasmids. Therefore, my work focuses on the validation of these existing reporter expression systems, especially on AV plasmids for recombinant TERRA sense expression, if correctly constructed. To digest pAD/CMV/V5-DEST and pAD/PL/DEST, vectors were cut with EcoRI restriction enzyme and compared with schematic maps obtained from the Clone Manager software **(Figure 3.2).** EcoRI was selected in clone manager as suitable enzyme for adeviral vector. First, bacterial cultures with plasmids were activated from stock collection (provided by group Holzmann) and grown overnight in the LB medium. The origin and construction of Plasmids used in this study is listed in table S3 **(Kreilmeier et al. 2016; Diplomarbeit, Christian Stern, 2011).** The DNA of recombinant pAD/CMV/V5-DEST and pAD/PL/DEST plasmids carrying the TERRA expression cassettes controlled by CMV and H1 promoters were isolated. Restriction fragment analyses was performed and gel electrophoresis was applied to determine existence of plasmid DNA with the expected molecular weight >34 kbps. The results with restriction enzyme EcoR1 were compared with the predicted plasmid size by Clone Manager software. DNA fragments of pAD/CMV/V5-DEST and pAD/PL/DEST adenoviral plasmids analysed on agarose gel showed the similar expected fragments with clone manager results **(Figure 3.3. lane 1 and lane 3).** These results confirmed that the pAD/CMV/V5-DEST and pAD/PL/DEST were positively characterized correct adenoviral plasmids. Further analyses of the TERRA expression cassette from plasmids by sequencing demonstrated that both pADs are correct (K.Holzmann, personal communication)

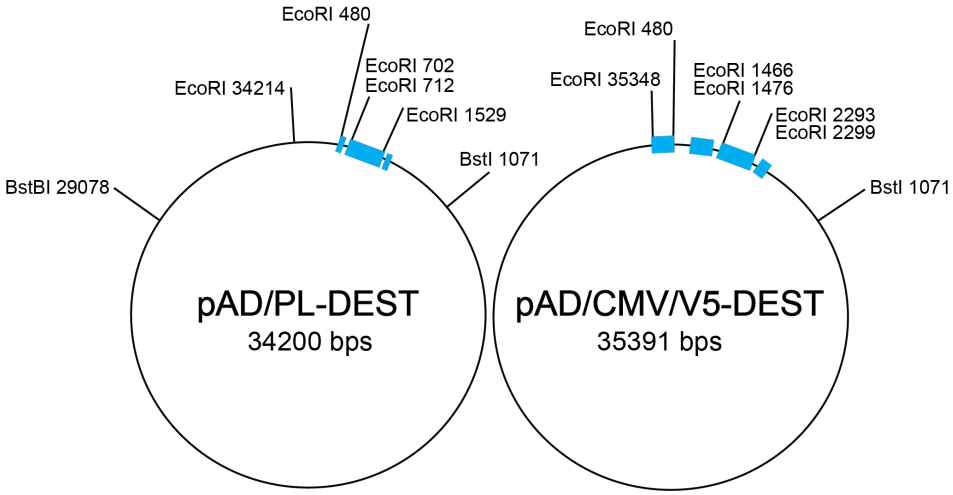
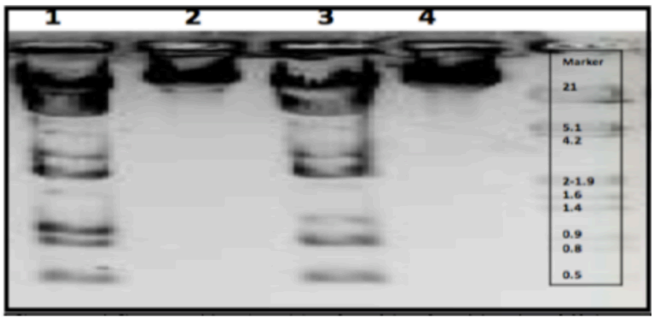


Figure 3.2. Schematic maps of plasmids from adenoviral vectors obtained from the Clone Manager for pAD/CMV/V5-DEST and pAD/PL/DEST. Integration of EcoRI restriction enzyme sites is indicated and TERRA expression cassette is shown in blue boxes. The four boxes in pAD/CMV/V5 indicates EcoRI recognition sites in TERRA gene.



Enzym	1 EcoRI	2 Cont.	3 EcoRI	4 Cont.
Konstrukt	pAdCMV-Terra-s	pAdCMV-Terra-s	pAdpL-Terra-s	pAdpL-Terra-s
Marker 21 5.1 4.2 2/1.9 1.6/1.4 0.9/0.8 0.5	24.2	>24	23.9	>24
	6.7		6.7	
	1.9		1.9	
	0.9		0.8	
	0.8		0.5	
	0.5			
C.manger	24.2/6.7/1.9 /0.9/0.8/0.5		23.9/6.7/ 1.9/0.8/0.5	
compare	Ok.		Ok.	

Figure 3.3. Analysis of the restriction digest of Adenoviral DNA plasmids on agarose-gel. pAD/CMV/V5-Terra sense and pAD/PL-Terra sense vectors were cut with EcoRI restriction enzyme or without restriction enzyme as a control. Lane1 shows pAD/CMV/V5-Terra sense digested fragments were compatible with the results from clone manager. Lane 3 shows pAD/PL-Terra sense digested fragments were positively in line with results from clone manager. Lane 2 and 4 show undigested controls. Units of marker is base pairs starting from 21000 bp to 500 bp.

Next, adenovirus packaging cell line HEK293 cells were used for adenovirus vector production. In brief, higher amounts of recombinant Adenovirus plasmid DNAs were isolated by endo-free Maxipreps from the characterised plasmids pAD/CMV/V5-Terra sense and pAD/PL-Terra sense. DNAs were cut by PacI and EcoRI restriction enzymes for liberation of linear dsDNA AV genome and confirmation of the inserts. Linearized recombinant dsDNA from AVs were transfected by lipofection into HEK293 cells to start the recombinant AV life cycle. HEK293 cells cultured in vitro and adhered to the surface of the culture flask. Infection of recombinant AV-eGFP with low multiplicity of infection (M.O.I.) to HEK293 cells was performed as control and GFP expression was monitored. The monolayers of transfected/infected HEK 293 cells start after two days to show a cytophatic effect by phase-contrast microscopy analysis (**Figure 3.4**). GFP signal of AV-eGFP was monitored by flourescence microscopy and revealed an appropriate density by GFP infected cells as shown.

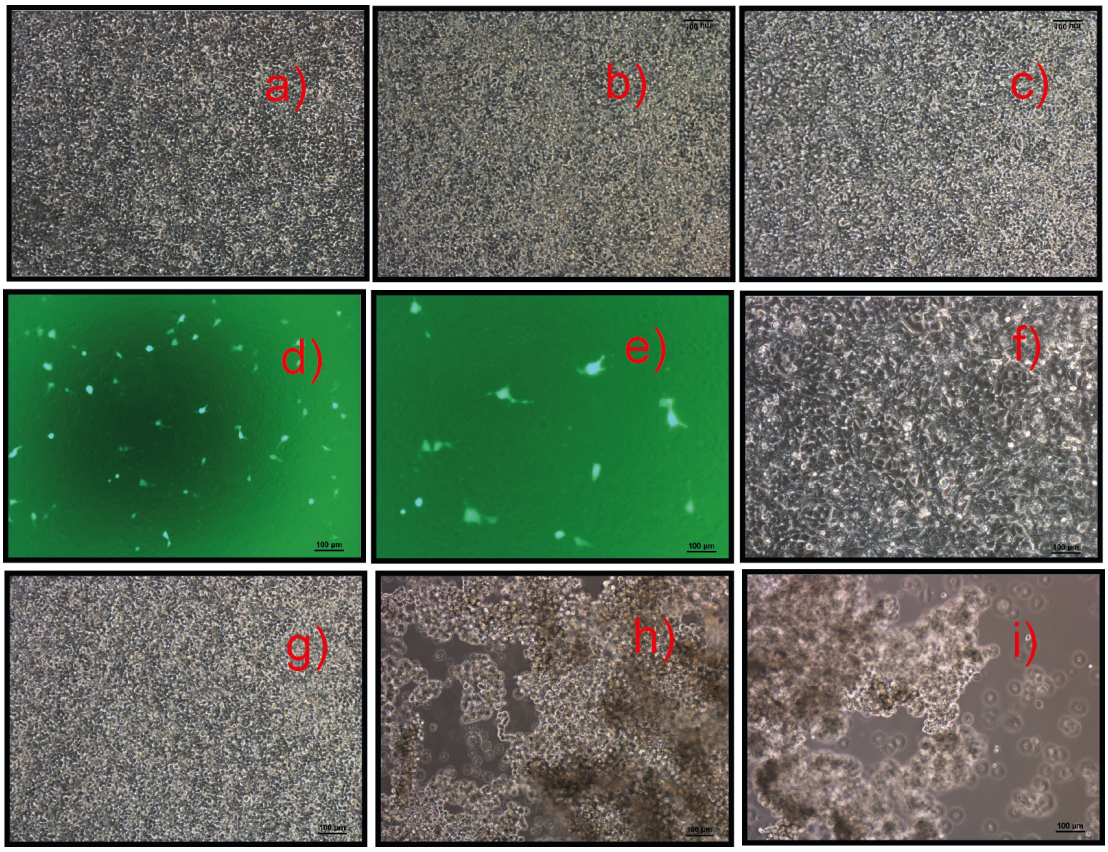


Figure 3.4. Transfection of linearized dsDNA of AVs and monitoring of cells by phase-contrast and GFP control signals monitored 2 days after transfection. a) untreated+ Tris and it is for control, 10x Objective, phase, settled. **b)** untreated, it is control, 10x objective, phases, settled. **c)** GFP, treatment, 10x objective, phases, settled. **d)** GFP, treatment, 10x objective, Fluorescence, settled **e).** GFP, treatment, 10x objective, Fluorescence, settled. **f)** GFP, treatment, 10x objective, phase, settled. **g)** GFP treatment, 10x objective, phases, settled. **h)** pAD/CMV5-Terra sense, treatment, 10x objective, phase, clusters. **i)** pAd/PL-Terra sense, treatment, 10x objective, phase, clusters

Taken together; these results indicate Adenovirus packaging cell line HEK293 cells produced recombinant Adenovirus plasmid DNAs pAD/CMV/V5-Terra sense and pAD/PL-Terra sense at an appropriate density by monitoring GFP expression by flourescence microscopy.

For this study, ectopic TERRA expression levels were analyzed in HEK 293 cell lines (recombinant AV-infected) with the primers for specific site exogenous TERRA **(Table 3.2).** Exogenous TERRA expressions were determined by quantitative PCR (qPCR-7500 FAST real-time PCR System). cDNA was synthesized with Random

Hexamer Primers and then TERRA gene expression levels were compared using these primers.

3.3. Recombinant exogenous TERRA gene levels detected in AV-infected HEK 293 cells.

The ectopic expression of recombinant TERRA was analysed by AVs with CMV and hH1 promoter. Primary transfected HEK 293 cells were analysed after the cytopathic effect took place **(Figure 3.4)**. AV-infected cells were harvested and RNA was isolated. Ectopic TERRA transcript expression by recombinant AVswas determined by qPCR (**Figures 3.5 and 3.6**) **.** The primers from 5 'and 3' multiple cloning specific sites for recombinant TERRA were used to quantify exogenous TERRA by qPCR **(Figure 3.1., Table 2.2.).** After infection with AV constructs, relative quantity (RQ) values of the exogenous TERRA transcript levels were determined **(Figure 3.5)**. The ribosomal housekeeping gene 36B4 and HEK293 cells infected with eGFP-AV and unb-dm (mock control) were used as referenceset as RQ=1 by ddCt method . Total RNA was isolated from HEK293 cells that showed a cytopathic effect indicating a high number of recombinant AVs and from HEK293 control cells without cytopathic effect (**Fig.3.4**). Isolated RNA was converted to cDNA and applied for Real-Time qPCR to generate amplification curves and cycle threshold (Ct) raw date (**Figure 3.6**). Ct and calculated RQ values with errors are listed in **suppl. Table S2**. All vectors did not show an increase in TERRA expression levels in HEK293 **(Figure 3.5)**. Ectopic TERRA sense vector with CMV and hH1 promoter resulted in an increase in TERRA expression **(Figure 3.5).** AV-infected HEK 293 cells with pAD/PL/hH1-Terra sense vector showed higly significant expression using exoTERRA1-5’ and thus indicates that recombinant hH1-TERRA sense transcripts exist in HEK 293 cells **(Figure 3.5A).** exoTERRA1-5’ did not show up TERRA transcripts in HEK2913 cells transfected with pAD/CMV/V5-Terra sense **(Figure 3.5A).** Recombinant TERRA gene expression levels with exoTerra2-3 'primer pairs

showed highly significant level of amplification in infected HEK293 cells with pAD/CMV/V5-Terra sense in comparison to the expression in pAD/PL-Terra sense (p=0,0117) **(Figure 3.5B).** No or low expression was observed in mock control and anti-terra sense **(Figure 3.5B).** Similarly, TERRA gene expression levels with total Terra1+2b primer pairs showed highly significant level of amplification in infected HEK293 cells with pAD/CMV/V5-Terra sense in comparison to the expression in pAD/PL-Terra sense (p=0,0002) **(Figure 3.5C).** Furthermore, by primers of exoTERRA1fwd+2rev total TERRA transcript expression was significantly detected **(Figure 3.5D).** Amplification plots with Ct values demonstrate absolute gene expression values (**suppl. Table 2).** Absolut recombinant TERRA expression values measured with exoTerra1-5' and exoTerra2-3' 2assay were highest in pAD/PL-Terra sense infected cells (Ct=16.82 to 15.41) as compared to pAD/CMV/V5-Terra sense (Ct=34.51 to 15.61) and anti-sense (Ct=32.2 to 23.99) TERRA infected cells **(suppl.Table S2).** RQ values of whole 0.8 kbps telomere sequence TERRA expression level are not accurate in this single experiment as the Ct values demonstrated that this qPCR assay failed to detect any products below Ct value of 30, indicting extreme low absolute amounts of transcripts or generally low efficiency of the qPCR assay for amplification of long and difficult G-quadruplex-structured DNA (**suppl.Table S2**). In summary, this result indicates that pAD/CMV/V5-Terra sense and pAD/PL-Terra sense are positive vectors for the expression of TERRA transcripts when specific primer pairs used such as pAD/PL/-Terra sense with exoTerra1-5 and exoTerrafwd-2rev, pAD/CMV/V5-Terra sense with exoTerra 2-3 and totTerra-tel1+2b primers.

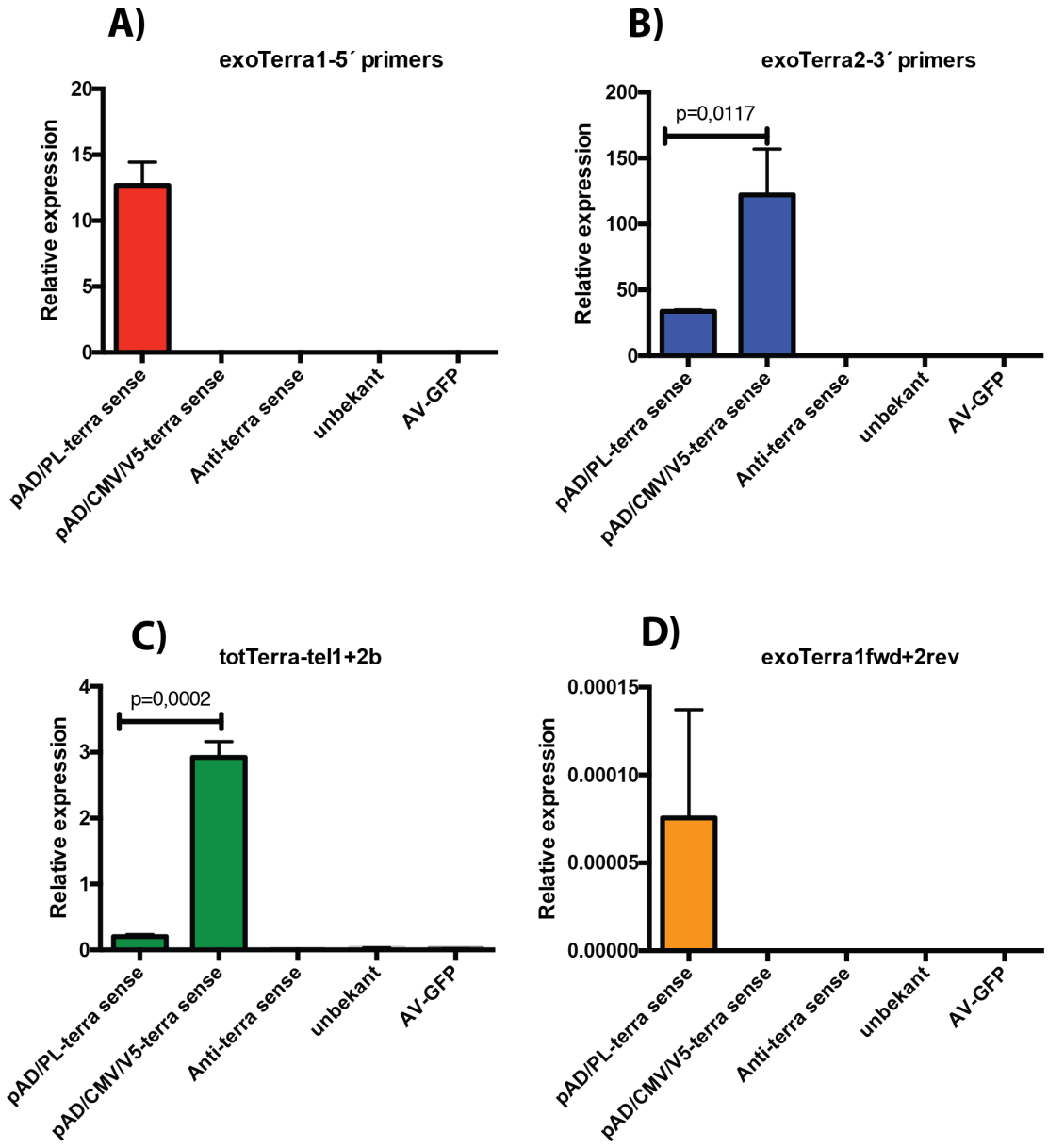


Figure 3.5. AV-infected HEK 293 cells line with relative quantity (RQ) transcript expression levels of exogenous and endogenous TERRA using specific primers. **A)** relative expression in adenoviral vectors and controls using exoTerra1-5' primers. **B)** Expression level using exoTerra2-3' primers. **C)** Expression level using totTerra tel1+-2b primers. **D)** Expression level using exoTerra tel1fwd+-2rev primers

3.4. Lentiviral vectors for recombinant stable expression of telomeric part of TERRA

The telomere fragment in the pSP73 plasmid was transferred to plasmids called pENTR/D as described (**Kreilmeier et al. 2016; Diplomarbeit, Christian Stern, 2011**). A set of available pENTR constructs with attL1 and attL2 recombination regions was used to transfer the inserts into pDEST expression vectors by using Gateway Cloning system (**Figure 2.1**)**(Jenn YC et al., 2015).** Such cloning technique allows a simple one-step procedure instead of complex restriction enzyme applications.

In this study, I used two types of pENTR for construction of plasmids for recombinant pENTR Vectors

1) pENTR hH1-TERRA
2) pENTR-hH1

The Lentiviral Destination Vectors used in this study are the strong CcdB product of attR1 and attR2 recombination regions, a potent cytokine of 101 amino acid residues whose activity is negatively regulated by the CcdA protein **(Bernard P 1996).** This gene was transferred to gene expression vectors in LR-Reaction-ending pENTR vectors. As a result, a recombinant target vector known as an expression clone was generated and at the same time the ccdB gene linked to pENTR vector **(Figure 2.1).** The aim here was to transfer the recombinant DNA plasmids into lentiviral recombinant DNA pDEST vectors:

pLenti4/V5/DestCMV-Tel-Region
pLenti6/UbCV5-Tel-Region
pLenti6/BLOCK-iT

Plasmid DNA from pENTR (1-4) and pDONR was isolated from stored bacterial stocks. pLENTI-DONR plasmids were already constructed in E.coli TOP10 cells

(Kreilmeier et al. 2016; Diplomarbeit, Christian Stern, 2011). E. coli Stbl3 competent cells were obtained from Heidi Huber (Group W.Mikulits). LR reactions were performed by Doris Mejri (Group K.Holzmann). The bacterial plasmids in E. coli (Stbl3) were transferred and selected in ampicillin-supplemented media. As a control I used pUC19, a relatively small and easily transferable plasmid. The transformation was confirmed by comparing the number of colonies produced per µg of DNA.

The number of colonies counted on plates. pUC control should be above 10^6, which is desirable for transformation efficiency. For this, 10 pg and 1 ng of pUC19-Plasmid-DNA were used.

10pg DNA……..10 clone 1ng DNA……1000 clone
1µgDNA…….x=1*10^6 clone

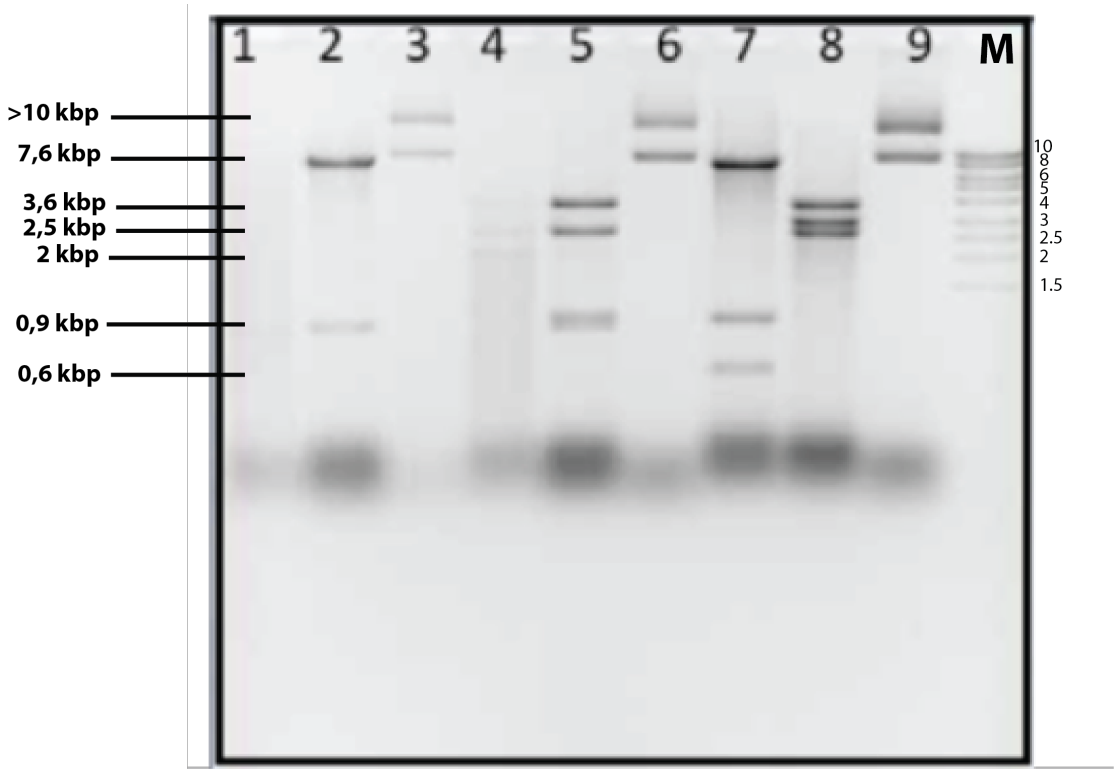
Transformation rate was calculated to be1*10^6 clone/1µg DNA **(Figure 3.6).**

Figure 3.6. Example of control pUC19 plasmid was used to demonstrate the number of viable colonies after transformation following ampicilin selection. **a)** 99/100 µl of bacterial cultures showed colony formation after plating and **b)** pUC19 plasmid showed a viable colony formation of 1/100 (=5µl =10pg). This experiment was performed several times. Appropriate number of colonies were gained in the experimen above.

3.5. Validation of lentiviral plasmid vectors plenti6/Ubc amplified in E.coli by digestion with EcoRV and SacI was confirmed

The aim here is to test whether the Gateway-cloned 0.8kbps telomeric insertis actually transferred correctly to the desired pDEST vector. These sizes were compared with the theoretical ones calculated by Clone Manager software. LV vectors containing the 0.8 kbps telomeric insert were excised with EcoRV and SacI restriction enzymes, and the visualized fragment sizes on gels were determined by

comparision with molecular markers. Agarose gel shows the fragmented DNA cut by EcoRV and SacI restriction enzymes and their respective bands (**Figure 3.7**). Minimum 2 colonies were picked for analysis. Detailed circular plasmid maps of vectors are illustrated below with characteristic restriction enzyme sites **(Figure 3.8).**



1 EcoRV	**2 EcoRV**	**3 Cont**	**4 SacI**	**5 SacI**	**6 Cont.**	**7 EcoRV**	**8 SacI**	**9 Cont.**	**Enzym**
pLenti6-UbC-s	pLenti6-UbC-as	pLenti6-UbC-s	pLenti6-UbC-s	pLenti6-UbC-as	pLenti6-UbC-as	pLenti6-UbC-GUS	pLenti6-UbC-GUS	pLenti6-UbC-GUS	Konstrukt
	7.6	>10		3.6	>10	7.6	3.6	>10	Marker
	0.8		2.5	2.5		1	2.9		10
			2	1		0.6	2.5		8
									6
									5
									4
									3-2.5
									2-1.5
7.6/0,9	7.6/0.8		3.6/2.5/2	3.6/2.5/1/.09		7.6/1/0.6	3.6/2.9/2.5		C.manger
No	Ok.	Ok.	Not all	Ok.	Ok.	Ok.	Ok.	Ok.	compare

Figure 3.7. Restriction analysis of lentiviral vectors digested with EcoRV and SacI on 1% agarose. Numbers are fragment sizes in kbps.
Lane 1. pLenti6/UbC/V5-sense was cut with EcoRV and compared with Clone manager resulting with no band.
Lane 2. pLenti6/Ubc + pENTR-antisense was cut with EcoRV and compared with Clonemanager, that is, a positive band was obtained.

Lane 4. pLenti6/UbC/V5-Tel-Region-sense plasmid DNA was cut with SacI restriction enzyme and the expected bands were compared with findings in clone manager and very weak signals were observed.
Lane 5. pLenti6/UbC/V5-Tel-Region-antisense recombinant DNA was cut with SacI enzyme, compared with in silico Clonermanager analysis and the result was positive.
Lane 7. GUS plasmid was used instead of pENTR vector for control. The pLenti6/Ubc + GUS recombinant plasmid was cut with EcoRV restriction enzyme was compared with clone manager results and positive bands were obtained.
Lane 8. The same plasmid was also digested with SacI restriction enzyme and the result of the comparison was confirmed positively.
Lane 3 and **9** are undigested controls.

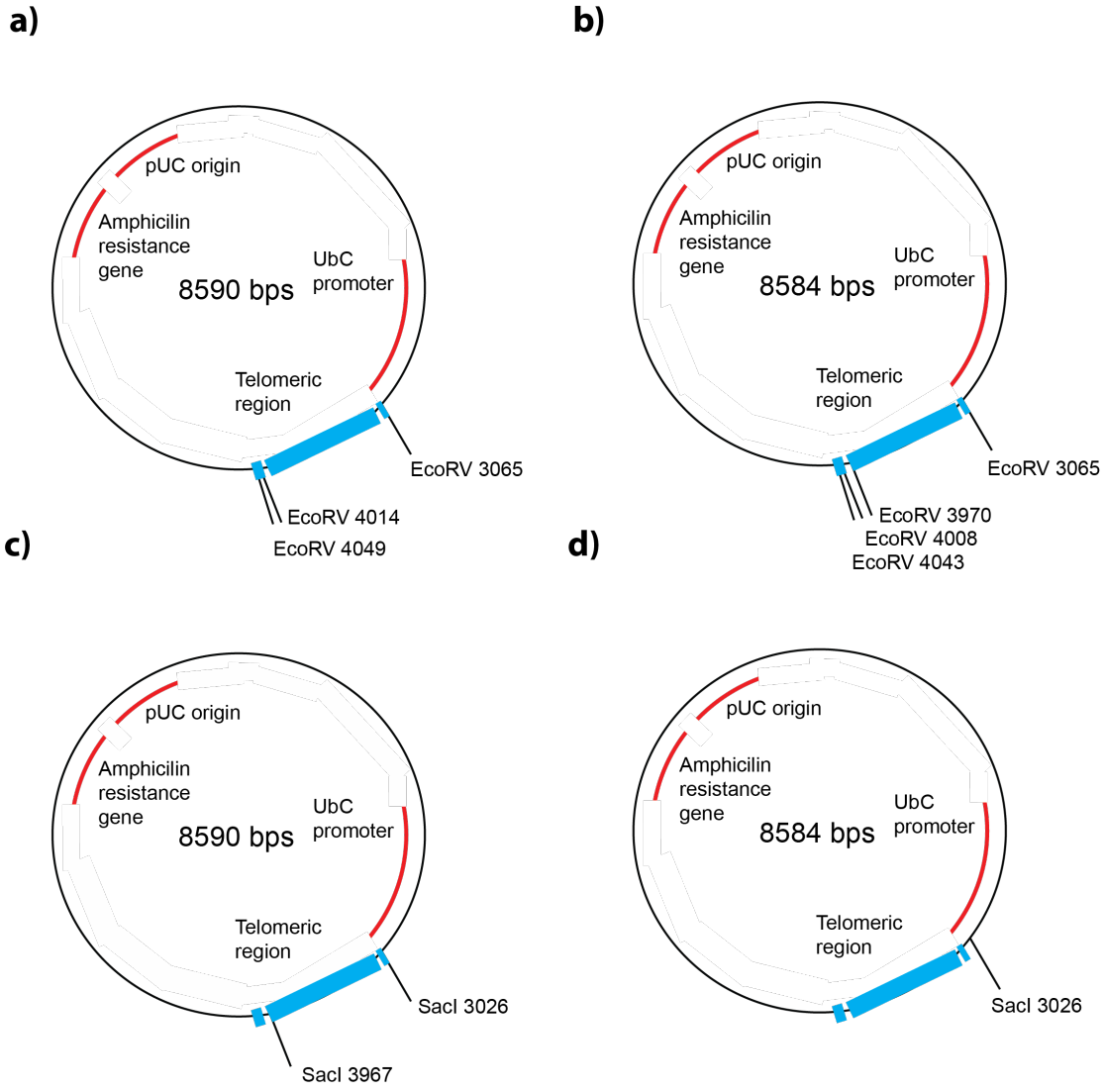


Figure 3.8. Maps of plenti6/Ubc plasmid DNA by Clone Manager software. a) and **b)** pLenti6/UbC/V5/Dest-Tel-region-antisense plasmid DNA in silico Clone manager program, EcoRV restriction enzymes sites and the fragments are shown (Box). **c)** and **d)** In the same clone, the regions and fragments of pLenti6-UbC-V5-Tel-Region-antisense/sense plasmid DNA are cleaved by the silico clone manager program and shows the enzyme SacI restriction enzyme sites (Box). Numbers in the middle of plasmid indicate the total base pairs and numbers on the enzyme show number of basepapirs for enzyme recognition sites. pUC: origin of replication. Ubiquitin C promoter (UbC) is for driving the expression.

pLenti6/UbC sense in lane 1 did not show fragmented sizes with EcoRV but showed expected results with SacI. Undigested controls showed no restriction cut (lane 3, lane 6 and lane 9). As a result of restriction analysis of LV vectors, pLenti6/UbC/V5-Tel-Region-antisense had the correct bands with clone manager analysis. pLenti6/Ubc-antisense with EcoRV and pLenti6/UbC/V5/-Tel-region-sense plasmid with SacI and pLenti6/UbC/V5-Tel-Region-antisense with SacI enzyme confirmed positive results with that of clone manager analysis **(Figure 3.7).**

3.6. PLenti4/CMV recombinant plasmid restriction analysis showed expected bands with that of clone manager.

In order to characterize recombinant plasmid DNA, the endonuclease enzyme analysis was performed and the results were compared with clone manager results. pLenti4/V5/CMV antisense/sense plasmid DNA was cut with the EcoRV and SacI restriction enzyme. The samples were run on 1% agarose and bands were compared to the clone manager analysis. The observed band size and number were confident and as a result all PLenti4/CMV vectors demonstrated positive results with clone manager analysis **(Figure 3.9).**

Maps of pLenti4/V5/Dest-CMV plasmid DNA obtained from clone manager are shown in figure 3.12. pLenti4/V5/DestCMV-antisense/sense plasmid DNA is analyzed in clone manager program. The regions excised with EcoRV restriction enzyme and the fragment sizes are shown in box **(Figure 3.10a and 3.10b).** Similarly **Figure 3.10c and 3.10d** show the restriction sites with SacI restriction enzyme and fragment sizes are illustrated (box). These results indicate pLenti4/V5/DestCMV vector confirmed positively with results from clone manager when it is digested with EcoRV and SacI restriction enzyme. This vector could be useful for TERRA sense and antisense expression in cells.

Enzym	1 EcoRV	2 SacI	3 Cont	4 EcoRV	5 SacI	6 Cont.
Konstrukt	pLenti4_s	pLenti4_s	pLenti4_s	GUS pLenti4_s	GUS pLenti4_s	GUS pLenti4_s
Marker	6.9	3.6	>10	6.9	3.6	>10
10	0.9 Weak	2.1		1	2.5	
8		1.8		0.6	2	
6						
5						
4						
3-2.5 2-1.5						
C.manger	6.9/0.9	3.6/2.1/1.8kb		6.9/1/0.6	3.6/3.1/1.8	
compare	Ok.	Ok.	Ok.	Ok.	Ok.	Ok.

Figure 3.9. Restriction analysis pLenti4/V/CMV-Tel-Region-antisense/sense plasmid DNA on agarose gel.
Lane 1. pLenti4/V/CMV-Tel-Region-antisense/sense plasmid DNA was digested with EcoRV restriction enzyme and compared to the clone manager analysis.
Lane 2. The same Recombinant DNA was cut with SacI restriction enzyme and the observed band size and number were compared with the clone manager results**.**
Lane 4 and 5 control. GUS plasmid was used instead of pLenti4/CMV. It was cut with EcoRV and SacI.

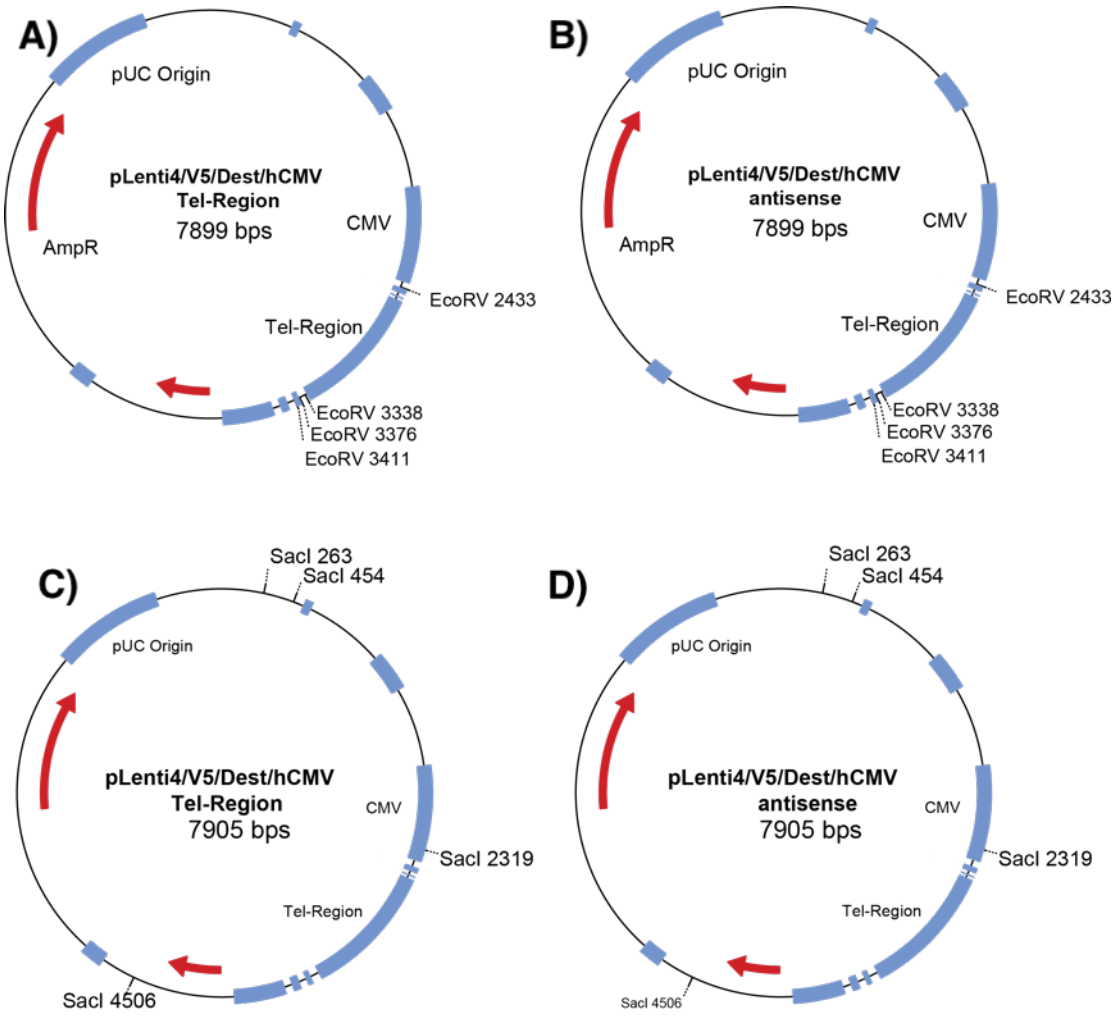
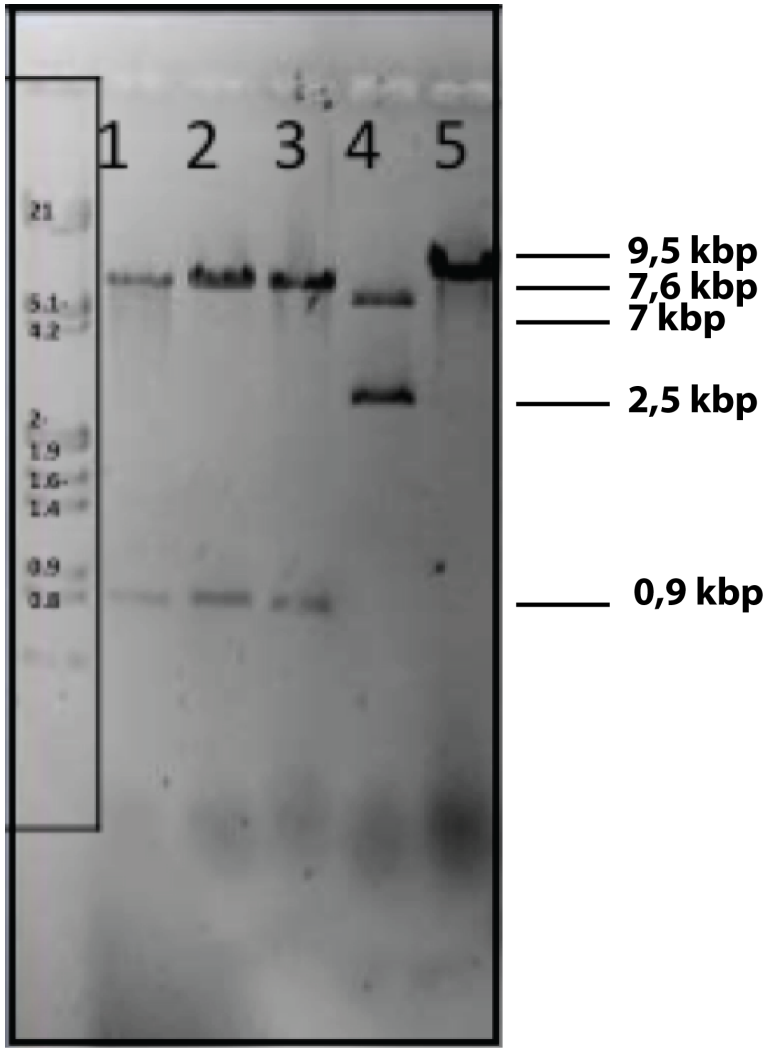


Figure 3.10. Maps of pLenti4_V5_Dest-CMV plasmid DNA on the Clone manager. A) and **B)** pLenti4_V5_Dest-CMV-Tel-Region-sense and antisense plasmid DNA was excised with EcoRV and SacI restriction enzyme **(C and D)**. Fragment sizes are illustrated in boxes.

3.7. PLenti6/Ubc of amplification with EcoRI was confirmed and validated

To characterize PLenti6/Ubc vector, I cut 3 PLenti6/Ubc vectors (pLenti6/Ubc sense, PLenti6/Ubc antisense and PLenti6/Ubc/GUS) with EcoRI restriction enzyme As a result, identification of the correct product in comparison with the results of in-silico clone manager analysis showed correct band. **Figure 3.11 Lane 1** shows pLenti6/Ubc-sense cut by restriction enzyme EcoRI and compared with Cloner Manager results and positive bands (7600 and 800 basepairs) were obtained. **Lane 2 and 3** show pLenti6/Ubc-antisense were obtained with the same restriction enzyme and positive results were obtained. **Lane 4** shows pLenti6/Ubc-GUS used as a control was cut with EcoRI and compared with clone manager results but no expected bands were obtained. pLenti6/Ubc-sense did not display the same results with the results of Clone Manager. **Lane 5** shows that negative results were gained with pLenti6/Ubc-GUS.

Additionally, **Figure 3.12** illustrates clone manager maps of pLenti6/Ubc and the regions cut by the EcoRI restriction enzyme in pLenti6/UbC-Tel-Region-antisense and sense recombinant plasmids (**Figure 3.12a and 3.12b).** Summing up, restriction analysis by EcoRI showed that pLenti6/UbC sense and pLenti6/UbC antisense were correctly validated in compatible with clone manager analysis.



Enzym	**1 EcoRI**	**2 EcoRI**	**3 EcoRI**	**4 EcoRI**	**5 EcoRI**
Konstrukt	pLenti6_Ubc_s	pLenti6_Ubc_as	pLenti6_Ubc_as	pLenti6_Ubc_GUS	pLenti6_Ubc_GUS
Marker	7.6	7.6	7.6	7.6	9.5
21	0.8	0.8	0.8	2.5	
5.1					
4.2					
2					
1.9					
1.6					
1.4-0.9-0.8					
C.manger	7.6-0.8	7.6-0.8	7.6-08	0.9	0.9
compare	Ok.	Ok.	Ok.	not	not

Figure 3.11. Testing of PLenti6/Ubc recombinant DNA with EcoRI enzyme.
Lane 1. pLenti6/Ubc-sense was cut by restriction enzyme EcoRI and compared with Cloner Manager results.
Lane 2 and 3. pLenti6/Ubc-antisense were obtained with the similar and positive results.
Lane 4. pLenti6/Ubc-GUS used as a control was cut with EcoRI and compared with Clonee manager results and giving negative results.
Lane 5. pLenti6/Ubc-GUS by EcoRI showed negative results in comparison with clone manager results.

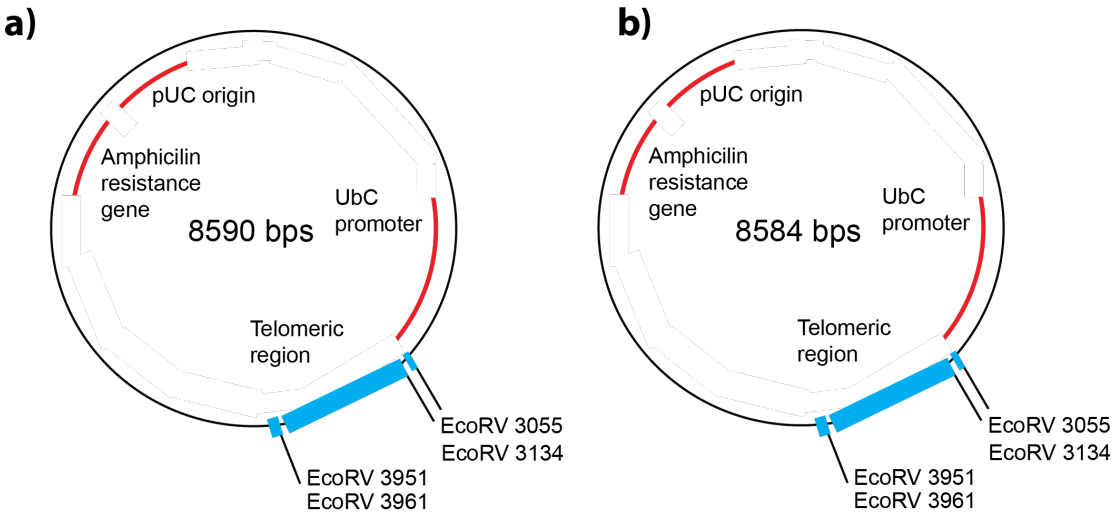
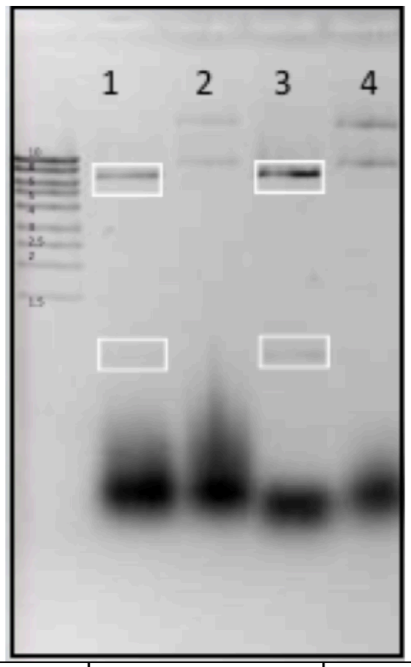


Figure 3.12. Cloner-Manger Maps of pLenti6/Ubc. The regions cut by the EcoRI restriction enzyme **(a and b).** (clone manager) (box).

3.8. Plenti4/CMV of amplification with EcoRI was validated.

In order to validate pLenti4/V5/CMV-Tel-Region, the vector was cut by the EcoRI restriction enzyme and their expected bands on the Agarose Gel showed similar size and number with that of Cloner manager results. **Figure 3.13.** shows Plenti4/CMV recombinant DNA cut with EcoRI. Lane 1 and lane 3 shows pLenti4/V5/DestCMV-Tel-Region-antisense was cut with EcoRI restriction enzyme and compared with cloner manager results and the expected band to be 0.8 kb in size was obtained. Lane 2 and 4 were used as undigested control. In silico clone manager program was used to generate the pLenti4/V5/DestCMV-Tel-Region -antisense plasmid with EcoRI restriction enzyme and mapped for the purpose of showing the excised regions **(Figure 3.14).** These results indicate pLenti4/V5/DestCMV-Tel-Region-antisense cut with EcoRI is correct plasmid and the bands were similar to results obtained from clone manager.



Enzym	**1** **EcoRI**	**2** **Cont.**	**3** **EcoRI**	**4** **Cont.**
Konstrukt	Plenti4/CMV-as	Plenti4/CMV-as	Plenti4/CMV-as	Plenti4/CMV-as
Marker	6.9	>10	6.9	>10
10	0.8	>10	0.8	>10
8				
6				
5/4				
3/2.5/2/1,5				
C.manger	6.9-0.8		6.9-0.8	
compare	Ok.		Ok.	

Figure 3.13. Plenti4/CMV recombinant DNA with EcoRI.
Lane 1 and 3. pLenti4/V5/DestCMV-Tel-Region-antisense cut with EcoRI.
Lane 2 and 4 were used as undigested control.

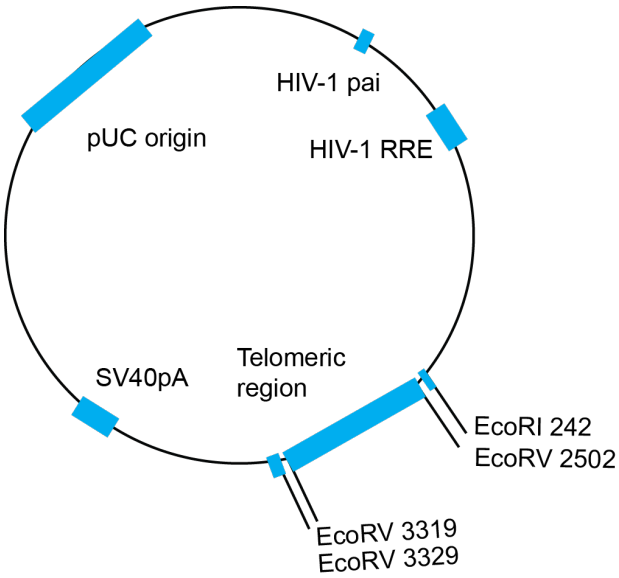


Figure 3.14. Clone Manager Maps of pLenti4/V5/Dest-CMV-Tel-Region-antisense plasmid with EcoRI restriction enzyme.

3.9. In silico analysis of endogenous TERRA showed splicing prediction in specific chromosomal regions.

In order to define possible splicing sites in endogenous TERRA, in silico analysis of TERRA was performed using Human Splicing Finder whether there is any splicing in 2p, 10q and X/Yq human chromosomal DNA (Nejc Kupper, 2015), Diploma thesis MUW). The one of the reason of choosing these chromosome ends out of around 20 is that they contain a conserved tandem repetitive region of 61-29-37 bp repeats in subtelomeres that function as TERRA promoters, epigenetic regulated CpG-islands and demonstration of TERRA transcript expression by qPCR (**Sampl S. 2012**) (**Nergadze, SG et al. 2009).** I repeated these in-silico analyses and compared my results with data from a previous Diploma thesis at the MUW **(Nejc Kupper, 2015).**

The two chromosome ends were studied with Human splicing Finder (http://www.umd.be/HSF/) version 2.4.1 **(Desmet FO et al. 2009).** I used Human splicing finder, an online bioinformatics tool to predict splicing signals, potential splice sites and branch points in Chr2p, 10q and X/Yq. I loaded the sequence from genome browser from 3´ to 5´. The sequence for Chromosome 2p model for in silico analysis deposited in GenBank database entry as NT_005334.17, for chromosome 10q model

M57752.1 and for Chr Xq/Yq in the GenBank database entry M57753.1. The human splicing Finder calculated the probability of splice site in the sequence of three chromosomes. Possibilities can be calculated giving a consensus values (CV). The consensus value is proportional to the probability of splice. Comparison of TERRA Chr2p, 10q and X/Yq sequences obtained from genome browser results with natural splice site, Aceptor/donor splice sites, and branch point CVs **(Table 3.2)** are listed.

Table 3.2. Comparison of mean and standard deviation consensus values (CV) for TERRA models and natural splicing.

Location	**Acceptor splice site**	**Donor splice site**	**Branch point**
Natural splice site 83.4±8.6	400.000	87.5±8.3	86.8±6.3
Chr 2p 100±70,36	388	89,51±65,86	92,64±65,08
Chr10q 98,37±70,31	162	90,37 65,19	92,64±65,08
Chr Xq/Yq 96,63±70,03	361	94,81±65,16	89,73±65,08

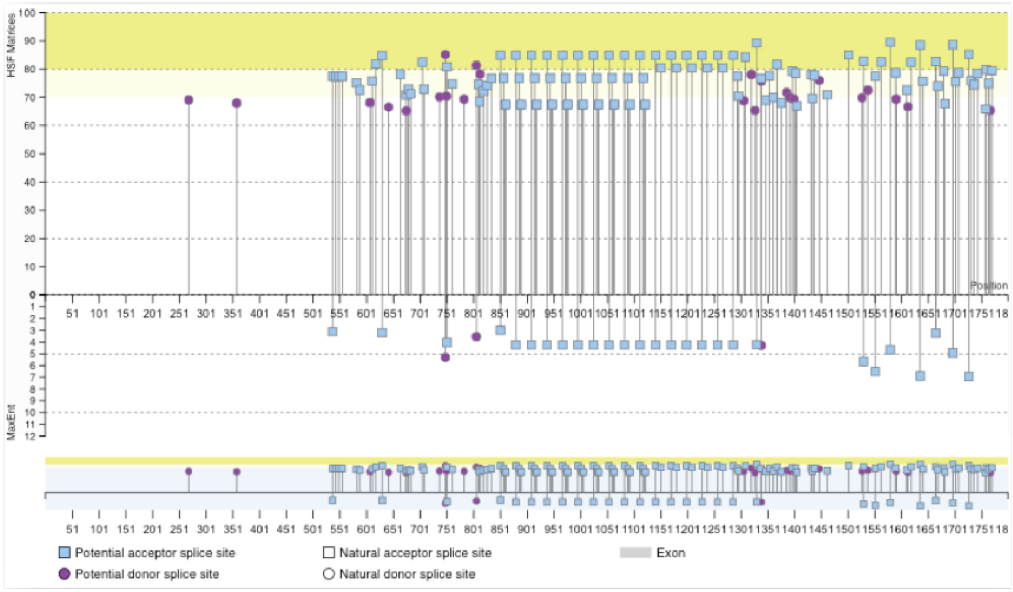
My results were different as compared to the first analyses performed by Nejc Kupper ("Splicing and Back-splicing of TERRA", approved 2015 at Medical University of Vienna). The reasons might be due to the different length of sequence of subtelomeric region of Chr 2p, 10q Chr and Xq/Yq in the previous analysis.

The *Human Splicing Finder* (HSF) *splice* site *prediction* algorithm displayed different *splice* site *prediction for TERRA.* I found 388 splicing sites with high rated CV values between the positions 1 and 1800 bp of the Chr2p model. **Figure 3.15A** shows strongest candidates are located between the exon boundary (Exon/intron boundaries are referred to the potential splice sites) and 50 nucleotides upstream of TERRA the number of splice sites are high and begins at 551 nucleotides. The **figure 15B** shows a panel for Chr2p where silencer sites (red and pink) and enhancer motifs site (panel below with blue and green) and both Brown graph were

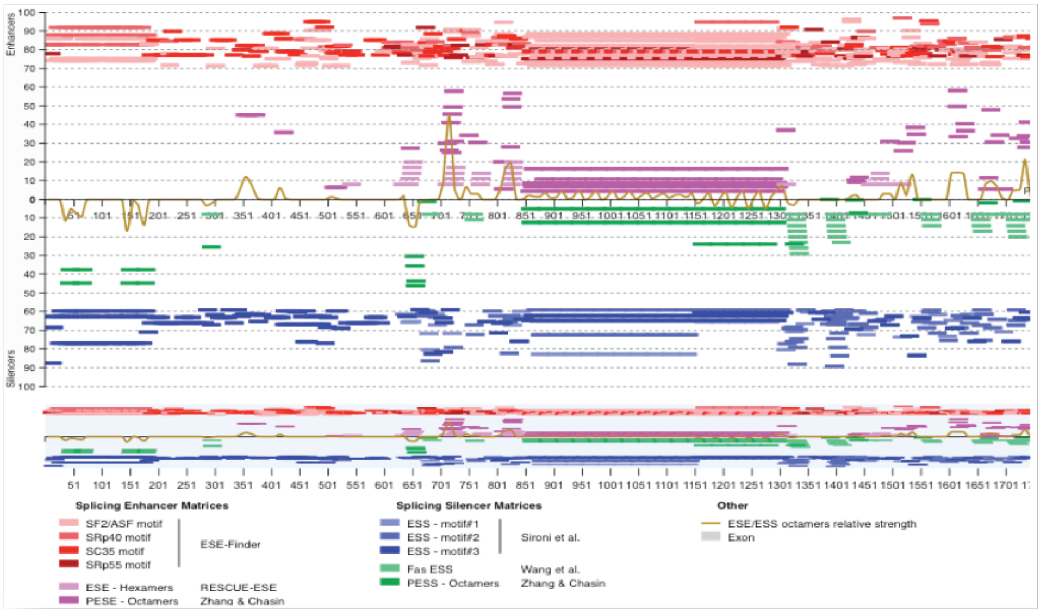
found in silico on the chromosome models. For Chr10q TERRA model sequences, splicing sites and motifs have been located between the positions 1 and 1700 bp **(Figure 15C).** DNA motifs are known short and repeating pattern in DNA sequence. And splicing site is special sequences at the intron ends recognized by spliceosome. A high number of splicing sites and motifs continues to 1001 from the 51st nucleotide. The non-splicing regions appear as a straight line and so there are degenerated and defects in splicing **(Figure 15D).** TERRA shows high splicing density between positions 1 and 2401 bp of the Xq/Yq model **(Figure 15F)**. Auxiliary splicing sequences or the splicing site of the silencer and enhancer show an equal splicing density **(Figure 15D).**

These results demonstrate splicing of TERRA and TERRA transcripts have multiple potential splicing sites within the subtelomeric region of Chr 2p, 10q Chr and Xq/Yq. To sum up, the analysis showed that TERRA transcripts of the telomeric regions show multiple potential splice sites und thus splicing between subtelomeric and telomeric regions may be possible.

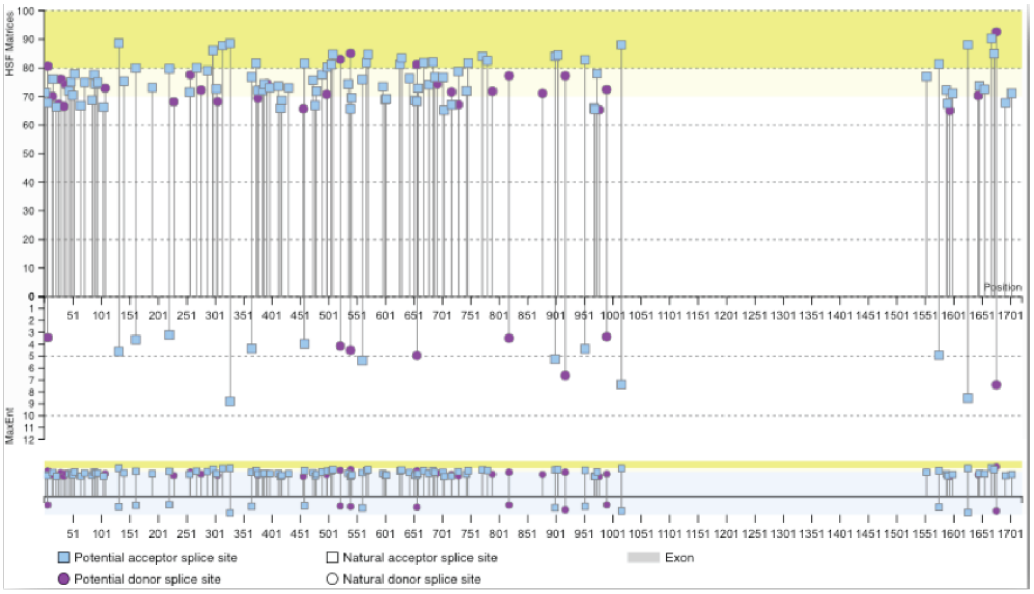
A)


HSF Matrices
MaxEnt
Position
Potential acceptor splice site
Natural acceptor splice site
Exon
Potential donor splice site
Natural donor splice site

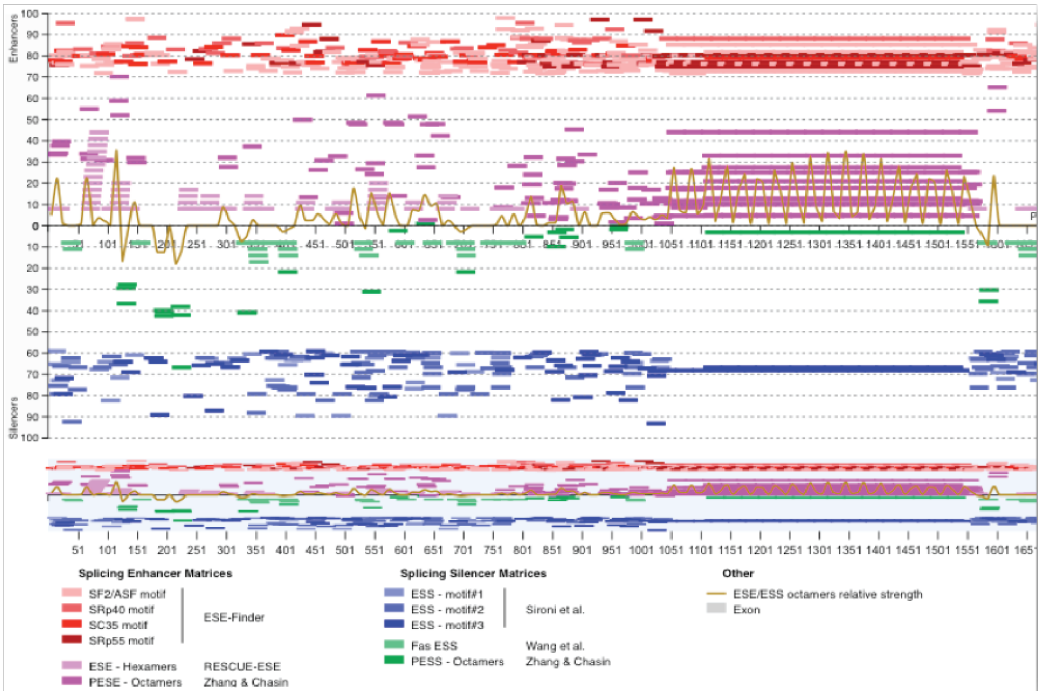
B)


Enhancers
Silencers
Splicing Enhancer Matrices
SF2/ASF motif
SRp40 motif
SC35 motif
SRp55 motif
ESE-Finder
ESE - Hexamers
PESE - Octamers
RESCUE-ESE
Zhang & Chasin
Splicing Silencer Matrices
ESS - motif#1
ESS - motif#2
ESS - motif#3
Sironi et al.
Fas ESS
PESS - Octamers
Wang et al.
Zhang & Chasin
Other
ESE/ESS octamers relative strength
Exon

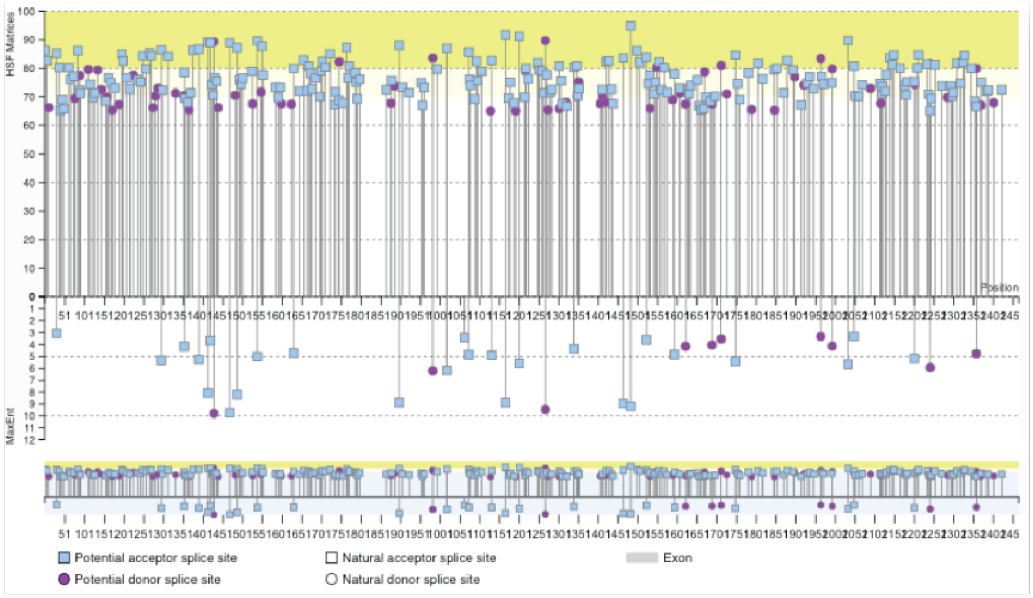
C)


HSF Matrices
MaxEnt
Position
Potential acceptor splice site
Natural acceptor splice site
Exon
Potential donor splice site
Natural donor splice site

D)


Enhancers
Silencers
Splicing Enhancer Matrices
SF2/ASF motif
SRp40 motif
SC35 motif
SRp55 motif
ESE-Finder
ESE - Hexamers
PESE - Octamers
RESCUE-ESE
Zhang & Chasin
Splicing Silencer Matrices
ESS - motif#1
ESS - motif#2
ESS - motif#3
Sironi et al.
Fas ESS
PESS - Octamers
Wang et al.
Zhang & Chasin
Other
ESE/ESS octamers relative strength
Exon

E)



F)

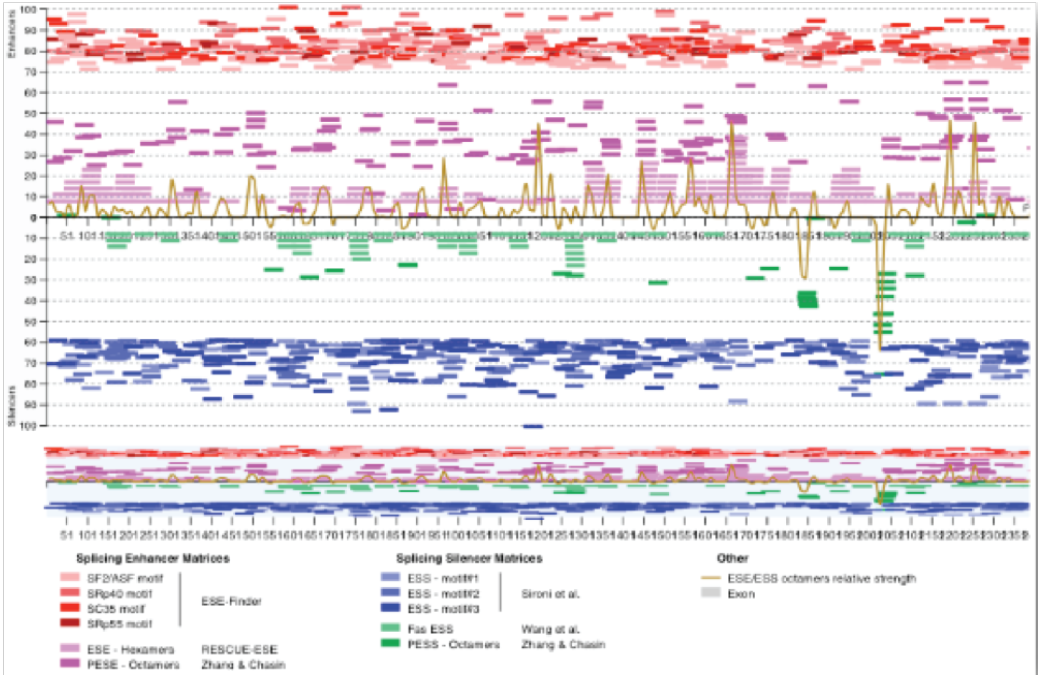


Figure 3.15. Visualization of the location of TERRA potential splices sites and splicing enhancer/silencer motifs in the Chr 2p, 10q Chr and Xq/Yq model sequences. Light blue squares represent recipient sites, Purple circles represent donor areas.
A) Splicing sites between 1 and 1800 bp of the Chr2p model. Strong intensity begins at 551 nucleotides.
B) The figure shows a panel where silencer sites (red and pink) and enhancer motifs site (panel below with blue and green) and both Brown graph were found in silico on the chromosome models. Chr2p (GenBank database entry NT_005334.17.)
C) Splicing sites are located between 1 and 1700 bp in Chr10q. (GenBank database entry M57753.1.). The splicing density continues to from the 51 to 1001 nucleotide.
D) In here, the non-splicing regions appear as a straight line, so there are degenerated arrays that are not the opposite of the exons sequences in Chr10q.
E) Picture shows high splicing density between positions 1 and 2401 bp of the Xq/Yq model (GenBank database entry M57752.1).
F) Auxiliary splicing sequences or the splicing site of the silencer and enhancer show an equal splicing density in Xq/Yq model.

3.10. In silico analysis for primer design for specific TERRA transcripts

To determine whether there are experimentially validated specific primers for detection of subtelomeric parts of TERRA from Chr2p, ChrXq/Yq and Chr10q, I first performed in silico analysis of the sub-telomeric primer pairs for TERRA in Chr2p, ChrXq/Yq, Chr10q which were chosen from previous studies (**Neijc Diploma thesis, MUW 2015**) . The primers which are shown in **table 3.3** were analyzed by Clone Manager software and as a result there was no specific primers for Chr2p, ChrXq/Yq, Chr10q of TERRA. However, new appropriate primers may be designed by taking these primer pairs as an example in future studies.

Table 3.3. Post evalution of PCR specific primers for detection of TERRA from chromosome ends from data of a previous study (Neijc Diploma thesis, MUW 2015)

Assay	Primer pairs	DNA Expected product length/ c.Manager	PN-primer Blast	Gel number/ figure	Cell line	gDNA(bps)
PCR1	1_Chr2p_Pos1_fwd 1_Chr2p_Pos303_rev	303bps	303bps	9.7	SW480	400.500/300-200
PCR2	2_Chr2p_Pos 255_fwd 2_Chr2p_Pos736_rev	482bps	482bps	9.7	SW480	1031-700/30(
PCR3	3_Chr2p_Pos 722_fwd 3_Chr2p_Pos 1370_rev	649bps	649bps	9.8	SW480	500-300/100-8(
PCR4	4_Chr2p_Pos1351_fwd 4_Chr2p_Pos1783_rev	433bps	433bps	9.8	SW480	500-300
PCR1	3_Chr10q_Pos44_fwd 3_Chr10q_Pos166_rev	123bps	123bps	9.8	SW480	123bps
PCR2	4_Chr10q_Pos133_fwd 4_Chr10q_Pos393_rev	261bps	520bps	9.8	SW480	261bps
PCR3	1_Chr10q_Pos330_fwd 1_Chr10q_Pos1284_rev	520bps	261bps	9.8	SW480	520bps
PCR4	2_Chr10q_Pos1174_fwd 2_Chr10q_Pos1705_rev	532bps	532bps	9.9	SW480	many band
PCR1	1_ChrXq_Yq_Pos8_fwd 1_ChrXq_Yq_Pos455_rev	448bps	448bps	9,9	SW480,	No bande
PCR2	2_ChrXq_Yq_Pos434_fwd 2_ChrXq_Yq_Pos1147_rev	714bps	714bps	9,9	SW480	400-500
PCR3	3_ChrXq_Yq_Pos1041_fwd 3_ChrXq_Yq_Pos1503_rev	463bps	463bps	9.9/9.10	SW480	>500
PCR4	4_ChrXq_Yq_Pos1390_fwd 4_ChrXq_Yq_Pos2235_rev	846bps	846bps	9.9/9.10	SW480	NO bande

In addition, in silico analysis of 61-29-37 repeats transcription starting sites were evaluated for 3 chromosomal models for TERRA splicing. **Figure 3.16** shows the length and location of the 61 (blue box), 29 (red box) and 37 (black box) repeats in the gDNA. The total length of the Chr 2p model is 1800 nts, of the Chr 10q model 1721 nts and of the Chr Xq/Yq model 2381 nts. The sequences were obtained online from the database entry NT_005334.17 (Chr 2p; 61 bp repeats span position 1779 to 1487, 29 bp repeats span position 1132 to 872, 37 bp repeats span position 1242 to 706) M57753.1 (Chr 10q; 61 bp repeats span position 408 to 764, 29 bp repeats

span position 1037 to 1555, 37 bp repeats span position 1159 to 1789) and M57752.1 (Chr Xq/Yq; 61 bp repeats span position 2110 to 2410, 29 bp repeats span position 2624 to 2884, 37 bp repeats span position 2888 to 3117). These results showed that the designed primers were no specific for 61-29-37 bp repeats in subtelomeres of Chr2p, ChrXq/Yq, Chr10q of TERRA.

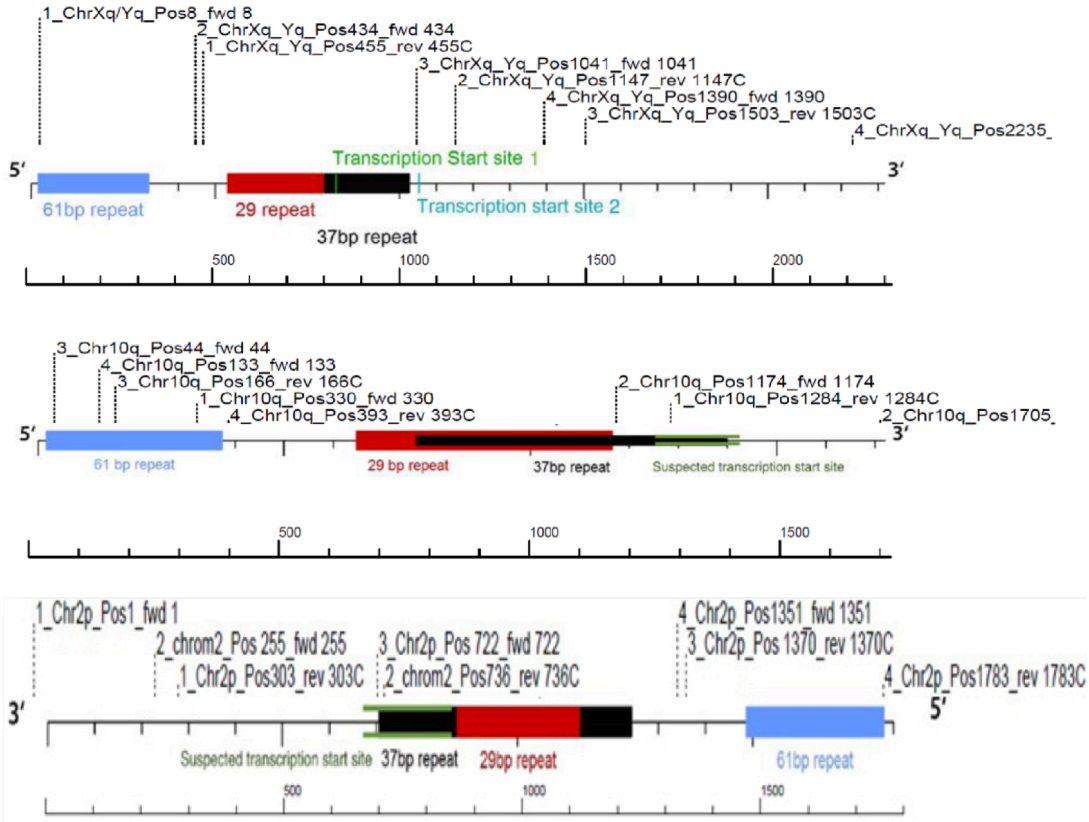


Figure 3.16. Visualization of gDNA model length, 61-29-37 repeats and transcription start sites. In silico analysis performed earlier, 61-29-37 repeats transcription starting sites are shown. The length and location of the 61 (blue box), 29 (red box) and 37 (black box) repeats in the gDNA. The total length of the Chr 2p model is 1800 nts, of the Chr 10q model 1721 nts and of the Chr Xq/Yq model 2381nts. The database entry NT_005334.17, M57753.1 and M57752.1 (Chr Xq/Yq). The pictures were obtained from Clone manager.

CHAPTER 4.

DISCUSSION

Normal RNA transcripts undergo post-transcriptional processing steps such as splicing and polyadenylation but some lncRNAs show alternative splicing forms of processing that distinguish them from other transcripts **(Quinn JJ , Chang HY 2016)** such as circular RNAs (circRNAs), small nucleolar RNA (snoRNA) and tRNA-like structures. MALAT1 (metastasis-associated lung adenocarcinoma transcript 1) and NEAT1 (nuclear enriched abundant transcript 1) can be given as example of alternative splicing of long non-coding RNAs **(Quinn JJ , Chang HY 2016).** These lncRNAs are processed from 3′end resulting with mature tRNA-like small lncRNAs **(Quinn JJ , Chang HY 2016).** Long non-coding RNAs (lncRNAs) has been reported in cancer as they undergo changes during the cancer development. For example, selected eight lncRNAs signatures were significantly associated with overall survival of hepatocellular carcinoma (HCC) patients **(Shi YM et al., 2018).** Splicing of hTERT has been demonstrated in human cells lines and might have a role in regulation of telomerase function and splicing patterns in TERT in a tissue- and cell type-specific manner. Regulation of hTERT alternative splicing showed a link with telomere length **(Bollmann FM, 2013).**

In this study; to investigate whether any evidences for processing of TERRA transcripts as performed in previous work and to try to answer to the question whether there is a process described in literature for non-coding RNAs and TERRA processed by splicing. I first validated and characterized adenoviral (AV) and lentiviral (LV) vectors containing the telomeric part of TERRA. Next, I studied with recombinant AVs the endogenous and exogenous expression from the telomeric part of TERRA. Further, I carefully searched the literature to answer the question whether there is splicing observed or a suitable vector system to study the splicing process

for non-coding RNAs, such as TERRA, similar to splicing in coding genes. I used molecular biology tools, such as transformation, selection with antibiotics, restriction enzyme digest, gel electrophorese and in silico sequence analyses and other techniques.

Although TERRA transcripts have a function on telomerase activity and on telomere stability and its function, thebiological functions of TERRA are still uncertain **(Oliva-Rico D , 2017)**. My work mainly focused on the validation of TERRA reporter expression systems for detection of RNA processing such as splicing. I validated AV and LV plasmid constructs for transient and stable expression of telomeric part of TERRA. I studied expression in human HEK 293 cells of recombinant TERRA transcripts and compared it with the expression of endogenous TERRA transcript levels.

The AV plasmid vectors pAD/CMW/V5-Tel region and pAD/PL/-Telregion, pAd/CMV-Terra-s were successfully validated by restriction enzyme analyses. I strongly claim that these vectors could provide a useful model system for TERRA expression studies in the future.

The LV plasmid vectors pLenti4/V5/CMV-Tel-Region, pLenti4/V5/CMV-Tel-Region-antisense, pLenti6/Ubc-antisense were successfully validated by restriction enzyme analysis. But weak band from weak band of pLenti6/UbC/V5-Tel-Region-antisense was observed. This vector may not be effective to study the molecular function of TERRA. On the other hand, 3 plenti vectors were analysed and one of them pLenti6/Ubc-sense did not show the same results with Clone Manager software. This vector could be eliminated from future experiments. Additionally, pLenti6/Ubc antisense with EcoRV, pLenti6/UbC/V5-Tel-Region-sense plasmid with SacI and pLenti6/UbC/V5-Tel-Region-antisense with SacI enzyme confirmed positive results

with that of clone manager. The successfully validated vectors could be effective to investigate TERRA in human cells.

To primary study functional expression of telomeric part of TERRA, HEK 293 cell lines were transfected with various AV vectors with CMV and hH1 promoter. HEK 293 cells with AVs were analysed after 2 days by qPCR for exogeneous TERRA transcripts. pAD/CMV/V5-Terra-sense and pAD/PL-Terra-sense are positive vectors for the expression of TERRA transcripts when specific primer pairs used such as pAD/PL/-terra sense with exoTerra1-5 and exoTerrafwd-2rev, pAD/CMV/V5-Terra-sense with exoTerra 2-3 and totTerra-tel1+2b primers. pAD/CMV/V5-Terra-sense and pAD/PL-Terra-sense vector could further be validated using other human cell models. Addition to adenoviral vectors, lentiviral vectors pLenti6/UbC sense and pLenti6/UbC antisense were correctly validated in compatible with clone manager analysis. Future studies should focus on these two adenoviral vectors to establish effective models for TERRA expression analysis, especially for analyses of post transcriptional processes, such as splicing.

Although it is very difficult to design primers for TERRA detection, due to the high similarity of DNA sequence at subtelomeres **(Riethman HA et al.,2005).** Therefore, accurate and specific primers to detect exogeneous TERRA transcripts from various chromosomal ends are still challenging but lingner has shown that TERRA is variable, cell type and species specific and also using Northern blot and RT-PCR analysis, they demonstrated that not only human telomeres but also subtelomeres are transcribed a fraction of TERRA molecules (**Azzalin CM, Lingner J 2008**). Such PCR assays may be usefull for the detection of TERRA splicing events.

I validated 3 DNA sequence models for chromosomal end 2p, 10q and Xq/Yq which might contain splicing sites/motifs. I used models of 2p, 10q and Xq/Yq and predicted in-silico various number of potential splicing sites. The model Xq/Yq showed high density of possible splicings within the subtelomeric part of TERRA. The results demonstrate that splicing of TERRA transcripts is generally possible and needs now to be experimentally proven.

Supplements

Table S1. The list of plasmids with names deposited at Addgene for research provided from Klaus Holzmann Lab (www.addgene.com)

ID	Plasmid	Gene/Insert	Vector Type	Publication
111375	pLV CMV-TERRA	TERRA polyA (Homo sapiens)	Lentiviral	Telomere Transcripts Target Telomerase in Human Cancer Cells. Genes (Basel). 2016 Aug 16;7(8). pii: genes7080046. doi: 10.3390/genes7080046.
111380	pAV hTERTsh	shRNA C3 against hTERT (Homo sapiens)	Adenoviral	Telomere Transcripts Target Telomerase in Human Cancer Cells. Genes (Basel). 2016 Aug 16;7(8). pii: genes7080046. doi: 10.3390/genes7080046.
111381	pENTR hH1-TERRA	hH1-TERRA (Homo sapiens)	Gateway Entry Cloning	Telomere Transcripts Target Telomerase in Human Cancer Cells. Genes (Basel). 2016 Aug 16;7(8). pii: genes7080046. doi: 10.3390/genes7080046.
111383	pENTR TERRA	TERRA (Homo sapiens)	Gateway Entry Cloning	Telomere Transcripts Target Telomerase in Human Cancer Cells. Genes (Basel). 2016 Aug 16;7(8). pii: genes7080046. doi: 10.3390/genes7080046.
111385	pENTR U6-hTERTsh	shRNA C3 against hTERT (Homo sapiens)	Gateway Entry Cloning	Telomere Transcripts Target Telomerase in Human Cancer Cells. Genes (Basel). 2016 Aug 16;7(8). pii: genes7080046. doi: 10.3390/genes7080046.
111374	pAV hH1-ARRET	ARRET w/o polyA (Homo sapiens)	Adenoviral	telomeric repeat-containing RNA (TERRA and ARRET) plasmids (unpublished)
111378	pLV UbC6-ARRET	ARRET polyA (Homo sapiens)	Lentiviral	telomeric repeat-containing RNA (TERRA and ARRET) plasmids (unpublished)
111382	pENTR hH1-ARRET	hH1-ARRET (Homo sapiens)	Gateway Entry Cloning	telomeric repeat-containing RNA (TERRA and ARRET) plasmids (unpublished)
111384	pENTR ARRET	ARRET (Homo sapiens)	Gateway Entry Cloning	Telomere Transcripts Target Telomerase in Human Cancer Cells. Genes (Basel). 2016 Aug 16;7(8). pii: genes7080046. doi: 10.3390/genes7080046.

Table S2. Gene expression qPCR data as Relative Quantity (RQ) and Cycle Threshold (Ct) values for analyses samples with error (SD:standerd deviation, Min-Max: 95% confidence interval) of technical replicates (n=3)

Sample Name	Target Name	RQ	RQ Min	RQ Max	Cт	Cт Mean	Cт SD
E3-s-Terra	exoTerra1-5´	370789.25	307318.72	447368.34	16.96	16.82	0.16
E3-s-Terra	exoTerra1-5´	370789.25	307318.72	447368.34	16.65	16.82	0.16
E3-s-Terra	exoTerra1-5´	370789.25	307318.72	447368.34	16.86	16.82	0.16
E3-s-Terra	exoTerra2-3´	43575.78	38367.21	49491.44	15.40	15.41	0.10
E3-s-Terra	exoTerra2-3´	43575.78	38367.21	49491.44	15.51	15.41	0.10
E3-s-Terra	exoTerra2-3´	43575.78	38367.21	49491.44	15.30	15.41	0.10
E3-s-Terra	totTerra-tel1+2b	10.94	8.68	13.79	22.60	22.82	0.20
E3-s-Terra	totTerra-tel1+2b	10.94	8.68	13.79	23.01	22.82	0.20
E3-s-Terra	totTerra-tel1+2b	10.94	8.68	13.79	22.84	22.82	0.20
E3-s-Terra	36B4				20.49	20.48	0.05
E3-s-Terra	36B4				20.52	20.48	0.05
E3-s-Terra	36B4				20.43	20.48	0.05
G3-s-Terra	exoTerra1-5´	6.66	3.54	12.55	34.43	34.51	0.57
G3-s-Terra	exoTerra1-5´	6.66	3.54	12.55	35.11	34.51	0.57
G3-s-Terra	exoTerra1-5´	6.66	3.54	12.55	33.98	34.51	0.57
G3-s-Terra	exoTerra2-3´	143304.98	91136.54	225335.72	15.66	15.61	0.40
G3-s-Terra	exoTerra2-3´	143304.98	91136.54	225335.72	15.18	15.61	0.40
G3-s-Terra	exoTerra2-3´	143304.98	91136.54	225335.72	15.98	15.61	0.40
G3-s-Terra	totTerra-tel1+2b	32.35	0.43	2430.57	27.69	23.18	3.91
G3-s-Terra	totTerra-tel1+2b	32.35	0.43	2430.57	20.85	23.18	3.91
G3-s-Terra	totTerra-tel1+2b	32.35	0.43	2430.57	20.98	23.18	3.91
G3-s-Terra	36B4				22.56	22.50	0.06
G3-s-Terra	36B4				22.48	22.50	0.06
G3-s-Terra	36B4				22.45	22.50	0.06
as Terra	exoTerra1-5´	2.38	1.83	3.09	32.21	32.02	0.22
as Terra	exoTerra1-5´	2.38	1.83	3.09	31.78	32.02	0.22
as Terra	exoTerra1-5´	2.38	1.83	3.09	32.06	32.02	0.22
as Terra	exoTerra2-3´	27.33	23.64	31.59	23.89	23.99	0.10
as Terra	exoTerra2-3´	27.33	23.64	31.59	23.99	23.99	0.10
as Terra	exoTerra2-3´	27.33	23.64	31.59	24.08	23.99	0.10
as Terra	totTerra-tel1+2b	0.23	0.10	0.53	27.20	26.34	0.74
as Terra	totTerra-tel1+2b	0.23	0.10	0.53	25.90	26.34	0.74
as Terra	totTerra-tel1+2b	0.23	0.10	0.53	25.93	26.34	0.74
as Terra	36B4				18.42	18.31	0.09
as Terra	36B4				18.28	18.31	0.09
as Terra	36B4				18.25	18.31	0.09
unb-dm	exoTerra1-5´	0.40	0.05	3.12	36.95	35.08	1.80
unb-dm	exoTerra1-5´	0.40	0.05	3.12	34.92	35.08	1.80
unb-dm	exoTerra1-5´	0.40	0.05	3.12	33.36	35.08	1.80
unb-dm	exoTerra2-3´	0.98	0.59	1.63	29.49	29.28	0.20
unb-dm	exoTerra2-3´	0.98	0.59	1.63	29.26	29.28	0.20
unb-dm	exoTerra2-3´	0.98	0.59	1.63	29.09	29.28	0.20
unb-dm	totTerra-tel1+2b	1.37	0.84	2.25	24.44	24.26	0.17
unb-dm	totTerra-tel1+2b	1.37	0.84	2.25	24.24	24.26	0.17
unb-dm	totTerra-tel1+2b	1.37	0.84	2.25	24.09	24.26	0.17
unb-dm	36B4				18.70	18.83	0.43

unb-dm	36B4				19.31	18.83	0.43
unb-dm	36B4				18.49	18.83	0.43

Table S2. continued

Sample Name	Target Name	RQ	RQ Min	RQ Max	Cт	Cт Mean	Cт SD
gfp-av	exoTerra1-5´	1.00	0.73	1.38	33.68	33.71	0.28
gfp-av	exoTerra1-5´	1.00	0.73	1.38	33.45	33.71	0.28
gfp-av	exoTerra2-3´	1.00	0.67	1.49	29.59	29.21	0.35
gfp-av	exoTerra2-3´	1.00	0.67	1.49	29.15	29.21	0.35
gfp-av	exoTerra2-3´	1.00	0.67	1.49	28.88	29.21	0.35
gfp-av	totTerra-tel1+2b	1.00	0.90	1.11	24.70	24.67	0.09
gfp-av	totTerra-tel1+2b	1.00	0.90	1.11	24.58	24.67	0.09
gfp-av	totTerra-tel1+2b	1.00	0.90	1.11	24.74	24.67	0.09
gfp-av	36B4				18.84	18.79	0.05
gfp-av	36B4				18.77	18.79	0.05
gfp-av	36B4				18.74	18.79	0.05
gfp-av	eGFP	1.00	0.18	5.62	24.61	22.81	1.55
gfp-av	eGFP	1.00	0.18	5.62	21.92	22.81	1.55
gfp-av	eGFP	1.00	0.18	5.62	21.92	22.81	1.55
E3-s-Terra	exoTerra1fwd+2rev	11.67	1.24	109.98	37.12	34.83	2.02
G3-s-Terra	exoTerra1fwd+2rev				ud		
as Terra	exoTerra1fwd+2rev				ud		
unb-dm	exoTerra1fwd+2rev				ud		
gfp-av	exoTerra1fwd+2rev	1.00			ud	36.76	
	exoTerra1-5´				33.25		
	exoTerra2-3´				30.15		
	totTerra-tel1+2b				31.05		
E3-s-Terra	exoTerra1fwd+2rev	11.67	1.24	109.98	34.04	34.83	2.02
G3-s-Terra	exoTerra1fwd+2rev				ud		
as Terra	exoTerra1fwd+2rev				ud		
unb-dm	exoTerra1fwd+2rev				ud		
gfp-av	exoTerra1fwd+2rev	1.00			36.76	36.76	
	36B4				32.06		
	eGFP				36.87		
	exoTerra1fwd+2rev				ud		
E3-s-Terra	exoTerra1fwd+2rev	11.67	1.24	109.98	33.32	34.83	2.02
G3-s-Terra	exoTerra1fwd+2rev				ud		
as Terra	exoTerra1fwd+2rev				ud		
unb-dm	exoTerra1fwd+2rev				ud		
gfp-av	exoTerra1fwd+2rev	1.00			ud	36.76	

ud, undetermind;

Table S3. Plasmids used in this study: pAd means Adenoviralvectors, Kan = Kanamycin, Amp = Ampicilin **(Christian Stern (2011).**

Plasmid:	**Origin/Construction:**	**Resistance:**
pENTR/D/hH1 Sense Tel	Insert of telomere fragment in sense orientation (5.1.3.2.)	Kan
pENTR/D/hH1 Antisense Tel	Insert of telomere fragment in antisense orientation (5.1.3.1.)	Kan
pENTR/D/Sense Tel hH1	Elimination of hH1 promoter of pENTR/D/hH1 Sense Tel (5.1.4.1.1.)	Kan
pENTR/D/Antisense Tel hH1	Elimination of hH1 promoter of pENTR/D/hH1 Antisense Tel (5.1.4.1.2.)	Kan
pAd/PL-DEST™ Gateway® Vector	Invitrogen, #V494-20. adenoviral promoterless gateway vector	Amp
pAd/pl Sense Tel	Gateway reaction of pENTR/D/hH1 Sense with pAd/pl (5.1.5.1.2.)	Amp
pAd/pl Antisense Tel	Gateway reaction of pENTR/D/hH1 Antisense with pAd/pl (5.1.5.1.2.)	Amp
pAd/CMV/V5-DEST™ Gateway® Vector	Invitrogen, #V493-20. adenoviral gateway vector with CMV promoter	Amp
pAd/CMV Sense –hH1 Tel	Gateway reaction of pENTR/D/Sense ΔhH1 with pAd/CMV (5.1.5.1.2.)	Amp
pLenti6/UbC/V5-DEST	Lentiviral expression vector with UbC promoter from Invitrogen (#V499-10)	Amp

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