Identification and characterization of Carboxylesterase variants

Master thesis for the degree Master of Pharmacy

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Preface

This thesis is made as a completion of the master education in Pharmacy and it took place at the Department of Pharmacology, Oslo University Hospital, Ullevål during the period of August 2015 to April 2016.

First of all, I would like to express my deep gratitude to my external supervisors Marianne K. Kringen and Kari Bente Foss Haug for all the guidance and support through the entire study. I have learnt so much and I am truely insipired. I would also like to thank Hege Gilbø Bakke for helping me with all the practical problems I had, and for her kindness and patience. Furthermore I would like to thank all the colleagues at the department for their help and for a delightful and warm working atomsphere. I am grateful to my internal supervisor Hege Thoresen for following the progress of my study and for her constructive comments on the thesis.

My thanks also go to the Class 2016 and my friends for all the support and for joyful memories through the years of my education.

Finally I would like to thank my parents for all the love and encouragement, I would have never completed my education without your support.

Ullevål, April 2016
Kathleen Nanding

Abstract

Alternative splicing is a naturally occuring process where different protein isoforms could be encoded from the same pre-mRNA. A single pre-mRNA could have a combination of multiple patterns of mechanisms such as exon skipping, intron retention and alternative splice site selection. Alternative splicing has contributed to protein diversity and could influence their biological functions in different aspects.

Carboxylesterases 1(CES1) and carboxylesterase 2(CES2) are members of carboxylesterase (CEs) family and are mainly expressed in liver and small intestine tissues. They are involved in drug metabolism, drug activation and other biological processes. Genetic variations of the *CES1* and *CES2* gene have shown to affect the drug metabolism in different drugs.

This study has focused on identification and characterization of carboxylesterase variants and was performed in three parts.

In this study, different methods and bioinformatics tools were used for detection, sequencing and analysing of splicing variants in CES1 and CES2. One new alternative splicing variant was found in the *CES1* gene with deletion of exon 12, exon 13 and most part of exon 11 (135bp). The deletion of exons has lead to missing of one active site and one mutagenesis site in the *CES1* gene. Alternative splicing variant that were known has also been detected, one variant in CES1 with deletion of exon 7 and one variant in CES2 with deletion of exon 2 and 3.

The expression level of alternative splicing variants of CES2 was studied with reverse transcriptase-quantitative PCR. The alternative splicing variant has shown highest expression in fetal brain tissue, where the original splicing variant has the highest expression level in liver tissue.

A pilot study for enzyme activity of cells with- and without insertion of alternative splicing variant of CES1 was performed where the original plan was to study the enzyme activity of both original- and alternative splicing variant of CES1 and CES2. It is difficult to make valuable conclusion based on the results.

Abbreviations:

4-MUBA	4-methylumbelliferyl acetate
A	Adenine
A-site	Acitivity site
AML	Acute myeloid leukemia
AraC	Cytarabine
Asn	Asparagine
Bcl-xl	B-cell lymphoma-extra large
bp	Base pair
C	Cytosine
C-terminus	Carboxyl-terminus
cDNA	Complementary DNA
CE	Carboxylesterase
CES2_EX2_3SPL	cDNA fragment with deletion of exon 2 and 3
CES2_EX8_SPL	cDNA fragment with deletion of exon 8
COX	Cyclooxygenase
Ct	Threshold cycle
Cys	Cysteine
dCK	Deoxycytidine kinase
DNA	Deoxyribonuclei acid
dNTP	Nucleoside triphosphate
dUTP	Deoxyruridine triphosphate
EDTA	Ethylenediaminetetraacetic acid
EMEM	Eagle's Minimum Essential Medium
ER	Endoplasmic reticulum
ESE	Exonic splicing enhancer
ESS	Exonic splicing suppressor
FAM	6-carboxyfluorescein
G	Guanine
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Glu	Glutamic acid
HEK	Human embryonic kidney

His	Histidine
ISE	Intronic splicing enhancer
ISS	Intronic splicing suppressor
kb	Kilo base-pair
LacZ	β-galactosidase
LB	Lysogeny broth
Leu	Leucine
mM	Milli molar concentration
mRNA	Mature RNA
N-terminus	Amino-terminus
ng	Nanogram
NKCC2	Sodium-potassium-chloride transporter 2
NMD	Nonsense-mediated decay
NSAIDs	Nonsteroid anti-inflammatory drug
PCR	Polymerase chain reaction
PGK1	Phosphoglycerate kinase 1
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
RT-qPCR	Reverse transcriptase - quantitative PCR
snRNP	Small nuclear ribonucleoprotein particle
SOC	Super optimal broth with Catabolite repression
SRE	Splicing regulatory elements
ss	Splice site
T	Thymidine
TBE	Tris/Borate/EDTA
UV	Ultraviolet
x-GAL	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
µl	Microliter
µM	Micro molar concentration

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1 Introduction

1.1 RNA splicing.

For most eukaryotic genes, the initial RNA that is transcribed from a gene's DNA template has to be processed to become a mature messenger RNA (mRNA) that can synthesis protein (1). RNA splicing that edits initial RNA and form mature mRNA is one of the important steps involved in this process. The initial RNA is known as pre-mRNA which is a primary transcript and functions as precursor of an mRNA(2). Pre-mRNAs are usually built up of alternative segments of introns and exons, where introns are noncoding segments and exons are coding segments that will be retained in mRNA and eventually be expressed (3). Both ends of a pre-mRNA are modified during or after transcription. The modification at the 5´end of the pre-mRNA, is known as the 5´cap which is modified form of a guanine nucleotide. The 3´ end is modified by an enzyme that adds 50 to 250 adenines and thereby forming a poly-A tail (3).

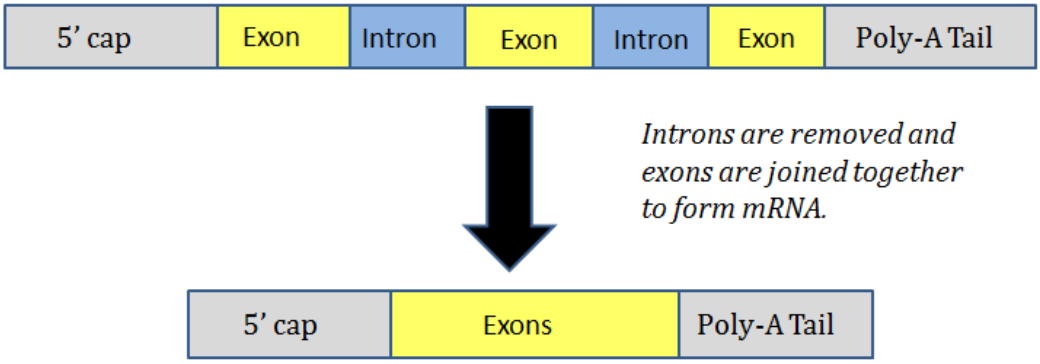


Figure 1.1A: The RNA splicing (4). During RNA splicing, the introns are removed from pre-mRNA and exons are thereby joined together to form mRNA that will be further translated into proteins (3).

However, the number of proteins synthesized is not necessarily equivalent to the number of genes involved (2). The same pre-mRNA can generate different proteins as variations in the incorporation of coding regions can lead to more than one mRNA transcripts. The mechanism behind this phenomenon is known as alternative splicing(5).

1.2 Alternative splicing

Alternative splicing determines which coding segments are included in the mRNA and thereby generating protein isoforms that differ in their peptide sequence(6). The generation of different protein isoforms from the same pre-mRNA expands protein diversity by generating proteins with subtle or opposing functional differences(2, 7). Alternative splicing is a common process in cells as it is estimated to be involved in the expression of minimum 60% of human genes (6).

1.2.1 Spliceosome and splicing regulatory elements.

Most exons in pre-mRNA are always included in the mRNA, and they are known as constitutive exons. The other exons which can be either included or excluded are called cassette exons (6). The decisions of which exons are included depend on splicing regulatory elements (SREs) (8). The SREs regulate splicing by recruiting splicing factors which are proteins of spliceosome(8). The spliceosome is a dynamic complex of five small nuclear ribonucleoprotein particles (snRNPs) and numerous proteins cooperate to recognize the splice sites and catalyze the splicing reaction(7). SREs are classified based on their location and activity: exonic splicing enhancers (ESEs), exonic splicing silencers (ESSs), intronic splicing enhancers (ISEs) and intronic splicing silencers (ISSs)(8). ESEs enhance splicing by recruiting members of the SR protein family, and ESSs are often bound by splicing repressors (9). ISEs are often found as GGG sequences that enhance recognition of adjacent 5'splice sites (5'ss) or 3'splice sites (3'ss), and ISSs contain binding sites for the splicing repressors (9).

1.2.2 Patterns of alternative splicing.

Many different patterns of alternative splicing are found, it is common for a single pre-mRNA to use a combination of multiple patterns to generate different mRNA (6).
Exon skipping where one or more cassette exons are spliced out together with introns is the most prevalent splicing pattern in human genes (5). The cassette exon can either be spliced out as a single exon or as part of mutually exclusive exons where only one exon of several adjacent cassette exons is included in mRNA at a time (Figure 1.2.2A,B) (6). Exon skipping usually happens when there are two or more introns in the pre-mRNA as all the splice sites are similar and it is therefore possible to join the wrong splice sites(10). The alteration of length of exons is also a common pattern of alternative splicing. Exons can be lengthened or

shortened due to recognition of two or more splice sites at one end of an exon(5, 6). It can happen either at the 5'end of the exon (alternative 5'ss selection which causing modification at the 3' site of the upstream exon, as shown in Figure 1.2.2C), or at the 3' end of the exon (alternative 3'ss selection causing modification at the 5' site of the downstream exon, (as shown in Figure 1.2.2D)(5, 11). Intron retention where one intron is remained in mRNA is the least prevalent pattern in human genes (Figure 1.2.2G) (5, 11). The intron retention is believed to be caused by intron, not exon (5). It can also be related with factors such as weaker splice sites of introns, shorter intron lengths, higher expression levels and lower density of both a set of ESSs and the ISEs (12).

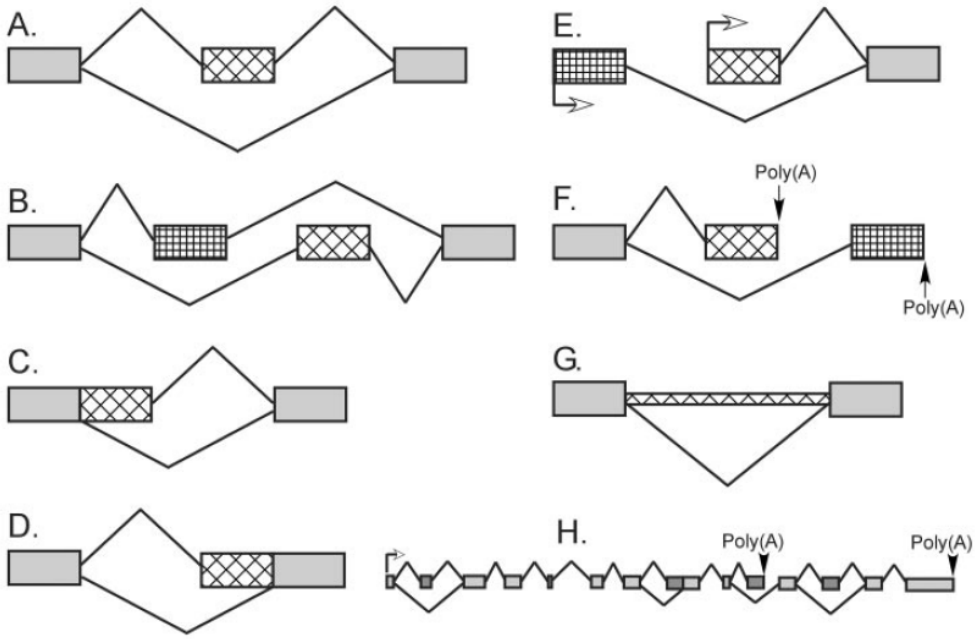


Figure 1.2.2: Overview of different patterns of alternative splicing (6). Grey boxes represent constitutive exons, and hatched boxes represent cassette exons or remained intron. A) Exon skipping where one cassette exon can either be included or excluded. B) Exon skipping with mutually exclusive exons where only one exon of the several adjacent exons is included in mRNA at a time. C) 5'ss selection. D) 3'ss selection. E) Alternative promotors switch the 5' most exons of a transcript. F) Alternative poly (A) sites switch the 3' most exons of a transcript. G) Intron retention. H) A single mRNA with multiple alternative splicing patterns(6).

1.2.3 Biological influences of alternative splicing.

Alternative splicing give rise to different mRNAs and the subsequent proteins encoded from the mRNA can be changed as a result. The changes in proteins can affect almost all aspects of protein functions and the effects can range from completely loss of function to effects that are difficult to detect(13). The changes of protein can be categorized into three types: introduction of stop codon; alteration of the protein structure; and changes in the untranslated region(13).

Introduction of stop codons is one of the changes that have the most obvious biological influences(13). Nonsense-mediated decay (NMD) is a pathway that helps to degrade mRNAs with premature stop codons. However the NMD is not able to perform its function if translation ends less than 50-55 nucleotides upstream of the 3´-most exon-exon junction or downstream of the junction(14). Therefore introduction of stop codon would generally switch off about 18-25% of the mRNA transcripts(15).

Most of the alternative splicing lead to changes in primary structure of the proteins and can thereby cause biological influences in different properties of the protein such as binding property, intracellular localization, enzymatic activity and stability(13).
Binding properties of protein isoforms can vary in large extent. For binding between proteins and small ligands, the structural change in proteins caused by alternative splicing can either delete binding domains or abolish the binding activity (13). For binding between proteins, alternative splicing can lead to creation or deletion of binding domains or disruption of binding domains by insertion of protein sequences. Thereby alternative splicing frequently modulates the binding affinity between the proteins (13). Alternative splicing can also cause loss of binding affinity between protein and nucleic acids (DNA and RNA) (16, 17).

The intracellular localization of the proteins can be determined by alternative splicing by either regulating the interaction between proteins and membranes or by influencing localization signals. For example, alternative splicing can generate non-membrane-bound protein isoforms by deleting or interrupting transmembrane domains. These proteins will be released into the blood or extracellular space or into a different intracellular compartment where the proteins either perform no function, become less stable or perform a different effect on immune system modulation(13, 18-20). Alternative splicing can determine the intracellular localization of the proteins by regulating their retention in specific organelles and produce protein isoforms that are tissue-specific(13). For example, B-cell antigens that usually expresses in the plasma membrane will accumulate inside endoplasmic reticulum if their extracellular domains are deleted by alternative splicing (13). Alternative splicing can also determine the tissue specificity of the proteins by regulating the nuclear localization and function of transcription factors of the protein. For example, protein casein kinase I can either target to the nucleus or in the cytoplasma depending on whether their protein isoforms contain a nuclear localization signal or not (21). Alternative splicing has also shown to

regulate the sublocalisation of proteins. Protein isoforms of estrogen receptor can present in nucleus, cytoplasm or both depending on which exons are present(22).

Several studies have shown that alternative splicing can alter the enzyme activity of proteins in different aspects such as affinity, substrate specificity, catalytic properties and activity regulation(13). Alternative splicing can lead to structural changes that affect protein stability as the inclusion of alternate protein domains can regulate the half-life of proteins(13). Some protein isoforms produced by alternative splicing can have much longer half-life than the original splicing variant and are thereby more stable(23). Alternative splicing can also lead to posttranslational modifications such as glycosylation, palmitoylation or sulfation in proteins(13). The permeability of ion channels in proteins can be altered as a result of alternative splicing. The influence ranges from completely abolishment to reduced permeability depending on the type of the channel (24, 25).

Compare with alternative splicing in coding region of the RNA, the alternative splicing in non-coding region can lead to changes in regulatory elements, such as translation enhancers or RNA stability domains(26). Structural changes in the 5´or 3´´untranslated regions of the gene are less studied. However, it has been shown that stability of the RNA can be affected by alternative splicing(13). Indeed, a study with HIV-1 virus suggested that alternative splicing could alter the stability of RNA(27).

1.2.4 Alternative splicing in pharmacogenetics.

Since alternative splicing can alter protein functions in different aspects, it is reasonable to suggest that the mechanism has potential pharmacogenetics value which is the study of drug response in relation to specific genes(28). Studies have shown that alternative splicing can affect both the efficacy and safety of drugs by altering the drug metabolism and thereby causing different responds of patients to the same drug(26).

Alternative splicing has also shown to be responsible for developing of drug resistance. Cytarabine (AraC) is the most effective drug in the treatment of acute myeloid leukemia (AML), and phosphorylation of AraC which is catalyzed by deoxycytidine kinase (dCK) is an essential step for drug activation (29). The gene encoding human dCK contains seven exons, and four alternative splicing variants of the gene with deletion of either exon 5, exons

3 to 4, exons 3 to 6 or exons 2 to 6 were detected in samples from patients with clinically resistant AML. Since none of the alternatively spliced dCK isoforms were detected in either patients who were sensitive to AraC or in T cells from healthy donors. and the protein isoforms that are encoded by alternative spliced dCK mRNA are shown to be inactive in vitro (29), it is reasonable to suggest that alternative splicing of dCK is correlated with developing of drug resistance in the treatment of AML.

Additionally, the pharmacokinetic properties of drug are found to be altered due to alternative splicing. The protein sodium-potassium-chloride transporter 2 (NKCC2) targeted by the drug furosemide has three splice variants which are dramatically different in their kinetic behaviors(26). The variant F has much lower affinity for sodium and potassium than the other two variants, and the affinities for chloride are also remarkably different between these three variants (30).

The same drug can specifically target alternative splicing isoforms. Cyclooxygenase isozymes (COX) are the targets of nonsteroidal anti-inflammatory drugs (NSAIDs). COX-3 is an alternative variant with intron 1 retained in its mRNA that is selectively inhibited by analgesic drugs and potentially inhibited by some NSAIDs when compared with other variants (31).

Conversely, drugs could also influence the alternative splicing process of certain genes. Take anticancer drugs as an example, they were found to influence the production of Bcl-x isoforms(28). Programmed cell death (apoptosis) is a naturally occurred process that is involved in balancing between cellular proliferation and cell death. In many types of cancer, Bcl-x that is an apoptotic regulator is shown to be overexpressed in the antiapoptotic Bcl-x isoform (28). Many anticancer drugs can shift the RNA splicing of Bcl-x and favor the proapoptotic isoform of Bcl-x (28).

1.3 Human Carboxylesterase

1.3.1 Carboxylesterase family.

Carboxylesterase (CEs) are members of the esterase class of proteins that are identified in different species. CEs cleave carboxylic esters into the corresponding alcohol and carboxylic acid (32). The mammalian carboxylesterase (*CES* for human, *Ces* for mouse and rat) genes usually contain 12-14 exons of DNA encoding CES enzymes which may be shuffled during mRNA synthesis(33). The *CES* genes encode enzymes of at least five gene families: *CES*1, the major liver enzyme (hCE1) (34); *CES*2, the major intestinal enzyme (hCE2) (35); *CES*3, the enzyme that is expressed in brain, liver and colon (36); *CES*5A, the major urinary protein (37) and *CES6* (also called *CES4*A), which is a predicted CES-like enzyme in brain(38). The major human CES are classified into the CES1 and CES2 families(39).

1.3.2 CES1 and CES2.

CEs are localized in the endoplasmic reticulum (ER) of many tissues (39). CEs have a hydrophobic signal peptide at the N-terminus of the proteins which marks them for trafficking through ER. The C-terminus contains a His-X-Glu-Leu sequence which allows them to retain in the luminal site of the ER (39, 40). Four Cys residues are involved in disulfide bonds of CEs (40). Most CEs are glycoproteins, hCE2 has a glycosylation site at two different positions (Asn_{103} and Asn_{267}), while hCE1 only contains one site at Asn_{79} (40). hCE1 is build up with a central 15-stranded β-sheet surrounded by several α-helix and β-strands including a central catalytic domain (αβ-domain) and a regulatory domain. The activity site cavity (A-site) is in the interface of several protein domains. Z-site which is a surface ligand binding site locates in the central catalytic domain (Figure 1.3.2A) (39).

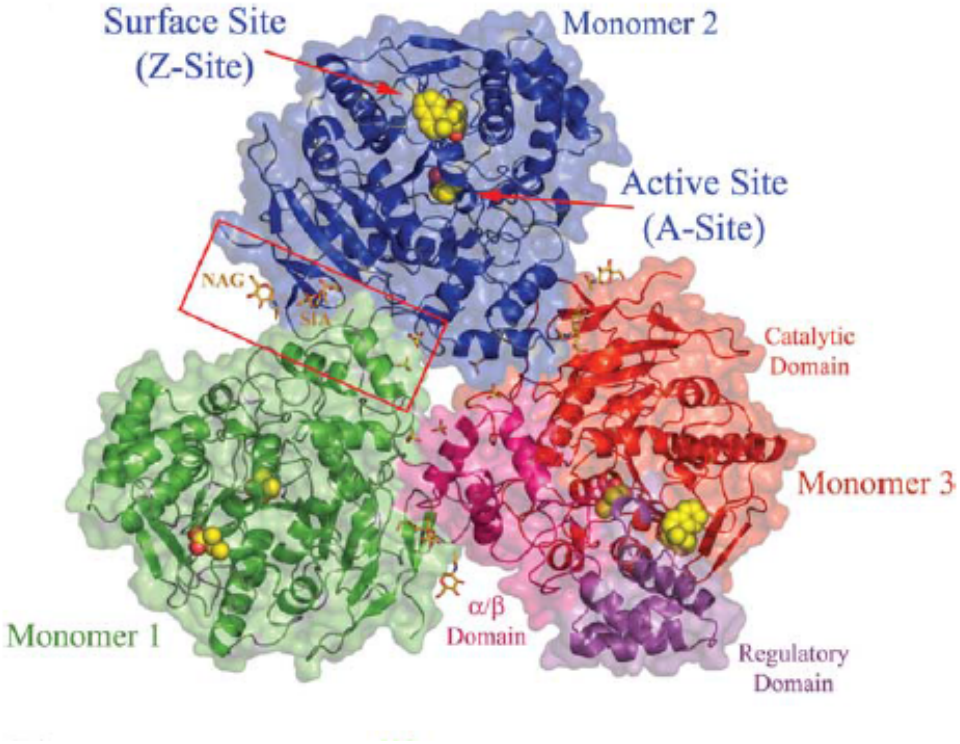


Figure 1.3.2A Trimetric structure of hCE1(41). Overall structure of hCE1 is in complex with mevastatin. Monomer 1 is in green, monomer 2 with A-site and Z-site is in blue and monomer 3 is the catalytic domain in red, separated by the regulatory domain in magenta and the α/β domain in pink (41).

The three dimensional structure of hCE2 has not been reported (39). Typically, hCE2 contains two glycosylation sites and hCE1 contain only one site. And the hCE1 has larger active site cavity which allows it to accommodate larger functional groups (39).
In general, the hCE1 favors substrates with relatively larger acyl group and small alcohol group, such as cocaine (methyl ester). Substrates such as heroin with relatively smaller acyl group and large alcohol group are more easily accepted by hCE2 (42).
hCE1 is mainly expressed in liver and also in lung, heart, testis and other tissues. hCE2 is expressed in small intestine, colon, kidney, liver, heart, brain and testis (39).

1.3.3 Biological roles of Carboxylesterase

CEs are essentially involved in drug metabolism, and also in drug activation, and other biological processes (43). CEs are able to cleave ester linkages in many clinically useful drugs, such as heroin, cocaine, mepridine, licodaine,etc (32). CEs are also involved in prodrug activation as they can hydrolyze prodrugs such as lovastatin to active metabolites (32, 43). The second biological role is involved in cholesterol metabolism (32). CEs,

especially hCE1 have a potential role to catalyze both the creation and the elimination of cholesteryl esters by transesterification and hydrolysis reactions depending on the cellular level of cholesteryl esters or free cholesterol (32, 43). It is also found that CEs is responsible for the trafficking and retention of proteins in ER (43), being able to bind and hold small proteins like C-reactive protein and release them into plasma due to the glycosylation sites when tissue injury occurs (43).

1.3.4 The pharmacogenetics of Carboxylesterase

Genetic variations of the *CES1* and *CES2* could lead to alterations in the catalytic functions of enzyme hCE1 and hCE2, thereby affect drug metabolism (44).

The antiplatelet drug clopidogrel is a part of dual therapy with aspirin for treatment and prevention of coronary heart diseases. One genetic variant of *CES1* showed better response on clopidogrel than the other variants (45). Alternative splicing could also affect response of hCE1 to imidapril which is a drug used for treating hypertension (46). Similarly, two genetic variants of *CES2* are functionally deficient and one genetic variant has shown low enzyme activity to irinotecan that is used in anticancer therapy (47).

1.4 Aim of the study

Human carboxylesterases are serine esterase involved in both drug and xenobiotic metabolism. Alternative splicing in genes *CES1* and *CES2* can cause functional alterations to enzymes that they encoded (hCE1 and hCE2) and thus lead to alterations in pharmacokinetics and drug responses.

This study is focused on the characterization of carboxylesterase variants in CES1 and CES2, caused by alternative splicing of the *CES1* and *CES2* genes.

Main objectives for this study are:

1.To identify alternative splicing variants in *CES1* and *CES2* genes

2. To determine the expression levels of identified alternative splicing variants in different tissues

3. To determine the enzyme activities of identified alternative splicing variants.

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals and Reagents

Chemicals and reagents used in this study are listed in Table 2.1.1A.

Table 2.1.1A Chemicals and reagents. Listed in alphabetical order.

Name	Supplier	Country
0,25% Trypsin-EDTA	Sigma	India
100mM 4-MuBA solution	Sigma	India
1kb Plus DNA ladder	Invitrogen	United States
Ampicillin 10ug/ul	Gibco LifeTech	United States
Canamycin 10ug/ul	Gibco LifeTech	United States
EMEM with L-Glutamin	ATCC®	United States
Gel loading solution 6x	Sigma	India
GelStar_Nucleic aicd gel stain	Cambrex Bio	Germany
Glyserol	Merck	United States
HEK 293 cells	ATCC®	United States
Isopropanol	Kemetyl Norge AS	Norway
LB-broth (medium)	Sigma	India
LB-Broth Agar	Sigma	India
OneShot®TOPO chemically component cell (exp. 01/2011)	Invitrogen	United States
OneShot®TOPO chemically component cell (exp. 06/2016)	Invitrogen	United States
Phosphate Buffer saline	Gibco LifeTech	United States
Seakem® LE Agarose	Lonza	Switzerland
SOC medium	Invitrogen	United States
TBE solution fo gel electrophoresis	AppliChem	Germany
Trypan Bluestain 0,4%	LifeTechnologies	United States
X-gal	LifeTechnologies	United States

Kits used in this study are listed in Table 2.1.1B.

Table 2.1.1B Kits used in this study. Listed in alphabetical order.

Name of Kit	**Supplier**	**Country**	**Cat.NO**
HotStar*Taq*®DNA Polymerase 1000 units	Qiagen	Germany	203205
Lipofectamine™3000 Trasfection Kit	Invitrogen by Life Science	United States	1576642
LongRange PCR Kit	Qiagen	Germany	206401/206402
pcDNA™*3.1*/*V5*-His TOPO™ *TA* Expression Kit	Invitrogen by Life Science	United States	
PCR Universal Mix	Applied Biosystems	United States	1505095
QI Aquick Gel Extraction Kit	Qiagen	Germany	28706
qScript cDNA Supermix	VWR	United States	20032
BamHI	New England Biolabs®Inc.	United States	R0136S
Xhol	Biolabs®Inc.	United States	RD1465
S.N.A.P.™ Plasmid DNA Midi Prep Kit	Termo Fisher	United States	K1901-01
S.N.A.P.™ Plasmid DNA MiniPrep Kit	Termo Fisher	United States	1626113
The original TA cloning kit	Invitrogen by Life Science	United States	
TOPO® XL PCR cloning Kit	Invitrogen by Life Science	United States	663284

2.1.2 Tools

Tools used in this study are listed in Table 2.1.2.

Table 2.1.2 Tools used in the study. Listed in alphabetical order.

Name	Supplier
1,5ml Biosphere® SafeSeal Tube	Sarstedt
1000µl Dualfilter	Eppendorf Reference
100µl Dualfilter	Eppendorf Reference
10ml Stripette disposable serological pipette	Corning Incorporated
10µl Dualfilter	Eppendorf Reference
15ml High-clarity Polypropylene cronical tube	Falcon® Corning Science
250 ml Erlenmeyer flask	Schott
25ml Stripette disposable serological pipette	Corning Incorporated
50 ml Polypropylene conial tube	Falcon® Corning Science
500 ml Erlenmeyer flask	Simax
50ml High-clarity Polypropylene cronical tube	Falcon® Corning Science
5ml Stripette disposable serological pipette	Corning Incorporated
96-wells Cell Culture Plate	Corning
Continental cooler -20^{o}C	ATLAS Storage for molecular biology
Countness™ cell counting chamber slides	Invitrogen
Gel comb /Gel tray	
Microplates	Lonza
Micropore surgical tape	3M
NeoTouch Prenium disposable Neoprene gloves	Ansell
Nunclon™surface -disposable for cell culture	Nunc Brand
Optically clear adhesive seal sheets	ThermoScientific
Pasteur pipette with cotton	VMR
Pipettboy	IBS IntegraBioscience
Pipette 0,5-10µl	Eppendorf Reference
Pipette 100-1000 µl	Eppendorf Reference
Pipette 10-100 µl	Eppendorf Reference

Pipette tips 10 µl	Eppendorf Reference
Pipette tips 100 µl	Eppendorf Reference
Pipette tips 1000 µl	Eppendorf Reference
Thermo-Fast 96 PCR detection plate	ThermoScientific

2.1.3 Instruments

Instruments used in this study are listed in Table 2.1.3.

Table 2.1.3 Instruments used in this study. Listed in alphabetical order.

Name	Supplier
Centrifuge 5430R	Eppendorf
Countness 11FL	Life technology
FireBoy eco	IBSM
Gene power supply GPS 200/400	Phamacia
HEPA filter	KOJAIR
Heracus Multifuge 3SR+ centrifuge	ThermoScientific
Horizon™ 11.14	BRL Bethesda Resources
Incubating Orbital Shakes	VMR
Incubator	Termaks
Incubator for cells	Grant Boekel
Integra Pipetboy	Intergra bioscience AG
Integra Vacuboy	IBS integra Bioscience
KOJAIR	Kojair Tech Oy, Finland
Mettler PM4800 DeltaRange	Mettler Toledo
Microscope 473012-2201	Zeiss West company
MiniSpin	Eppendorf
MS2 Minishaker	IKA®
NanoDrop	Fischer Scientific
SubAqua 25 Plus	Grant
UV Transilluminator	UVPinc
Vacusafe comfort	IBS integra Bioscience
Verti 96well Thermal cycle	AB Applied Biosystems Veriti
Victor² 1420 Multilabel Counter	Perkin Elmer Life science
ViiA7	Life technology

2.2 Polymerase chain reaction – PCR

PCR is an efficient way which enables rapid amplification of a specific segment of DNA (48). Essential components for a PCR assay are: a template DNA, a pair of primers, deoxy-nucleotides, DNA polymerase and Mg^{2+}. Primers are short strands of DNA which are complementary to the target DNA and serve as initiation point for DNA synthesis. They will allow DNA polymerase to link individual deoxy-nucleotides (adenine, thymine, cytosine and guanine) together and form the PCR product (48).

The PCR assay will reach sufficient amplification after 20 to 40 thermal cycles with each cycle usually contain three steps: 1. Denaturation step (95°C): temperature is raised above the melting point of the template DNA to separate the double-strand (3). 2.Annealing step (55-65°C): a cooling step to allow binding of the primers to the target DNA segment which is complementary (3). 3.Elongation step (72°C): raising temperature again allows extension of the primers by adding nucleotides to the 3' end of each primer (3). The variation in temperature in thermal cycles are summarized in Figure 2.2.

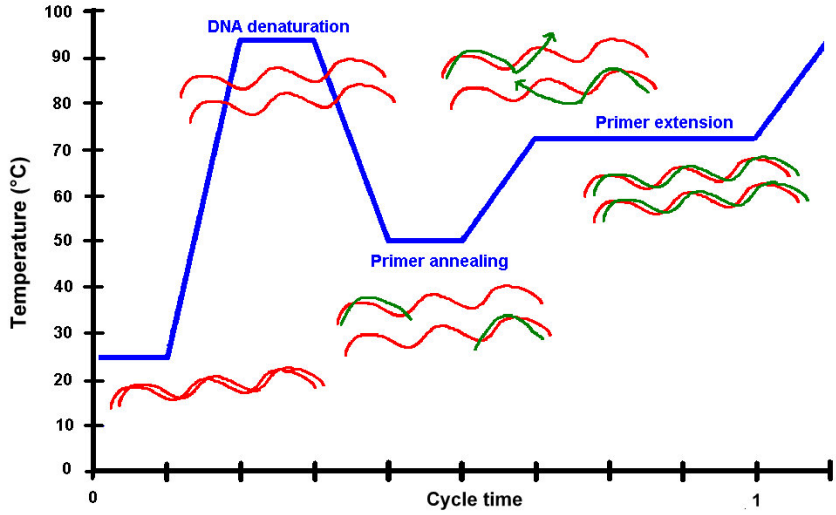


Figure 2.2: A summary of PCR thermal cycles (49) . During DNA denaturation the DNA double-strands are separated when temperature reaches the melting point, and each strand is bind by a complementary primer during annealing step. The extension is allowed during elongation step, forming two double-strands as the product of one PCR thermal cycle.

There are several modified versions of PCR. In this study, Long-Range PCR, Hot Star PCR and RT-qPCR (described in chapter 2.6) were also used.

2.2.1 Template RNAs

RNAs (purchased from Clontech and Biochain) used in this study are listed in Table 2.2.1.

Table 2.2.1 Template RNAs.

TISSUE	FIRMA
Human Adrenal gland total RNA	Clontech
Human Brain, cerebellum total RNA	Clontech
Human Brain, whole total RNA	Clontech
Human Fetal brain total RNA	Clontech
Human Heart total RNA	Clontech
Human Kidney total RNA	Clontech
Human Trachea total RNA	Clontech
Human Lung total RNA	Clontech
Human Placenta total RNA	Clontech
Human Prostate total RNA	Clontech
Human salivary gland total RNA	Clontech
Human Skeletal muscle total RNA	Clontech
Human Spleen total RNA	Clontech
Human Thymus total RNA	Clontech
Human Thyroid total RNA	Clontech
Human Uterus total RNA	Clontech
Human Colon total RNA	Clontech
Human Small intestine total RNA	Clontech
Human Stomach total RNA	Clontech
Human Liver total RNA	Clontech
Total RNA - Human Adipose tissue	Biochain
Total RNA - Human Peripheral blood leukocyte tissue	Biochain
Total RNA - Human Liver tissue	Biochain
Total RNA - Human Skin tissue	Biochain

2.2.2 cDNA – synthesis

A complementary cDNA was converted from template RNA by a reverse transcription reaction (10). qScript™ cDNA SuperMix which is a mixture of buffer, dNTPs, $MgCl_2$, primers, RNase inhibitor protein, qScript reverse transcriptase and stabilizers was used to carry out the reaction (50). 1µl of each RNA template was added in a cold 1,5 mL micro-tube on ice. To each sample, 4µl of qScript cDNA SuperMix and 15µl of DNase free water were added to reach the final volume of 20µl. The mixture was then vortexed gently and processed with following reactions: 5 minutes at 25°C, 30 minutes at 42°C, and 5 minutes at 85°C and hold at 4°C(50). The details of reaction mixture are listed in Appendix 6.1.1.

As the optimal amount of template DNA used for LR-PCR should be within 50-500 ng (51), all of the cDNA products listed in Table 2.2.1 except the liver cDNA were diluted 10 times in RNase-free water (1:10), and the liver cDNA was diluted 50 times (1:50) in RNase-free water. The procedure of cDNA dilution is listed in Appendix 6.1.2.

2.2.3 Long-Range PCR (LR-PCR)

The conventional PCR are generally used for DNA fragments that are less than 1kb. Long-Range PCR is designed for amplification of DNA fragment with size up to 40kb (52). Instead of only *Taq* DNA polymerase, the Long-Range PCR requires a mixture of thermostable DNA polymerases to facilitate the amplification and ensure efficiency of DNA extension (52). The mixture contains proofreading enzymes which prevent the formation of new mutations under PCR, comparing with the conventional PCR, the parameters of thermal cycle for Long-Range PCR are also adjusted (52).
Long-Range PCR Kit from Qiagen which is suitable for DNA fragments with size between 0,1 – 10 kb was used in the study (51).

Template cDNAs

cDNA from human blood, spleen, skin, heart and liver tissues were synthesized as described in chapter 2.2.2 and were amplified using LR-PCR.

Primers

Primer-295, 420,405,407,417 and 418 are forward primers and primer-297, 421,406,408 and 419 are reverse primers. The concentrations of stock primer solutions are 100μM and they need to be diluted 10 times with RNase-free water before use. The ratio of dilution is listed in Appendix 6.1.3.

Table 2.2.3A Primers used for Long-Range PCR of CES 1 and CES 2

Primers used for CES 1		
Primer Number	**Name**	**Sequence**
295	CES1A1_mrna_atg_F	5'-ATGTGGCTCCGTGCCTTTA-3'
297	CES1_mrna_utr_R_1	5'-GAACCTGCAATCCCTTTCGC-3'
420	CES1A1_atg_Koz	5′ - ACC ATG TGG CTC CGT GCC TTT A - 3′
421	CES1A1_uten stop_TA_klon	5′ - CAG CTC TAT GTG TTC TGT CTG G - 3′
Primers used for CES 2		
Primer Number	**Name**	**Sequence**
405	CES2_atg_frw	5'-ATGACTGCTCAGTCCCGCTC-3'
406	CES2_stop_rev	5'-CTACAGCTCTGTGTGTCTCTCTTCA-3'
407	CES2_atg_frw_2	5'-ATGACTGCTCAGTCCCG-3'
408	CES2_atg_rev_2	5'-CTACAGCTCTGTGTGTCTCTCTT-3'
417	CES2_atg_Koz	5′- ACC ATG ACT GCT CAG TCC CGC TC - 3′
418	CES2_ex4_Koz	5′- ACC ATG GTG TGG ATC CAC GGT GGT -3′
419	CES2_uten stop_TA_klon	5′- CAG CTC TGT GTG TCT CTC TTC A -3′

Reaction mixture and thermal cycle parameters

For 2µl of cDNA sample, 0,2µl of LR-PCR enzyme mix which is a mixture of thermostable DNA polymerases was added to ensure high extension rate and a proofreading ability (51). 2,52µl of LR-PCR buffer was added to ensure PCR reaction with no optimization required (51). For annealing and elongation of DNA strands, 0,5µl of both forward and reverse primer and 1,25µl of dNTP mix were added (51). Magnesium ions form a complex together with nucleotides and the complex was used as substrate for the DNA polymerase. Therefore the amount of magnesium ions should be sufficient to ensure incorporation (53). In this case, 0,35µl of 25mM of $MgCl_2$ would be sufficient for 2µl of cDNA. 17,7µl of RNase-free water

was added to fill the total volume to 25µl. The detail of reaction mixture is listed in Appendix 6.1.4. All of the procedures were carried out on ice (51).

The thermal cycle parameters were adjusted to minimum denaturation step and maximum annealing and elongation conditions (51, 52). The thermal cycle parameters used are shown in Table 2.2.3B.

Table 2.2.3B Thermal cycles of Long-Range PCR

Step	Temperature (°C)	Time	Number of cycles
Initialization	93	15 min	1
Denaturation	93	35 sec	
Annealing	55	1 min	35
Elongation	68	5 min	
Elongation	68	10 min	1
Final hold	8	Infinite	Store

2.2.4 Hot-Start PCR

Hot-Start PCR is a modified version of PCR which improves the performance of PCR by reducing off-target amplifications. Off-target amplification usually happens during cooler temperatures, where primer dimer and mis-priming extension products can form. They will compete with amplification of the desired target during PCR cycles (54). Hot Start PCR works by blocking DNA polymerase extension until the initial denaturation temperature is reached (54). The PCR buffer used in this kit is able to improve the performance of PCR by allowing a high ratio of specific-to-nonspecific primer binding during the annealing step (55).

Template cDNA

cDNA from human blood, spleen, and heart tissues were synthesized as described in chapter 2.2.2 and were amplified using LR-PCR.

Primers

Primer-162, 329 and 335 are forward primers and primer-163, 330 and 336 are reverse primers. All the primers were diluted with RNase-free water before use. The ratio of dilution is listed in Appendix 6.1.3.

Table 2.2.4A Primers used for Hot-Start PCR of CES 1 and CES 2

Primers used for CES 1		
Primer Number	**Name**	**Sequence**
162	CES1_ex5_ex9_F	5'-CTTTGGAGAGTCAGCGGGAG-3'
163	CES1_ex5_ex9_R	5'-TCCCATCAATCACAGTGCCC-3'
Primers used for CES 2		
Primer Number	**Name**	**Sequence**
329	CES2_E_1_5_F	5'-GCTCAGTCCCGCTCTCCTA-3'
330	CES2_E_1_5_R	5'-CAACAAGCGAAGACACACTCG-3'
335	CES2_E_7_11_F	5'-CCTGTCCCTAGCATTGTTGGT-3'
336	CES2_E_7_11_R	5'-CTCGCAAAGTTGGCCCAGTA-3'

Reaction mixture and thermal cycle parameters

The components of reaction mixture of Hot-Start PCR are similar with LR-PCR. The differences are the buffer and DNA polymerase used(55). Hot-Start PCR uses HotStar *Taq* DNA polymerase which is a modified form of *Taq* DNA polymerase(55). It prevents misprimed products and primer-dimers at low temperatures by remain in an inactive state at such temperatures (55). In this study, 0,25µl of HotStar *Taq* DNA polymerase was used for each sample. 2,5µl of 10x PCR buffer was used for this reaction, it contains a balanced combination of KCl and $(NH_4)_2SO_4$ and $MgCl_2$, ensures a high ratio of specific-to-nonspecific primer binding (55). 1µl of forward primer, 1µl of reverse primer and 0,5µl of dNTP mix were added to the reaction mixture. 18,75µl of RNase-free water was added to fill the final volume of the reaction to 2µl. At the end, 1µl of DNA template was added(55). The detail of reaction mixture is listed in Appendix 6.1.5.

The reaction mix was then mixed thoroughly and processed using the thermal cycle parameters listed in Table 2.2.4B.

Table 2.2.4B Thermal cycles of Hot-Start PCR

Step	Temperature (°C)	Time	Number of cycles
Initialization	95	15 min	1
Denaturation	94	30 sec	
Annealing	55	30 sec	35
Elongation	72	1 min	
Elongation	72	10 min	1
Final hold	4	Infinite	Store

2.2.5 Analyzing PCR products – Agarose gel electrophoresis

Agarose gel electrophoresis was used frequently in the study to separate and identify DNA/RNA fragments. Agarose gel electrophoresis can separate DNA fragments of sizes ranging from 100bp to 25kb (56). During gelation, agarose polymers can form a network with pores which biomolecules can pass through (57). The DNA and RNA molecules will migrate towards the positively charged anode as the molecules are negatively charged when the gel is placed in an electric field (56). While migrating, they are separated by size in a pattern such that the distance traveled is inversely proportional to the log of its molecular weight (58).

The concentration of the gel can vary from 0, 3% to 2% (59), and 1% gel was used in the study. The gel was prepared by weighing out 1g of agarose powder into a 500ml Erlenmeyer flask, mixed with 100mL of TBE buffer, and then dissolved by heating with microwave for 2 minutes (3 minutes if the gel was not completely dissolved). Placing under running tap water then cooled down the dissolved agarose. 10uL of highly sensitive fluorescent stain, GelStar nucleic acid gel stain was added after cooling for detecting of DNA or RNA molecules (60). The gel solution was poured carefully into a gel tray, and an appropriate comb (with either 12 or 20 teeth) was inserted correctly into its position. Any bubbles present was pushed away using the comb. The gel was then covered with an aluminum lid and left for 30-45 minutes to become rigid (61). When the gel was rigid enough, loaded the first well with 1kb plus DNA ladder and loaded following wells with DNA samples which have been mixed with appropriate amount of loading buffer (1μL of loading buffer into 5μL of sample or 2μL of loading buffer into 10μL of sample). The gel tray was then placed into the gel tank and run with electrophoresis for 45 – 60 minutes under 120 volts. The DNA/RNA fragments would be separated according to the difference in their sizes, and they were visualized by GelStar stain when placing under UV-light.

2.3 DNA extraction – QIAquick gel extraction

The DNA fragments of interest were purified from the agarose gel using QIAquick Gel Extraction Kit. Good quality of DNA is important for downstream procedures as impure or contaminated DNA can lead to suboptimal results, for example contaminants such as salts, proteins, ethanol and other detergents can interfere performance of DNA in following procedures (62). This procedure enables removal of impurities such as primers, nucleotides,

enzymes, salts, agarose, ethidium bromide and etc. from sample and ensures up to 80% recovery of DNA (63).

The gel surrounding the fragment was first excised using clean scalpel under UV light, and then dissolved in three volumes of Buffer QC by incubating at 50°C for at least 10 minutes. A yellow color was expected for the dissolved gel as it would show the pH of the mixture was optimal (64). One gel volume of isopropanol was added to the gel solution. The gel mixture was then separated within QI Aquick spin column under centrifuging by successively adding 500µl of buffer QG (to bind DNA) and 750µl buffer PE (to wash off contaminants and impurities) (65). The purified DNA could be eluted into a clean 1,5ml microcentrifuge tube using Buffer EB which breaks the hydrogen bonds that hold DNA on the membrane (64, 65).

2.4 TOPO Cloning

The purified DNA products from chapter 2.3 were cloned into plasmid vectors using a highly efficient one-step cloning technique called TOPO cloning. This technique is based on the nontemplate-dependent activity of Taq polymerase that allows adding of single deoxyadenosine (A) to the 3' ends of PCR products. Since the vectors used in TOPO cloning has a single overhanging 3' deoxythymidine (T) residues which is complementary to the 3' Adenine (A) end of PCR fragment, PCR fragments can be inserted and ligated with the plasmid vectors efficiently (66, 67). DNA topoisomerase I functions both as a restriction enzyme and as a ligase in this reaction; it binds to DNA at specific sites and cleaves the phosphodiester backbone after 5'-CCCTT in one strand. The enzyme is released after ligating vectors to the PCR insertion (66, 68).

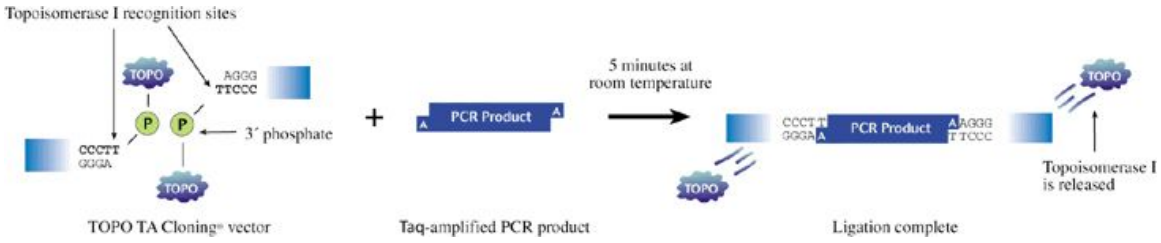


Figure 2.4 Overview of TOPO cloning (68). Topoisomerase I binds to DNA at specific site 5'-(C/T)CCTT-3' and forms a covalent bond with the phosphate group attached to the 3' thymidine. The DNA becomes unwind due to cleavage. The enzyme is released after successful ligation of PCR product with vectors (68).

In this study, TOPO TA cloning and TA XL cloning were used where TOPO TA cloning is suitable for relatively shorter PCR products and TOPO XL cloning can be used for the cloning of long PCR products (69).

2.4.1 Vectors

Vectors used in TOPO cloning are listed in Table 2.4.1. And the maps of vectors are shown in Figures 2.4.1 A, B, and C.

Table 2.4.1 TOPO cloning vectors.

Cloning method	Vector	Length of vector (kb)
TOPO TA cloning	pCR®2.1 -TOPO®	3,9
	pcDNA™3.1/V5-His-TOPO®	5,5
TOPO XL cloning	pCR-XL-TOPO®	3,5

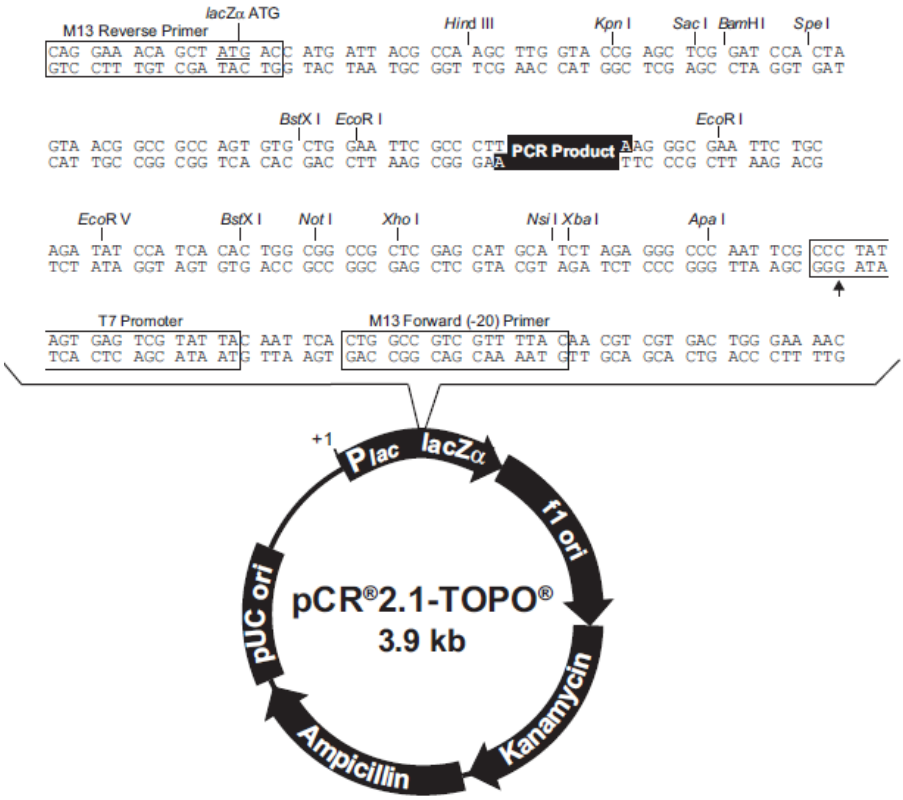


Figure 2.4.1A pCR®2.1 -TOPO® Map (66). Restriction sites are labeled to indicate the actual cleavage site and the arrow shows the start of transcription (66).

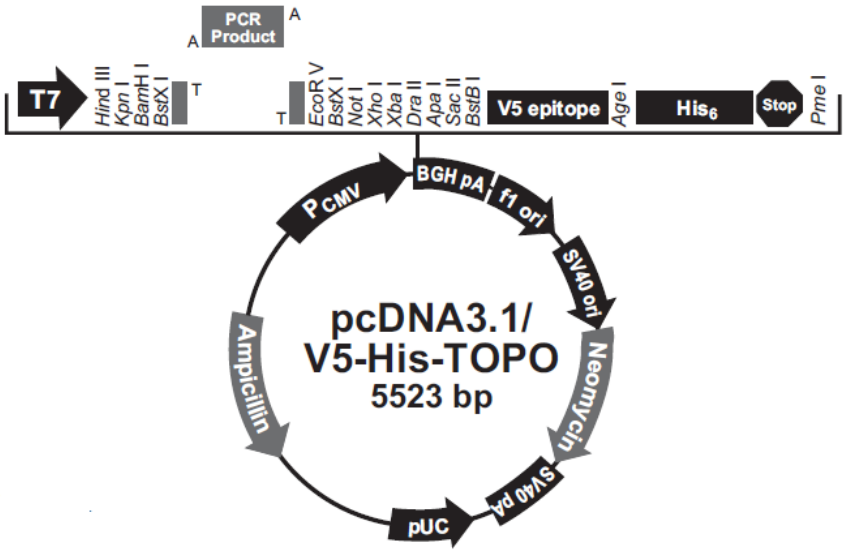


Figure 2.4.1B pcDNA™3.1/V5-His -TOPO® Map (70). The vector is supplied linearized between base 953 and 954 which is the cloning site, and the arrow shows the start of transcription (70).

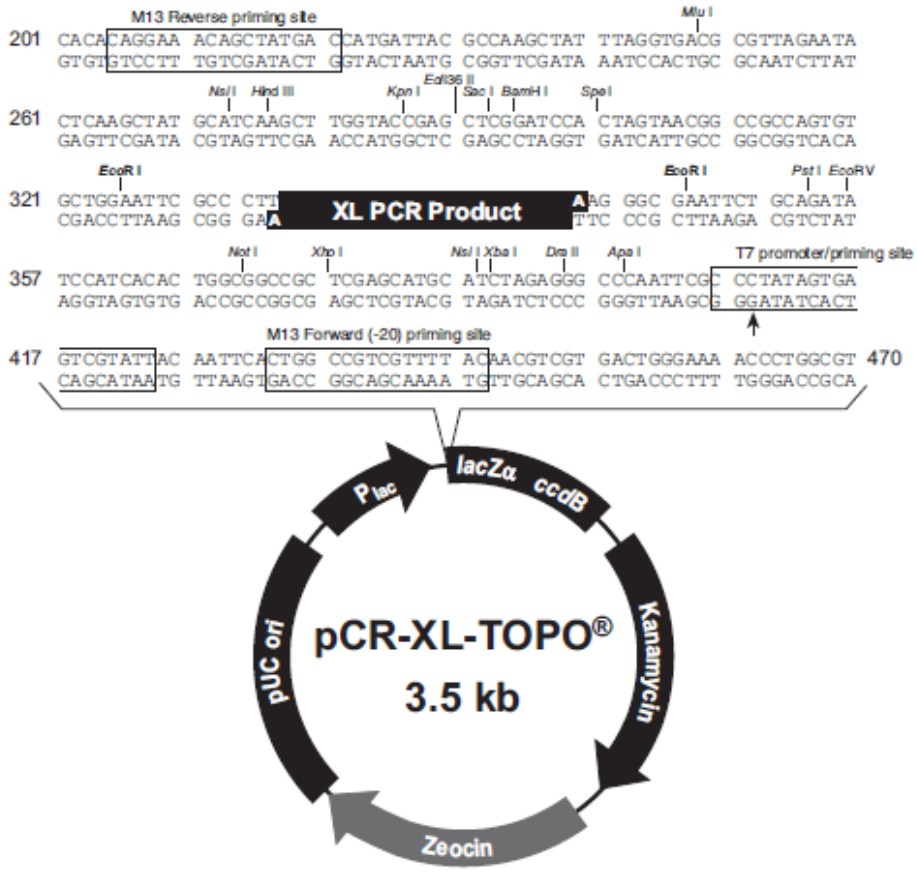


Figure 2.4.1C pCR- XL -TOPO® Map (69). Restriction sites are labeled to indicate the actual cleavage site and the arrow shows the start of transcription (69).

2.4.2 PCR products with adenine at the 3'end

The PCR products were processed with HotStar *Taq* DNA polymerase for adding A (adenine)- overhangs at the 3'ends of the PCR fragments before TA cloning. HotStar *Taq* DNA polymerase provides high PCR specificity and often increases the yield of the PCR product (55). 0,1µl of HotStar *Taq* DNA polymerase, 0,7µl of 2mM dNTPs and 0,7µl of 10x PCR buffer were added to 5µl of DNA template. The mixture was mixed thoroughly and incubated with optimal temperature. Details of reaction mixture and thermal parameters are listed in Appendix 6.1.6.

2.4.3 TOPO cloning reaction

The following preparations were made before transformation: 1.Equilibrate a water bath to 42 ^{o}C, 2.Pre-warm agar plates (Ampicillin plates for TOPO TA cloning and Kanamycin plates for TOPO XL cloning*)* and S.O.C. medium at 37^{o}C for 30 minutes (or until ready for use) in an incubator 3.Thaw Once Shot® competent cells on ice (1 cell for each transformation) (66, 69, 70). The cloning reaction as following was set up and incubated for 5 minutes at room temperature after mixed gently (66, 69, 70):

TA cloning: 1µl of 5xT4 ligase buffer, TOPO vector, ExpressLink T4 DNA ligase were added to 2µl of PCR product, then 2µl of RNase-free water was added to fill the total volume to 10µl (66, 70).

XL cloning: 1µl of pCR-XL-TOPO vector was added to 5µl of PCR product(69).

The overview of components in TA- and XL-cloning is listed in Appendix 6.1.7.

After incubation, 1µl of the 6X TOPO® Cloning Stop Solution was added to the reaction mixture and mixed for several seconds at room temperature. The mixture was then centrifuged briefly and placed on ice before transformed into competent cells (66, 69, 70).

2.4.4 Transformation into OneShot component cells

2µl of the cloning reaction mixture (as described in 2.4.3) was added to each OneShot competent cell, and then stood on ice for 30 minutes. The cells were then heat-shocked at 42^{o}C for 30 seconds using the pre-warmed water bath; and replaced on ice immediately after heat-shock. 250µl of the pre-warmed S.O.C medium was added to each cell tube and incubated with horizontal shaking at 37^{o}C for 1 hour at speed 200rpm.

After horizontal shaking, 80µl of the transformation mixture from each sample was poured onto pre-warmed plates. And the plates were incubated overnight at 37°C (66, 69, 70).

2.4.5 Recombinant selection

Recombinant selection is an important step of cloning as it is necessary to identify and separate the clones with desired insert from the unsuccessful transformed ones (71).
TA – and XL-cloning are different when it comes to selection principle of recombinants:

TOPO TA cloning: blue-white screen.

After overnight incubation, *E.coli* cells with the presence of recombinant could be shown as white colonies, and the visual screening method is known as blue-white screen (59, 71, 72). The method is based on activity of an enzyme in *E.coli* called β-galactosidase. The vector encodes α subunit of the *LacZ* protein with a multiple cloning site (MCS), and the host strain encodes the omega subunit to form the β-galactosidase upon complementation (59, 71, 72). When the enzyme is produced, X-gal (a colorless galactose sugar) in agar plates will be hydrolyzed to form 5-bromo-4-chloro-indoxyl which can further produce an insoluble blue pigment and thereby functions as an indicator of non-recombinant cells (59, 71, 72). If foreign DNA inserts into the MCS in the *LacZ* gene, the formation of β-galactosidase will be disrupted and X-gal will remain in its original form and shown as white colonies (59, 71, 72).

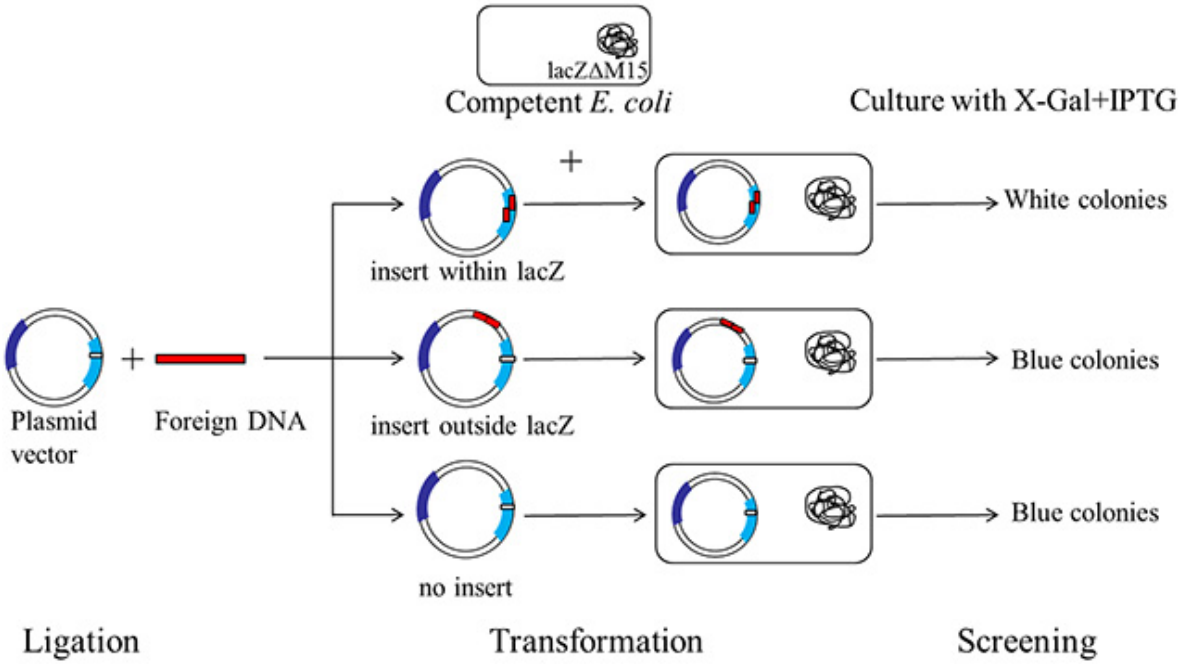


Figure 2.4.5A Overview of a typical blue-white screen (72).

TOPO XL cloning: direction selection.

The selection principle is different from blue-white screening as the cells that contain non-recombinant vectors will be killed upon transformation. So the only colonies that will grow on the plates are the positive recombinants (white colonies) (69).

The positive colonies with recombinant vectors were analyzed using PCR and gel electrophoresis. Both original and alternative splicing variants were selected for sequencing and further analysis with bioinformatics tools.

2.5 Sequencing and bioinformatics tools

2.5.1 Miniprep

Before sequencing, the pure plasmid DNA needs to be isolated. To confirm correct cloning of target gene into the vector, pipette tips with small amount of wanted colonies were incubated overnight in 3 ml of ampicillin/kanamycin medium at 37^{o}C by vertical shaking at 225 rpm. Subsequently, the recombinant plasmid DNA was isolated by the S.N.A.P™ Miniprep Kit from Invitrogen™ LifeTechnology(73).

Miniprep was carried in three steps:

1. **Lysis and precipitation:** 1,5ml of the overnight culture was centrifuged to pellet the cells. 150µl of resuspension buffer was used to suspend the cell pellet. Then 150µl of lysis buffer was added to the mixture and mixed gently by inverting. The mixture was then incubated at room temperature for 3 minutes. Then 150µl of ice-cold precipitation salt was added to the mixture and centrifuged at room temperature at 14000 x g for 5 minutes (73).

2.**Binding of plasmid DNA to column**: after the centrifugation as described above, the supernatant was transferred to a new sterile micro centrifuge tube and mixed with 600µl of binding buffer. The entire solution was transferred onto the S.N.A.P™ Miniprep column which placed inside the collection tube. The plasmid DNA binds to the column with high affinity by centrifuge the columns at 3000 x g for 30 seconds. The solution was then washed successively with 500µl of wash buffer, then 900µl of 1X Final wash with centrifugation at 3000 x g for 30 seconds between adding wash buffer. To dry the resin, the columns were centrifuged at room temperature at 14000 x g for 1 minute (73).

3. **Elution of plasmid DNA**: to elute the plasmid DNA, the plasmid bound column was transferred to a new sterile micro centrifuge tube and 60µl of sterile water was applied to the center of the column. The solution incubated at room temperature for 3 minutes. The plasmid

DNA was then eluted to the microcentrifuge tube underneath by centrifugation at room temperature at 14000 x g for 30 seconds (73).

2.5.2 Sequencing

The pure plasmid DNAs was sequenced by Tube sequencing service and Value-read service at Eurofins Scientific Company. Several bioinformatics software and online tools were used to analyze results from sequencing.

2.5.3 BLAT (BLAST – like alignment tool)

The DNA sequences obtained from TOPO cloning were compared with entries in UCSC genome bioinformatics server using BLAT. BLAT (short for "BLAST-like alignment tool) is a multiple algorithm used for annotate and assembly of the human genome and it was developed by James Kent (74, 75). Using BLAT the DNA sequences with none amino acid mutations (may contain few missense mutations) were aligned onto the human reference genes and the exon structure of mRNA was determined (75, 76). Thereby the exons in suspected alternative splicing variant were compared with the reference human genes.

2.5.4 ATGpr

The full amino acid sequences of both reference human gene and alternative splicing variants were predicted using ATGpr which is a computer program developed by Helix Research Institute in Japan for predicting whether a cDNA contains an initiation codon or not (77).

2.5.5 Clustal omega

The predicted amino acid sequences were then aligned using Clustal omega which is an online multiple sequence alignment program (78). By using this program, we were able to identify the length of missing exons.

2.5.6 Uniprot

The biological function of missing exons in the alternative splicing variants was identified using UniProt. UniProt (Universal Protein Knowledgebase) is an online database of protein sequences with accurate, consistent and rich sequence and functional annotation (function of the protein, active sites, biologically relevant domains and sites, post-translational modification, etc) (79). Using information provided by UniProt, it is easy to identify if the

missing exons would contain any active sites or/and cause any mutagenesis with biological meaning.

2.5.7 NEBcutter

NEBcutter which is a program freely available via a webserver (http://nc2.neb.com/NEBcutter2/) was used to analyze the restriction sites of the restriction enzymes that will cleave the input DNA sequence. The input sequence can be pasted in, picked up from a local file or from NCBI via accession number (80).

2.6 Reverse transcriptase – quantitative PCR (RT-qPCR)

Reverse transcriptase quantitative PCR (RT-qPCR) is a quantitative PCR method used in molecular medicine, biotechnology, microbiology and diagnostics for quantification of mRNA (81). It combines RT-PCR which converts RNA into cDNA together with qPCR which amplifies, detects and measures PCR products generated in real time (when it is synthesized) (82). In this study RT-qPCR was used to determine the expression levels of the alternative spliced variants in different human tissues.

2.6.1 Reverse transcriptase PCR (RT-PCR)

RNA samples which are listed in chapter 2.2.1 were reversely transcribed using reverse transcriptase PCR described in chapter 2.2.2.

2.6.2 Real-time quantitative PCR (qPCR)

Real-time quantitative PCR (qPCR) detects and measures the PCR products at each cycle of PCR using hydrolysis probe (82). The hydrolysis probes are dual-labeled oligonucleotides that can be labeled and thereby used for detection of the nucleic acid sequence that are complementary (83). The *Taq*Man probes used in this study are labelled with the fluorescent reporter dye that is bound covalently at the 5' end, and a quencher dye that is bound at the 3' end of the probe (82). A quencher dye functions as a 'dark absorber' which means that it can extinguish fluorescence of the fluorescent reporter dye when they are within certain distance (84). Therefore when the probe is intact, the fluorescent reporter dye is quenched by the quencher dye and no fluorescent emission signal will be detected (82). When the probe hybridizes to the target sequence of interest, and is cleaved by the 5' endonuclease activity of

the *Taq* DNA polymerase. The reporter dye will be separated from quencher dye and emits fluorescent signals which can be detected (82). The amount of fluorescent signal detected is directly proportional to the amount of PCR products during each PCR cycle (Figure 2.6.2A) (81).

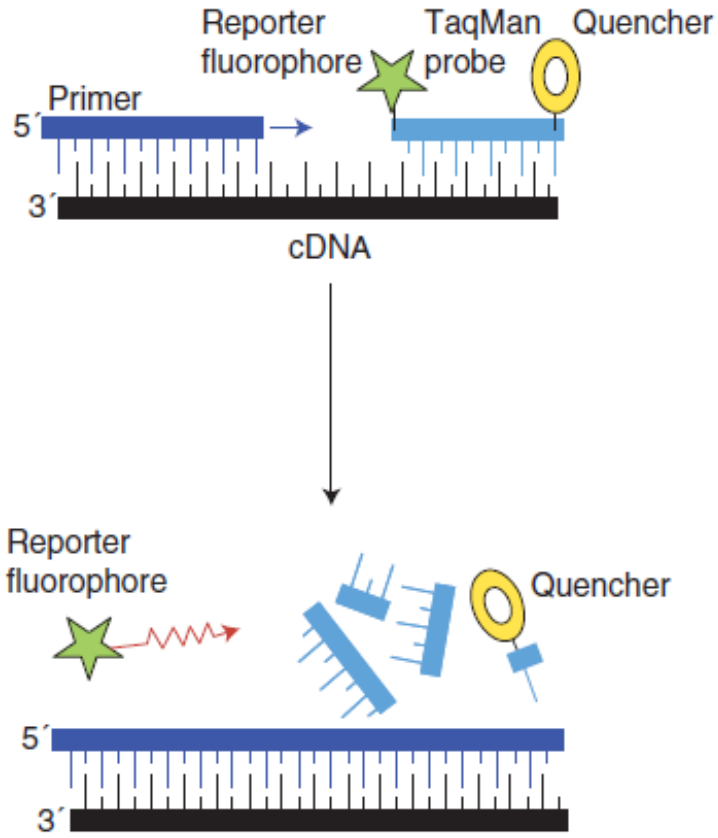


Figure 2.6.2A Hydrolysis probes (*Taq*Man® assay) (82). The fluorescent reporter dye (reporter fluorophore) is extinguished by quencher dye while the *Taq*Man is intact. The primer and probe anneal to the DNA strand following denaturation. During the extension phase, the probe is cleaved by *Taq* DNA polymerase and the fluorescence signal can be detected as the two dyes are separated (82).

In this study, *Taq*Man Universal PCR Master mix was used. It is a mixture of AmpliTaq Gold DNA polymerase, dNTPs with dUTP, passive reference and optimized buffer (85). To each reaction mixture of qPCR, 7µl of DNase free water, 10µl of *Taq*Man master mix, 1µl of probe were added to 2µl of DNA product of reverse transcription (as described in chapter 2.6.1). The reaction mixtures were then placed in a 96-wells PCR plate and sealed with Optically clear adhesive seal sheets. After centrifugation, the qPCR was performed using ViiA™ 7 Real-time PCR system. The details of reaction mixture and thermal cycle parameters of this method are listed in Appendix 6.1.8.

Hydrolysis probes used

FAM (6-carboxyfluorescein) probes (CES2_EX2_3SPL and CES2_EX8_SPL) were used in this study, where probe CES2_EX2_3SPL was designed to span the exon 1- exon 4 junction and probe CES2_EX8_SPL was designed to span exon 7 – exon 9 junction. These two probes were chosen as previous studies have found alternative splicing variants of CES2 with deletion of exon 2 and 3 or exon 8 (86).

Reference genes

Reference genes are used as internal references for normalization in qPCR. Theoretically, these genes are expressed at a constant level among different tissues of an organism, at all stages of development and under different experimental conditions. Therefore they are suitable to minimize the errors generated by differences in RNA samples and correct sample-to-sample variation during the qPCR reaction (82).

Reference genes used in the study were GAPDH and PGK1. We chose to use these two genes as they are two of the five genes which give the most suitable normalization factors for quantification of relative expression levels in different tissues (87).

Relative quantification

The data from qPCR can be analyzed using two different methods: absolute quantification that uses a standard curve and relative quantification that describes and compares the change in gene expression of the target gene relative to the reference group (88). In this study, the relative quantification was used to analyze the expression level of the target genes. The method used for relative quantification is called the comparative threshold method ($2^{-\Delta\Delta Ct}$ method). This method allows calculation and comparison of the relative expression levels of the target gene to a reference gene use following equation (89):

$\mathbf{2^{-\Delta\Delta Ct}}$: the normalized target amount in the sample

$\mathbf{\Delta\Delta Ct} = (Ct_{target} - Ct_{reference})_{calibrator} - (Ct_{target} - Ct_{reference)sample}$

$\mathbf{\Delta Ct} = Ct_{target} - Ct_{reference\ gene}$

Where $\mathbf{2^{-\Delta\Delta Ct}}$ is the amount of target gene in the sample, normalized to the reference gene and relative to the normalized calibrator, a calibrator is a reference sample used in qPCR to which all other samples are compared to determine the relative expression level of a gene (83). In this study, the tissue with the highest expression level of the target gene was chosen to use as

the calibrator. **Ct (threshold cycle**) is the fractional PCR cycle number at which the reporter fluorescence is greater than the minimal detection level (threshold) (Figure 2.6.2B) (82).

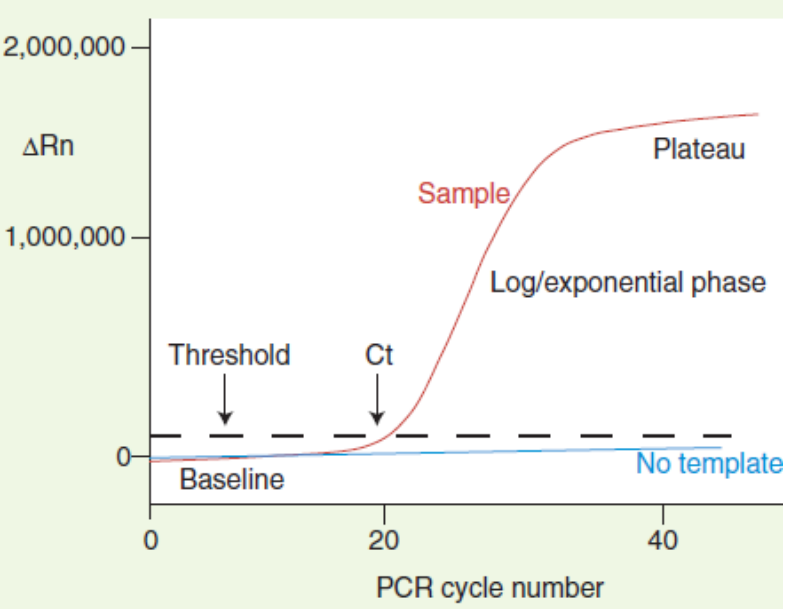


Figure 2.6.2B Model of a single amplification plot illustrating the nomenclature commonly used in real-time quantitative PCR (82). The baseline is when the fluorescent signal is lower than the limits of detection of the instrument. The ΔRn is the fluorescence emission of product at each time point. The threshold indicates the baseline for fluorescence signal, only signals above the threshold can be considered as a real signal (82).

In this study, the $2^{-\Delta\Delta Ct}$ value was calculated using software Excel. First of all, the samples with Ct SD larger than 0,5 were excluded. Then the sample with the highest Ct value was chosen as calibrator. The calculation of ΔCt, ΔΔCt and $2^{-\Delta\Delta Ct}$ for all samples was performed using Excel.

2.7 Measurement of enzyme activity

One original and one alternative splicing variant from CES1 and CES2 which have shown good results with sequencing were used for measurement of enzyme activities. The samples above were first amplified using LR-PCR (described in chapter 2.2.3) with primers that are listed in table 2.7, then separated using agarose gel electrophoresis (described in chapter 2.2.5) and extraction with DNA extraction (described in chapter 2.3).

Table 2.7 Primers used:

cDNA	PRIMER USED		
	Forward primer (#)	Reverse primer (#)	Amplify exons:
CES1	420	421	1 to 14
CES2	417	419	1 to 12
CES2	418	419	4 to 12

2.7.1 Cloning of the PCR products into pcDNA™3.1/V5-His-TOPO® vector

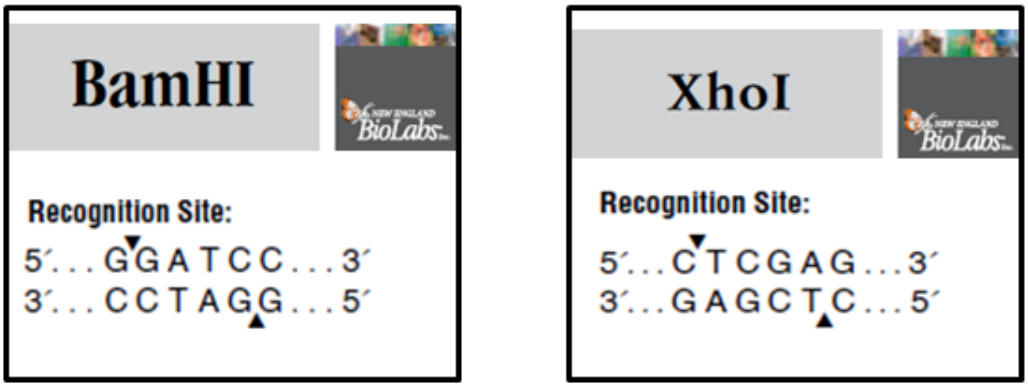
The purified PCR products were then cloned into vector pcDNA™3.1/V5-His-TOPO® using the method described in chapter 2.4. The amount of PCR product used in cloning was adjusted according to the brightness of the band under UV-light. 1µl of PCR product and 2µl of RNase-free water were used for the PCR products with bright band; 2µl of PCR product and 1µl of RNase-free water were used for the PCR products with significant weaker band. The overviews of reagents are listed in Appendix 6.1.9. The recombinants were selected based on direct selection where only positive recombinants grow on the plates, represented as white colonies.

2.7.2 Quantification of DNA by Nanodrop spectrophotometer

It is necessary to measure the concentration of nucleic acid in the samples before proceeding to next step since it is recommended to use approximately the same amount of DNA when comparing different samples (90). In this study, the Nanodrop spectrophotometer was used for quantification of DNA samples. The system was blanked by applying 1µl of sterile water, then 1µl of DNA from each sample was successively and measured using software NanoDrop ND 1000 (81).

2.7.3 Restriction enzymes

The restriction enzymes XhoI and BamI were used to identify the orientation of PCR insertion. Restriction enzymes are endodeoxyribonucleases that recognize specific nucleotide sequences in double stranded DNA and cleave both strands of the duplex. Both XhoI and BamI are class II restriction enzymes which cleave at specific recognition sites (Figure 2.7.3A) (91).



Reference: Datasheet for **BamHI**(R0136S) , NEB Reference: Datasheet for **XhoI**(RD1465) , NEB

Figure 2.7.3a Recognition site for the restriction enzymes BamHI and XhoI (92, 93). BamHI binds as a dimer to the symmetrical DNA sequence 5'-GGATCC-3' (94) and XhoI binds as a dimer to the symmetrical DNA sequence 5'-CTCGAG-3'.

In this study, the enzyme BamHI was used for CES1 and the enzyme XhoI was used for CES2. To 1 µg of DNA (required volume was calculated based on measurement from NanoDrop), 1µl of restriction enzyme and 5µl of 10X NEB buffer were added. The final volume was adjusted to 50µl with RNase-free water. Details are listed in Appendix 6.1.10. Using map of vector (Figure 2.4.1a) and NEBcutter (described in chapter 2.5.7) that detects the cutting site in PCR product, we could predict the expected lengths of DNA fragments produced after cutting in both orientations. By using gel electrophoresis, it would be possible to identify if the PCR products were correctly inserted or not.

2.7.4 Midiprep of recombinant plasmids and subsequent sequencing

Pipette tips with small amount of wanted colonies were incubated overnight in 3 ml of ampicillin medium at 37°C by vertical shaking at 225 rpm. The plasmid of the vector with CES1 or CES2 insertion in correct orientation was isolated using S.N.A.P™ Midiprep Kit from Invitrogen™ LifeTechnology(95).

Plasimd Midiprep were carried out in three steps:

1. Lysis and precipitation: 100 ml of the overnight culture was centrifuged to pellet the cells at 4000 x g for 10 minutes at 4°C, and 4 ml of resuspension buffer was used to suspend the cell pellet. Then 4 ml of lysis buffer was added to the mixture and mixed gently by inverting. The mixture was incubated at room temperature for 3 minutes, and 4ml of ice-cold precipitation salt was added. The mixture was incubated on ice for 5 minutes, then transferred to Midiprep column A and centrifuged at room temperature at 3000 x g for 5 minutes (95).

2. Binding of plasmid: After centrifugation, the column A was removed. To the filtrate in

column B 12ml of binding buffer was added. The column B with supernatant was transferred onto a new conical tube and centrifuged at 1000 x g for 2 minutes to bind the plasmid DNA to the column. The column bound with plasmid DNA was then washed successively with 5 ml of wash buffer, 5ml of 1X Final wash and 10 ml of 1X Final wash and centrifuged at 2000 x g for 2 minutes. To dry the resin, the columns were centrifuged at room temperature at 4100 x g for 5 minute (95).

3.Elution of plasmid DNA: before elution, the column B was transferred to a new sterile conical tube and 750µl of sterile water was applied to the center of the column. The column was incubated at room temperature for 3 minutes. The plasmid DNA was then eluted to the conical tube underneath by centrifugation at room temperature at 41000 x g for 5 minutes (95).

The purity of plasmid DNAs were determined by Value-Read service at Eurofins Scientific Company.

2.7.5 Cell culture and transfection by the plasmid purified

HEK293 cell line was used for this cloning study. The HEK293 cell line is an expression tool which generated by transformation of human embryonic kidney (HEK) cells following exposure to sheared fragments of human adenovirus type 5 (Ad5) DNA (96). The HEK293 cells were routinely maintained in EMEM medium with L-glutamine at 37^{o}C, and subcultured twice a week. The concentration of cell was maintained within 1-9 * 10^5 /ml (97). After the 8th subculture, the cells were prepared for transfection by cultivation in EMEM medium with L-glutamine at 37^{o}C for 1 hour. Cells were then transfected with the plasmid DNA using the Lipofectamine™ 3000 reagent by adding 5µl of Opti-Mem medium and 0,3 µl Lipofectamin™ 3000 reagent to 0,2 µg (mass unit) of DNA (98). The details of Lipofectamine transfection reagent protocol are listed in Appendix 6.1.11.

The 96-wells plate containing the samples was incubated at 37^{o}C.

2.7.6 Measurement of enzyme activity

The enzyme activity was measured at 24, 48, 72 and 96 hours after transfection by following procedures:

Cell medium preparation: 150µl of cell medium was transferred to new tubes and centrifuged at 1500 rpm for 10 minutes, then 75µl of the cell medium was transferred to microplates and mixed with 75µl of 1mM 4-MUBA solution (97).

Cell preparation: the cells were dissociated by adding 50µl of trypsin and further diluted in 100µl of EMEM medium with L-glutamine before transferred into a microplate. The cell solutions were then incubated at 37C for 1 hour. After incubation, 150µl of 0,5mM 4-MUBA solution was added to the cells (97).

The Victor² 1420 Multilabel Counter from Perkin Elmer Life Science was used to measure enzyme activity in 50 seconds interval for 15 minutes (97).

3 Results

3.1 Alternative splicing variants found by previous studies

Bachelor studies by students from HiOA in 2014 and 2015 have found several alternative splicing variants of CES1 and CES2 that are listed in Table 3.1 (86, 99).

Table 3.1 Results of previous studies.

Alternative splicing variants	Missing exons	Missing amino acids
CES1		
CES1_5_7	exon 6	232-267
CES1_6_8	exon 7	267-302
CES1_6_8.5	exon 7 and half of exon 8	267-310
CES2		
CES2_1_4	exons 2 and 3	1-216
CES2_7_9	exon 8	436-469
CES2_ 9_10.5	half of exon 10	557-573

Table 3.1 Alternative splicing variants detected in previous studies (86, 99) For CES1, a study in 2014 has detected three alternative splicing variants: CES1_5_7 which is missing entire exon 6, CES1_6_8 which is missing exon 7 and CES1_6_8,5 where entire exon 7 and half of exon 8 are spliced out. For CES2, a study in 2015 has detected three alternative splicing variants: CES2_1_4 which is missing entire exon 2 and 3, CES1_7_9 which is missing exon 8 and CES2_9_10,5 which is missing half of exon 10(86, 99).

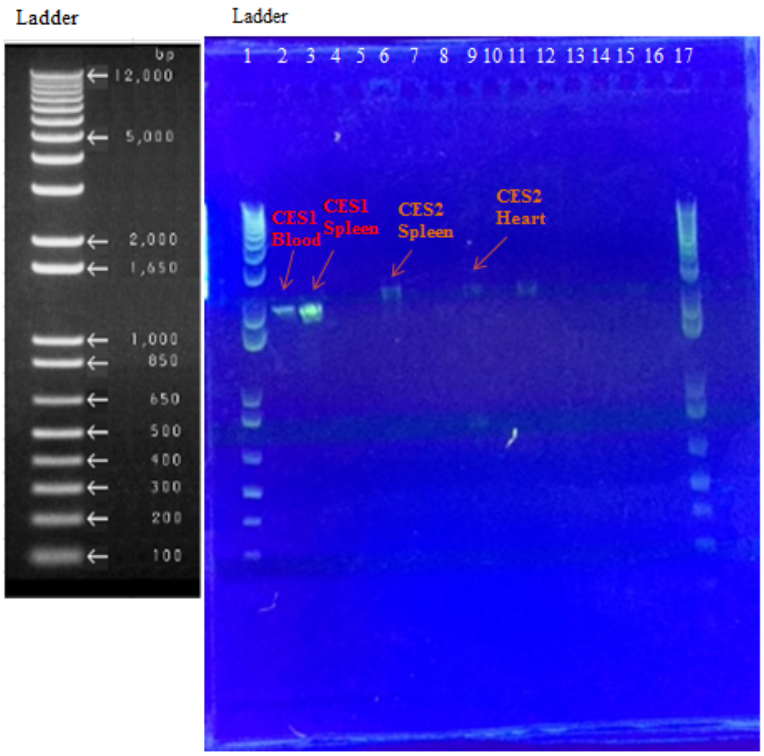
3.2 New alternative splicing variants

3.2.1 Detection of new alternative splicing variants in CES1 and CES2

cDNA from human blood, skin, heart, liver and spleen tissues were amplified using Long-Range PCR. Primers were designed to amplify cDNA fragments from exon 1 to exon 14 for CES1, and from exon 1 to exon 12 for CES2 as following (Table 3.2.1).

Table 3.2.1 Primers used for LR-PCR.

Gene	**PRIMER USED**			
	Primer pair	Forward primer (#)	Reverse primer (#)	Amplify exons:
CES1	1	295	297	1 to 14
CES2	2	405	406	1 to 12
CES2	3	407	408	1 to 12



WELL #		Tissue	Primer
1	Ladder (1kb plus)	-	-
2	CES 1	Blood	295+297
3	CES 1	Spleen	295+297
4	CES 1	Water	295+297
5	CES 2	Blood	405+406
6	CES 2	Spleen	405+406
7	CES 2	Liver	405+406
8	CES 2	Skin	405+406
9	CES 2	Heart	405+406
10	CES 2	Water	405+406
11	CES 2	Blood	407+408
12	CES 2	Spleen	407+408
13	CES 2	Liver	407+408
14	CES 2	Skin	407+408
15	CES 2	Heart	407+408
16	CES 2	Water	407+408
17	Ladder	-	-

Figure 3.2.1A Agarose electrophoresis analysis of LR-PCR. The analysis of PCR products from Long Range-PCR is shown in the picture in the middle. The table on the right summarizes the cDNAs and primers used in the samples in each well. Primer pair 1: #295 and #297 (exon 1-14) ; Primer pair 2: #405 and #406 (exon 1-12) ; Primer pair 3: #407 and #408 (exon 1-12). The agarose gel electrophoresis ran for 1 hour 50 minutes at 120 volts, each well was loaded with mixture of 10µl of PCR product and 1µl of loading buffer.

As seen in Figure 3.2.1A, more than one PCR fragment were observed in well 2,3,6 and 9. These four PCR products all shown to contain one main band between 2000 – 3000 bp and one or several weaker bands those are shorter. The weaker bands are slightly more obvious under UV-light than in gel picture. The result indicates that these DNA fragments might contain potential alternative splicing variants. For further analysis, the agarose gel electrophoresis was repeated using gel with wider wells and the PCR products of interest were purified from the agarose gel using QIAquick gel extraction kit (results of PCR products are shown in Figure 3.2.1B).

PCR fragments of interest: **PCR fragments:**

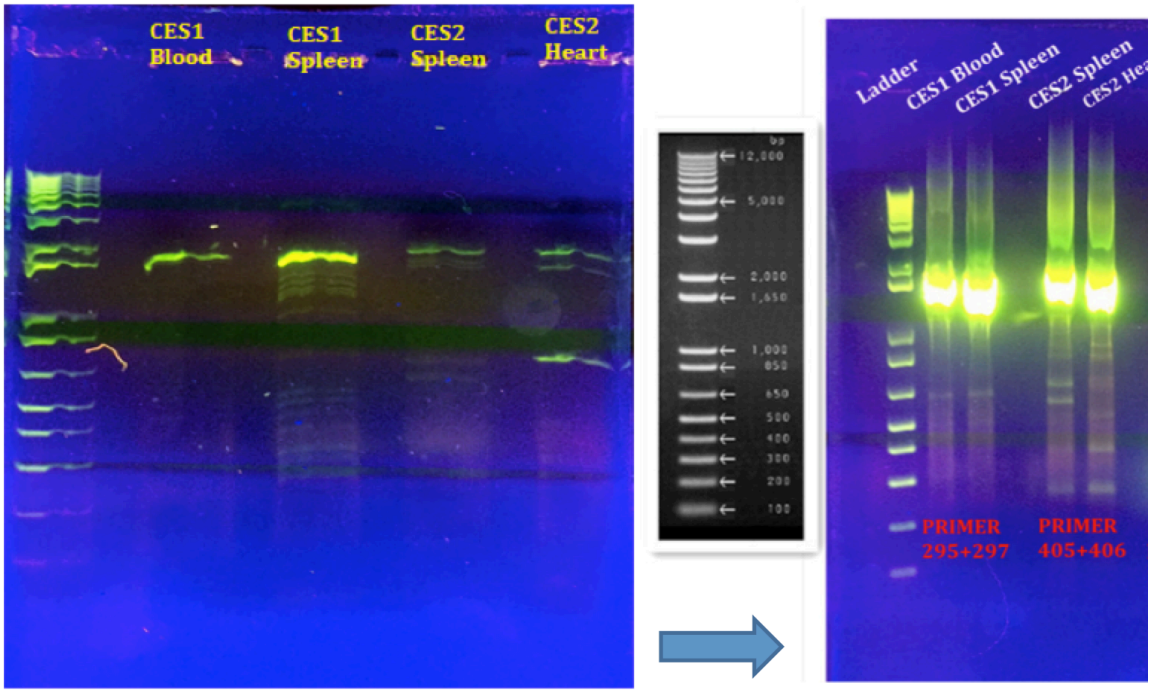


Figure 3.2.1B Agarose electrophoresis analysis of PCR fragments of interest (left). The PCR products of interest (Figure 3.2.1A) were selected and analyzed using agarose gel electrophoresis. The agarose gel electrophoresis ran for 1 hour at 120 volts, each well was loaded with mixture of 15µl of PCR product and 2µl of loading buffer. **Agarose electrophoresis analysis of purified PCR fragments (right).** The gel containing PCR fragments of interest were excised and extracted using QIAquick Gel Extraction Kit. The purified DNA products were analyzed using agarose gel electrophoresis. The agarose gel electrophoresis ran for 1 hour at 120 volts, each well was loaded with mixture of 10µl of PCR product and 1µl of loading buffer.

The five purified PCR products (two samples of PCR-product from CES1 blood) were then cloned into plasmid vectors using cloning kit *pCR® 2.1- TOPO®* (TOPO TA cloning with ampicillin antibody) and cloning kit *pCR-XL-TOPO®* (TOPO XL cloning with kanamycin antibody).

The resultant transformants with recombinant vector were selected by the blue-white screening method. The transformants with insertion of CES DNA into the vector are shown as white colonies and blue colonies represent transformants with non-recombinant vector (Figure 3.2.1C).

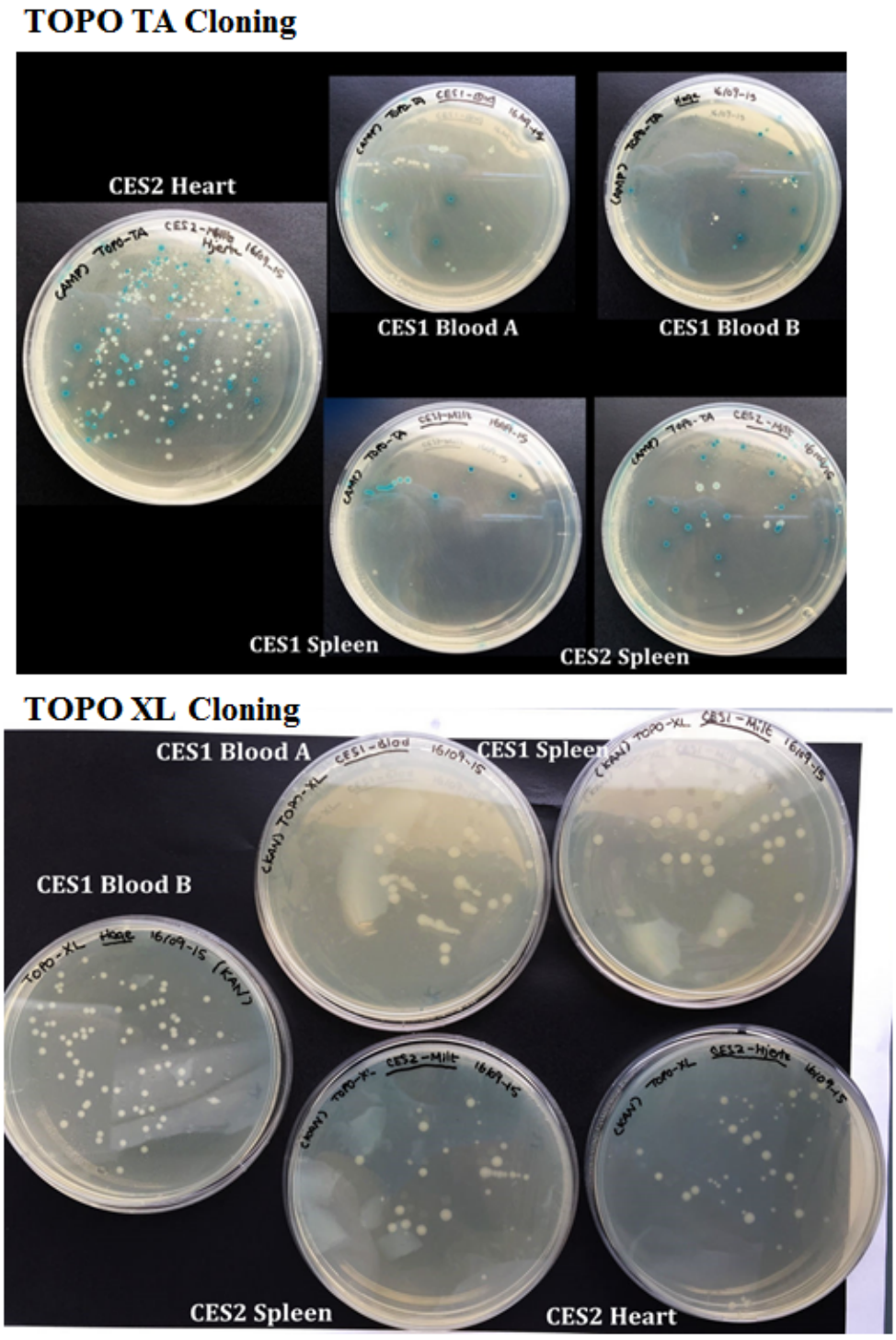


Figure 3.2.1C Overview of ampicillin agar plates with TOPO TA cloning (top). The five purified PCR products were cloned into pCR®2.1-TOPO® vector using TOPO TA cloning. The transformation mixture were poured onto ampicillin agar plates and incubated overnight at 37°C. The recombinant bacteria were selected by blue-white screen where presence of recombinant bacteria are shown as white colonies and blue colonies represent non-recombinant bacteria. **Overview of kanamycin agar plates with TOPO XL cloning(bottom).** The five purified PCR products were cloned into pCR XL-TOPO® vector using TOPO XL cloning. The transformation mixture were poured onto kanamycin agar plates and incubated overnight at 37°C. The recombinant bacteria were selected by direction selection where only recombinant bacteria grow on the plates and are shown as white colonies. The CES1 and CES2 cDNAs and organs from which the cDNA was isolated are as indicated.

To find the positive clones for original- and alternative splicing variants, the sample with the most positive colonies (recombinant bacteria) was selected: CES2_heart for TA-cloning and CES1_blood b for XL-cloning. Eight white colonies represent positive transformation were collected from each sample and amplified using Hot-Start PCR with following primers (Table 3.2.1B):

Table 3.2.1B Primers used for Hot-Start PCR before TA cloning.

cDNA	PRIMER USED			
	Primer pair	Forward primer (#)	Reverse primer (#)	Amplify exons:
CES1	1	162	163	5 to 9
CES2	2	329	330	1 to 5
CES2	3	335	336	7 to 11

Three pairs of primers were chose based on previous studies (86, 99). The PCR products were analyzed with agarose gel electrophoresis (Figure 3.2.1D).

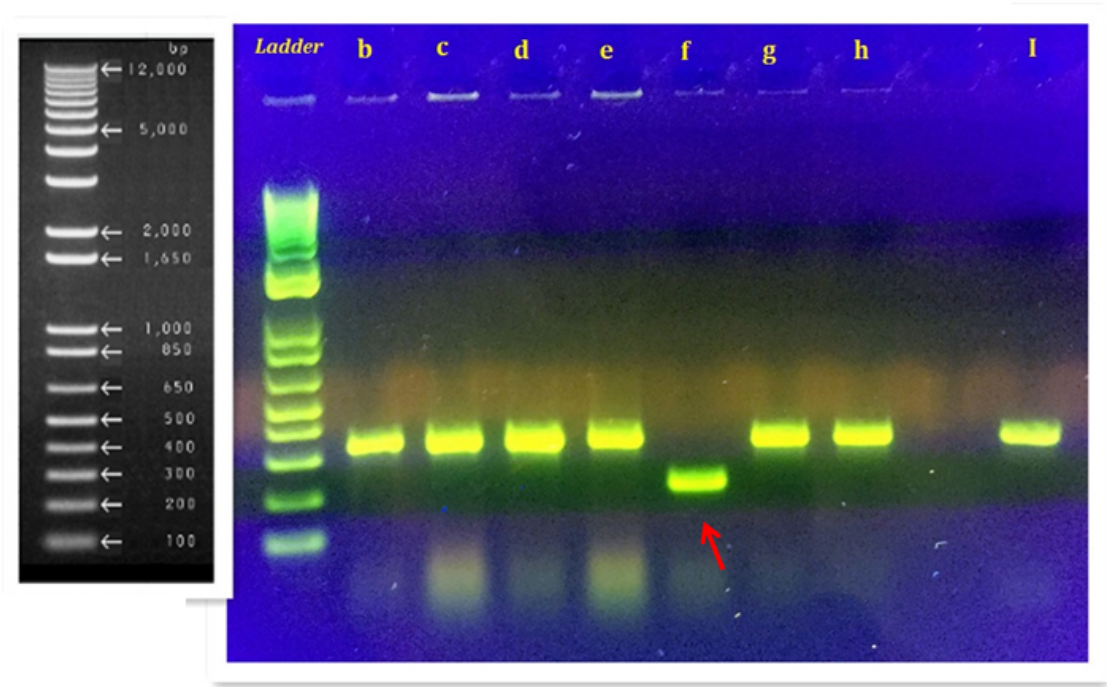


Figure 3.2.1D Electrophoresis analysis of PCR products with CES1-blood using primer pair (#162 and #163) which amplifies exon 5-9. The PCR products of positive transformations from CES1_blood were analyzed using agarose gel electrophoresis that ran for 1 hour at 120 volts; each well was loaded with mixture of 10µl of PCR product and 1µl of loading buffer. Well b-h are PCR products of positive colonies and well I is cDNA from liver tissue that was used as positive control. The red arrow indicates a DNA fragment that is shorter than the positive control.

Alternative splicing variants of CES1 were found with primer pair 1#162 and #163 that amplifies DNA fragment from exon 5 to 9. Alternative splicing variants of CES2 were found

with primer pair 2 #329 and #330 (exon 1 to 5) and primer pair 3 #335 and #336 (exon 7 to 11) (Figure 3.2.1D).

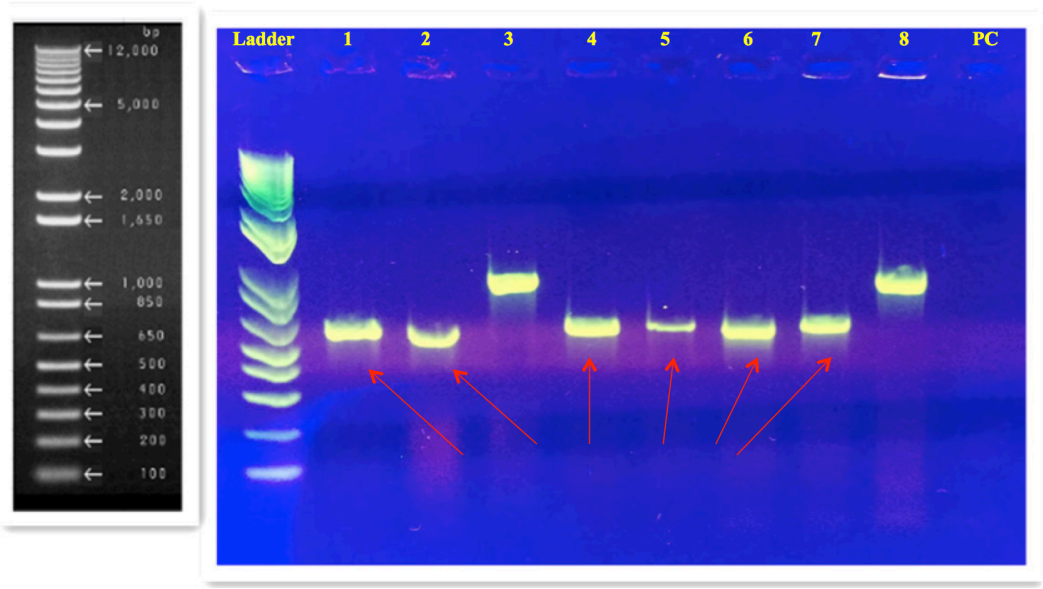


Figure 3.2.1E Electrophoresis analysis of PCR products with CES2-heart using primer pair (#329 and #330) which amplifies exon 1-5. The PCR products of positive transformations from CES2_heart were analyzed using agarose gel electrophoresis which ran for 1 hour at 120 volts; each well was loaded with mixture of 10µl of PCR product and 1µl of loading buffer. Well 1-8 are PCR products of positive colonies and well 9(PC) is cDNA from heart tissue that was used as positive control (No visible band. The samples were compared with positive control in figure 3.2.1F as it was the same sample ran under same condition.) The red arrows indicate DNA fragments which are shorter than the positive control.

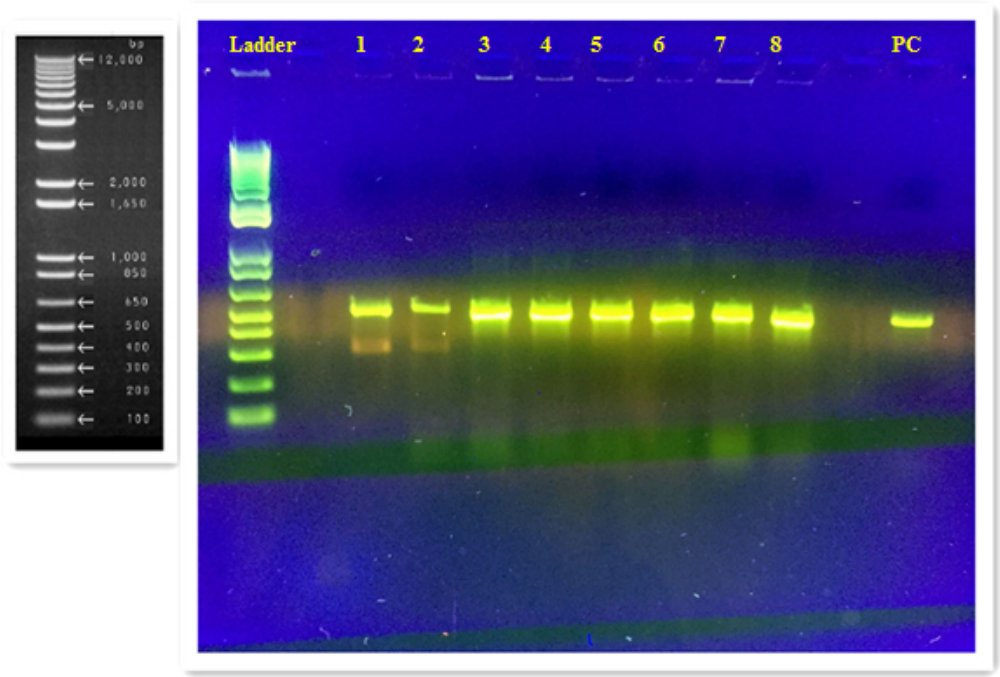


Figure 3.2.1F Electrophoresis analysis of PCR products with CES2-heart using primer pair (#335 and #336) which amplifies exon 7-11. The PCR products of positive transformations from CES2_heart were analyzed using agarose gel electrophoresis which ran for 1 hour at 120 volts; each well was loaded with mixture of 10µl of PCR product and 1µl of loading buffer. Well 1-8 are PCR products of positive colonies and well 9(PC) is cDNA from heart tissue that was used as positive control.

For CES1, the primer pair used would amplify DNA fragment from exon 5 to 9, the PCR product from colony f (Figure 3.2.1D) was shorter than the positive control, indicating an alternative splicing variant that is missing one or several exons between exon 5 to 9. For CES2, DNA fragments of different length were observed by amplifying the same colonies with two different pairs of primers. With primer pair 2 (#329 and #330) that amplifies DNA fragment between exon 1 to 5, six fragments were shorter than the positive control (Figure 3.2.1E). The results suggest that these DNA fragments might be alternative splicing variants that are missing one or several exons between exon 1 to 5. With primer pair 3 (#335 and #336) that amplifies DNA fragment between exon 7 to 11, all fragments were in the same length as the positive control (Figure 3.2.1F). The detected alternative splicing variants were summed up in Table 3.2.1C.

Table 3.2.1C Overview of alternative splicing variants in CES1 and CES2

DNA	Primer	Same as positive control	Shorter than positive control
CES1	Exon5-9	Colony- b,c,d,e,g,h	Colony- f
CES2	Exon1-5	Colony- 3,8	Colony- 1,2,4,5,6,7
CES2	Exon7-11	Colony- 1,2,3,4,5,6,7,8	None

The purified plasmid DNA of these samples was then isolated using the S.N.A.P™ Miniprep Kit. The purified plasmid DNA of colony- b, c, d, f of CES1 and colony- 1, 3, 4, 5, 6, 8 of CES2 with primer pair 2 (amplifies exon 1-5) were selected and sent to Eurofins Scientific Company for sequencing.

3.2.2 Analysis of alternative splicing variants of CES1

To understand function of the alternative splicing variants of CES1, following DNA sequences of the clones were further analyzed using bioinformatics software and online tools. The sequences were first aligned onto human reference genes using alignment tool BLAT to compare the exons in potential alternative splicing variants with the human reference genes (Figures 3.2.2A,B, C).

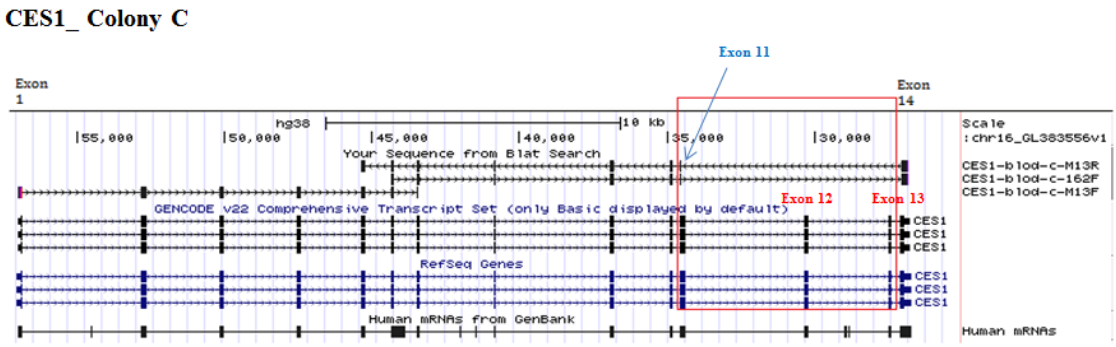


Figure 3.2.2A Screen print of analysis of CES1 _colony c by BLAT. The three lines on top represent the DNA sequence from CES1_colony c, the three blue lines at the bottom are human CES1 genes from genome bank which are used as reference genes. The black/blue blocks on the lines represent the exons present in the sequence. By comparing them, we found that exon 12, 13 and the most part of exon 11 (it is obviously thinner than the exon 11 in reference gene) are missing in CES1_colony c.

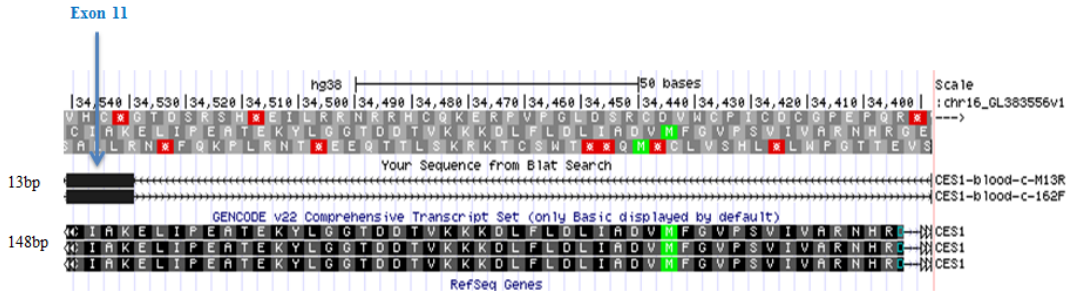


Figure 3.2.2B Screen print of analysis of exon 11 by BLAT which was displayed in zoomed view. By display the region of exon 11 in a zoomed view, we found that the exon 11 in CES1_colony c is 13bp in length (TGCATTGCCTAAG) and the reference genes are 148bp in length (34393-34541). Therefore the exon 11 on CES1_colony c is 135 bases shorter than the reference gene.

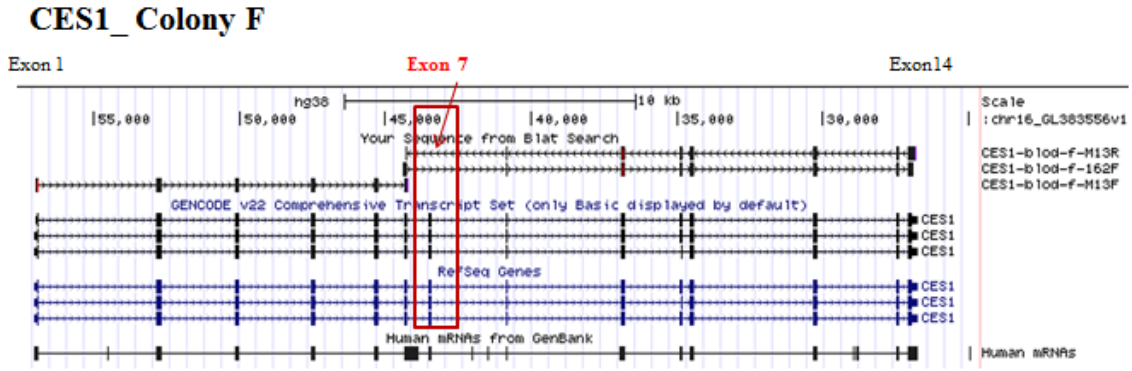


Figure 3.2.2C Screen print of analysis of CES1 _colony f by BLAT. The three lines on top represent the DNA sequence from CES1_colony f, the three blue lines at the bottom are human CES1 genes from genome bank which are used as reference genes. The black/blue blocks on the lines represent the exons present in the sequence. By comparing them, we found that exon 7 is missing in CES1_colony f.

As the alternative splicing variants of CES1_colony b, d are missing the exon 12 and 13 as colony c does, the screen prints of these two colonies are omitted. The only difference between these colonies is locations of several unmatched amino acids on the sequence. They are listed in the Table 3.2.2A below.

Table 3.2.2A Overview of missing exons and unmatched amino acids in CES1

Gene	Colony Number	Missing exons	Exons which are short than the Human gene	Location of Unmatched Amino acids	The amino acid on colony	The original amino acid human gen	Silent mutation?
CES1 Blood	B	12 &13	Exon 11: ~150 bases shorter	Exon 2	Arginine Alanine Phenylalanine Isoleucine Serine	Proline Alanine Leucine Valine Alanine	No Yes No No No
				Exon 7	Serine	Serine	Yes
				Exon 14	Isoleucine	Threonine	No
CES1 Blood	C	12&13	Exon 11: ~150 bases shorter	Exon 2	Arginine Alanine Phenylalanine Isoleucine Serine	Proline Alanine Leucine Valine Alanine	No Yes No No No
CES1 Blood	D	12&13	Exon 11: ~150 bases shorter	Exon 2	Arginine Alanine Phenylalanine Isoleucine Serine	Proline Alanine Leucine Valine Alanine	No Yes No No No
				Exon 7	Glutamic acid	Glutamic acid	Yes
CES1 Blood	F	7		Exon 2	Arginine Alanine Phenylalanine Isoleucine Serine	Proline Alanine Leucine Valine Alanine	No Yes No No No
				Exon 9	Isoleucine	Methionine Start codon	No

After confirming which exons are missing, the ATGpr was used to predict the full amino acid sequence of alternative splicing variant. The amino acid sequences of alternative splicing variants were then compared with the amino acid sequence of reference gene using Clustal Omega. We found that the absence of exons 11(partly), 12 and 13 in CES1_colony c led to difference in amino acid 439-575 when comparing with the reference gene (alignment is shown in Appendix 6.2.1.A). As for CES1_colony f, the absence of exon 7 led to difference in the amino acid 267-302 when comparing with the reference gene (alignment is shown in Appendix 6.2.1.B).

Functional annotation of CES1 accordning to **Uniprot**:

Sites

Feature key	Position(s)	Length	Description
Active site	221 – 221	1	Acyl-ester intermediate PROSITE-ProRule annotation 1 Publication
Active site	354 – 354	1	Charge relay system 1 Publication
Active site	468 – 468	1	Charge relay system 1 Publication

Mutagenesis

Feature key	Position(s)	Length	Description
Mutagenesis	79 – 79	1	N → A: Abolishes glycosylation. 1 Publication
Mutagenesis	221 – 221	1	S → A: Loss of activity. 1 Publication
Mutagenesis	354 – 354	1	E → A: Loss of activity. 1 Publication
Mutagenesis	468 – 468	1	H → A: Loss of activity. 1 Publication
Mutagenesis	564 – 567	4	Missing : Does not result in secretion. 1 Publication

Figure 3.2.2C The characterization of CES1. A list of functional annotation of CES1 was obtained according to Uniprot.

Further, the functional properties of alternative splicing variant in CES1_colony c was analyzed by using Uniprot based on the missing amino acids at the end of the sequence. Mutation at the active site on position 468 was found to result in loss of enzyme activity, and mutagenesis on position 554-567 could lead to non-secretion of the CES1 variant. For alternative splicing variant in CES1_colony f, the missing amino acids are not located within the active sites and mutagenesis sites.

3.2.3 Analysis of alternative splicing variants in CES2

To understand function of the alternative splicing variants of CES2, the DNA sequences were also further analyzed using bioinformatics software and online tools. The sequences were first aligned onto human reference genes using alignment tool BLAT to compare the exons in potential alternative splicing variants with the human reference genes (Figure 3.2.3A, B).

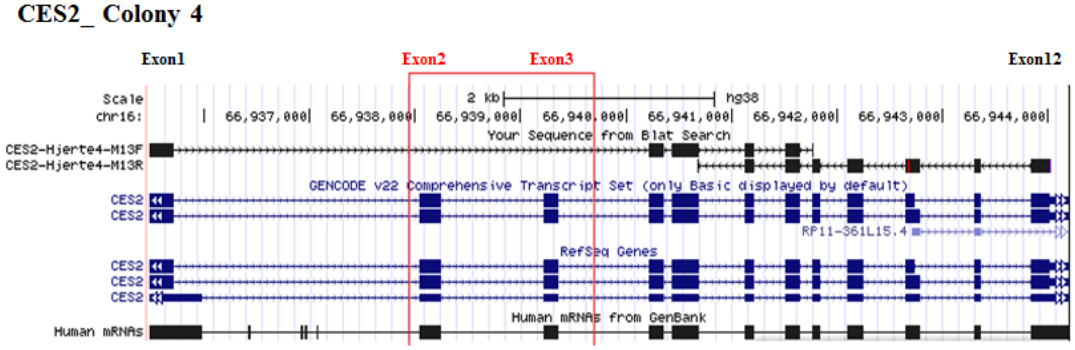


Figure 3.2.3A Screen print of analysis of CES2 _colony 4 by BLAT. The two lines on top represent the DNA sequence from CES2_colony 4, the three blue lines at the bottom are human CES2 genes from genome bank which are used as reference genes. The black/blue blocks on the lines represent the exons present in the sequence. By comparing them, we found that exons 2 and 3 are missing in CES2_colony 4.

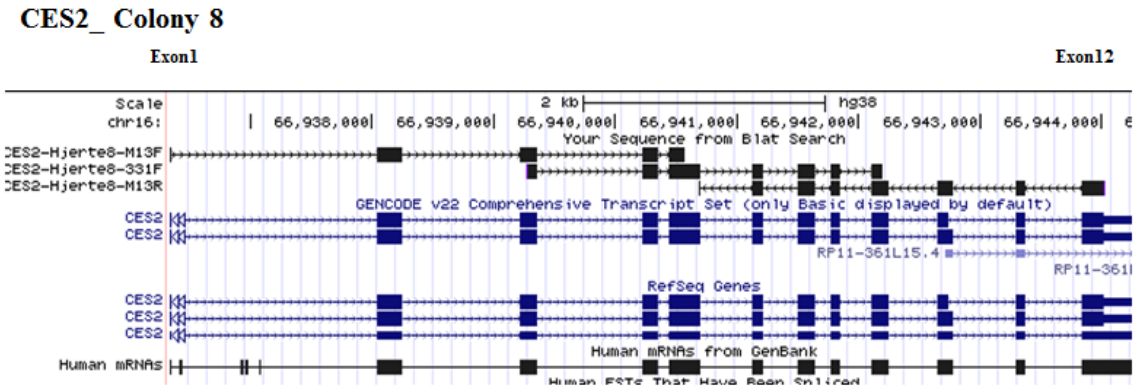


Figure 3.2.3B Screen print of analysis of CES2 _colony 8 by BLAT. The three lines on top represent the DNA sequence from CES2_colony 8, the three blue lines at the bottom are human CES2 genes from genome bank which are used as reference genes. The black/blue blocks on the lines represent the exons present in the sequence. By comparing them, we found that DNA sequences CES2_colony 4 contain the same amount of exons as the reference gene.

As the BLAT analysis of CES2_colony 1,5,6 were very similar with CES2_colony 4 and the analysis of CES2_colony 3 was the same as CES2_colony8, the screen prints of these colonies were omitted. The only difference between these colonies was several unmatched amino acids on the sequence. They are listed in the Table 3.2.3.

Table 3.2.3 Overview of missing exons and unmatched amino acids in CES2

Gene	Colony Number	Missing exons	Location of Unmatched Amino acids	The amino acid on colony	The original amino acid on human gen	Silent mutation?
CES 2 Heart	1	2 & 3	None	-	-	-
CES 2 Heart	3	None	Exon 4	Serine	Glycine	No
CES 2 Heart	4	2 & 3	Exon 10	Glycine	Glutamic acid	No
CES 2 Heart	5	2 & 3	None	-	-	-
CES 2 Heart	6	2 & 3	None	-	-	-
CES 2 Heart	8	None	None	-	-	-

By using ATGpr and Clustal Omega, we found that the alternative splicing variant of CES2_colony 4 were missing 207 amino acids at the beginning of the sequence due to absence of exon 2 and 3.

Functional annotation of CES2 accordning to **Uniprot**:

Sites

Feature key	Position(s)	Length	Description	Graphical view	Feature identifier	Actions
Active site	228 – 228	1	Acyl-ester intermediate PROSITE-ProRule annotation			
Active site	345 – 345	1	Charge relay system By similarity			
Active site	457 – 457	1	Charge relay system By similarity			

Figure 3.2.3C The characterization of CES2. A list of functional annotation of CES2 was obtained according to Uniprot.

According to Uniprot (Figure 3.2.3C), the three active sites of CES2 locate at position 228, 345 and 457. Since the amino acid sequence of alternative splicing variant was the same as the reference gene from position 207, it is suggested that the absence of exon 2 and 3 in alternative splicing variant does not lead to changes in biological function of the enzyme.

3.3 Quantification of original- and alternative splicing variants

In this study, RT-qPCR was used for quantification of expression level of RNA from CES1 and CES2 in different tissues (the tissues which were used are listed in chapter 2.2.1). Our intention was to test the expression level in both CES1 and CES2, but it was difficult to design probes that are specific for CES1. Therefore only expression level of CES2 was tested with hydrolysis probes CES2_EX2_3SPL and CES_EX8_SPL (described in chapert 2.6.2). GAPDH and PGK1 were used as reference genes in RT-qPCR as they would give suitable normalization factors for quantification.

The normalized target amount ($\mathbf{2^{-\Delta\Delta Ct}}$) was calculated based on measurements of RT-qPCR. (The measurements and calculation are shown in Appendix 6.2.2.) Relative to the expression level in fetal brain tissues, expression of splicing variants without exon 2 and 3 was dramatically decreased in all other tissues tested (Figure 3.3A). Also expression of splicing variants without exon 8 was significantly decreased in all tissues investigated relative to the expression level in liver B tissue (Figure 3.3B).

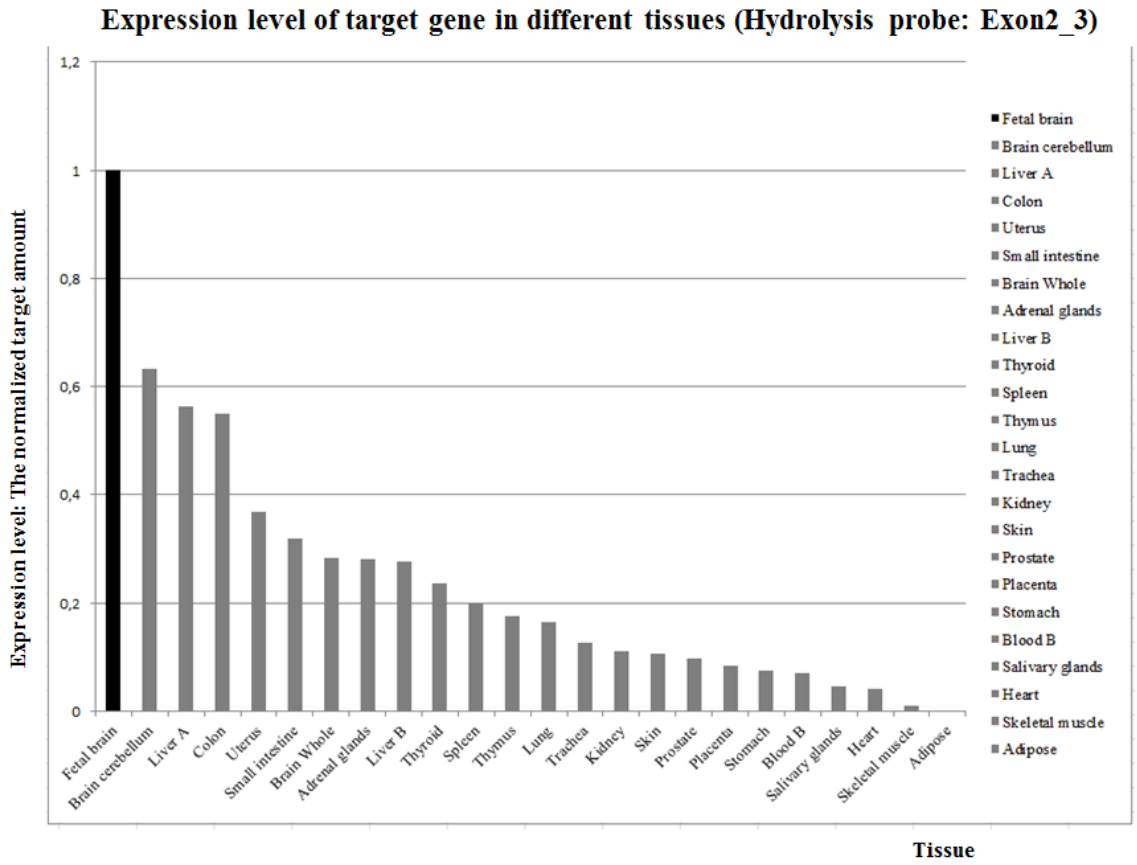


Figure 3.3A Expression levels of CES2 in different tissues with hydrolysis probe (CES2_EX2_3SPL). Y-axis: CES2 expression level; X-axis: tissues. Fetal brain tissues with the lowest ΔCt were used as reference (expression level = 100%), the expression levels of other tissues were compared to the expression level in Fetal brain tissue (used as reference).

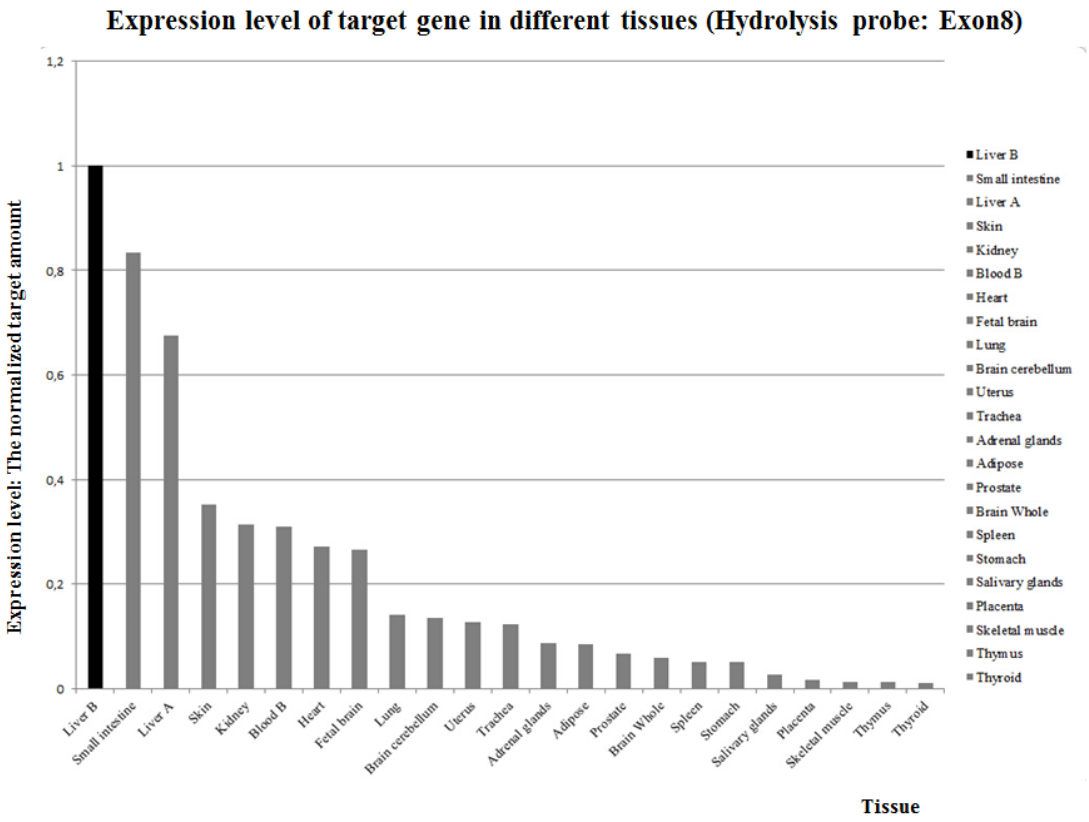


Figure 3.3B Expression levels of CES2 in different tissues with hydrolysis probe (CES2_EX8_SPL). Y-axis: CES2 expression level; X-axis: tissues. Liver tissues with the lowest ΔCt were used as reference (expression level = 100%), the expression levels of other tissues were compared to the expression level in liver tissue (reference).

3.4 Enzyme activity of alternatively spliced CES

To measure enzyme activity, alternative splicing variant needed to be cloned into an expression vector first.

3.4.1 Construction of expression plasmid

For enzyme activity measurement of alternatively spliced CES1 and CES2, two samples of each variant were used for PCR-product insertion, cloning and protein production. Since no original splicing variant of CES1 was detected in this study, cDNA from liver tissue was used for cloning for CES1 (Table 3.4.1A).

Table 3.4.1A Samples used for measuring enzyme activity.

DNA	Original splicing variant	Alternative splicing variant
CES1	Liver cDNA	Colony- c, f
CES2	Heart cDNA	Colony- 4, 8

The samples of plasmids were first amplified using LR_PCR (described in chapter 2.2.3) with following primers:

cDNA	PRIMER USED			
	Primer pair	Forward primer (#)	Reverse primer (#)	Amplify exons:
CES1	1	420	421	1 to 14
CES2	2	417	419	1 to 12
CES2	3	418	419	4 to 12

The PCR products were characterized and purified using agarose gel electrophoresis and DNA extraction (Figure 3.4.1A).

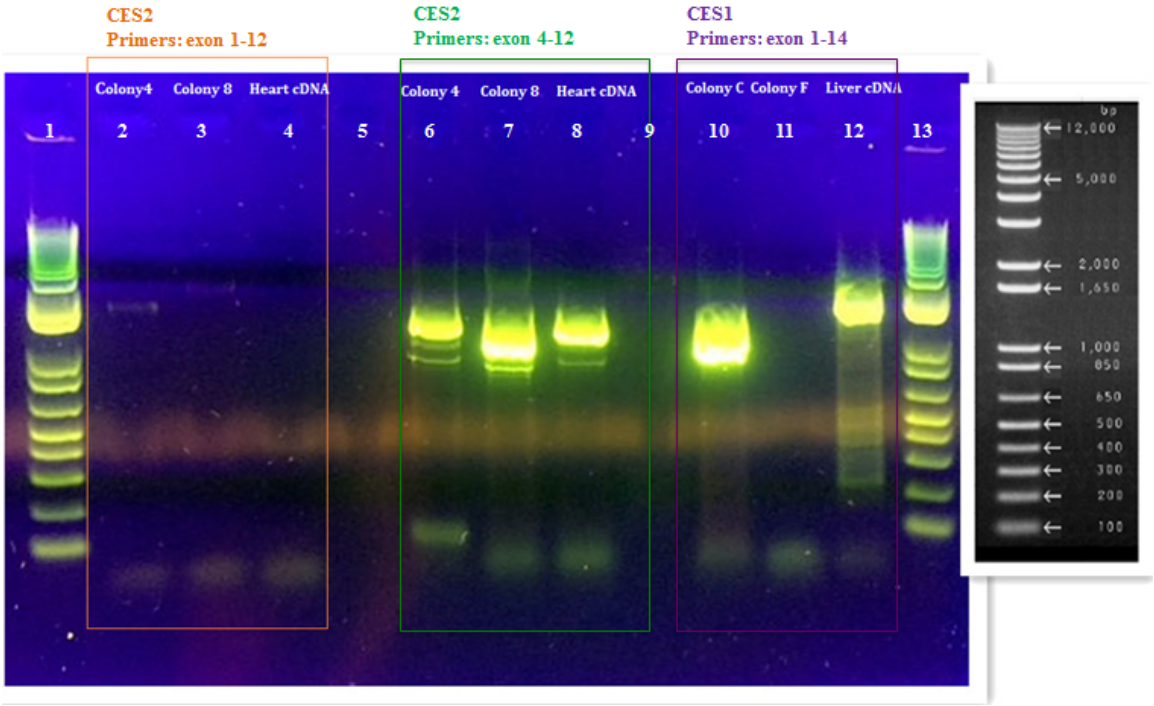


Figure 3.4.1A LR- PCR amplification and characterization of CES1 and CES2. The PCR products were analyzed using agarose gel electrophoresis that ran for 45 minutes at 120 volts; each well was loaded with mixture of 10µl of PCR product and 1µl of loading buffer. Well 1 and 13 were loaded with ladder. Well 2-4 (inside orange box) were loaded with PCR products of alternative splicing variants of CES2 with primer pair (#417 and 419) that amplifies exon 1 to 12. Well 6-8 (inside green box) were loaded with PCR products of original- and alternative splicing variants of CES2 with primer pair (# 418 and 419) that amplifies exon 4-12. Well 10-12 (inside purple box) were loaded with PCR products of alternative splicing variants of CES1 with primer pair (# 420 and 421) that amplifies exon 1-14.

The LR-PCR was repeated for CES2 as there was no result for CES2 with primer pair that amplifies exon 1-12 in the first PCR (Figure 3.4.1B).

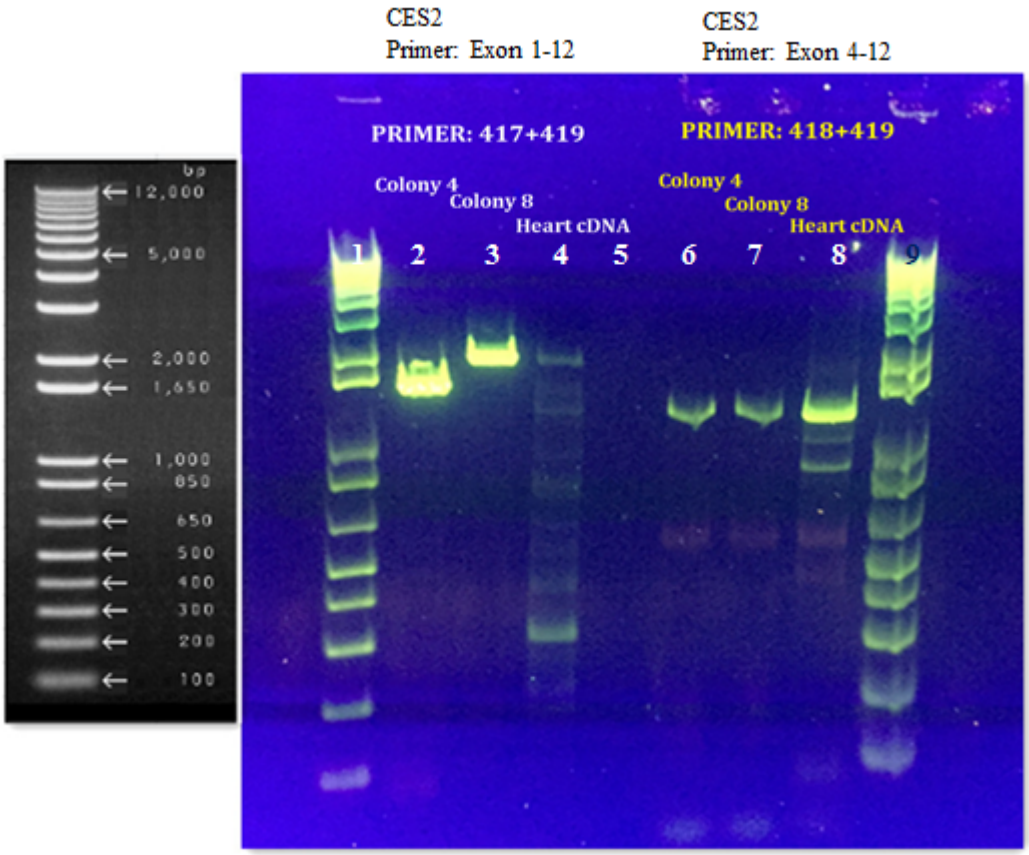


Figure 3.4.1B LR-PCR amplification and characterization of CES2. The PCR products were analyzed using agarose gel electrophoresis which ran for 45 minutes at 120 volts; each well was loaded with mixture of 10µl of PCR product and 1µl of loading buffer. Well 1 and 9 were loaded with ladder. Well 2-4 were loaded with PCR products of alternative splicing variants of CES2 with primer pair (#417 and 419) that amplifies exon 1 to 12. Well 6-8 were loaded with PCR products of original- and alternative splicing variants of CES2 with primer pair (# 418 and 419) that amplifies exon 4-12.

With primer pair 1 (#417 and 419) that amplifies exon 1-12 in CES2, the DNA fragment of colony 4 was shorter than colony 8. It is reasonable as colony 4 lacks exon 2 and 3, where exon 8 contains all 12 exons (described in chapter 3.2.3). With primer pair 2 (#418 and 419) that amplifies exon 4-12 in CES2, the DNA fragments from colony 4 and colony 8 were same in length, suggesting that they contain all the exons 4-12. Since we were not able to extract optimal DNA sample from colony f of CES1 (Figure 3.4.1A well 11, no DNA band present), five samples were used for further analysis: Liver cDNA, plasmid from alternative splicing variant found in CES1_colony c, plasmids from alternative splicing variant found in CES2_colony 4,8 (with primer that amplifies exon 1-12) and plasmid from alternative splicing variant found in CES2_colony 4 (with primer pair that amplifies exon 4-12).

The purified PCR products were then cloned into vector pcDNA™3.1/V5-His-TOPO® using TA cloning method described in chapter 2.4. The resultant transformants were plated on LB agar plate containing ampicillin for selection.

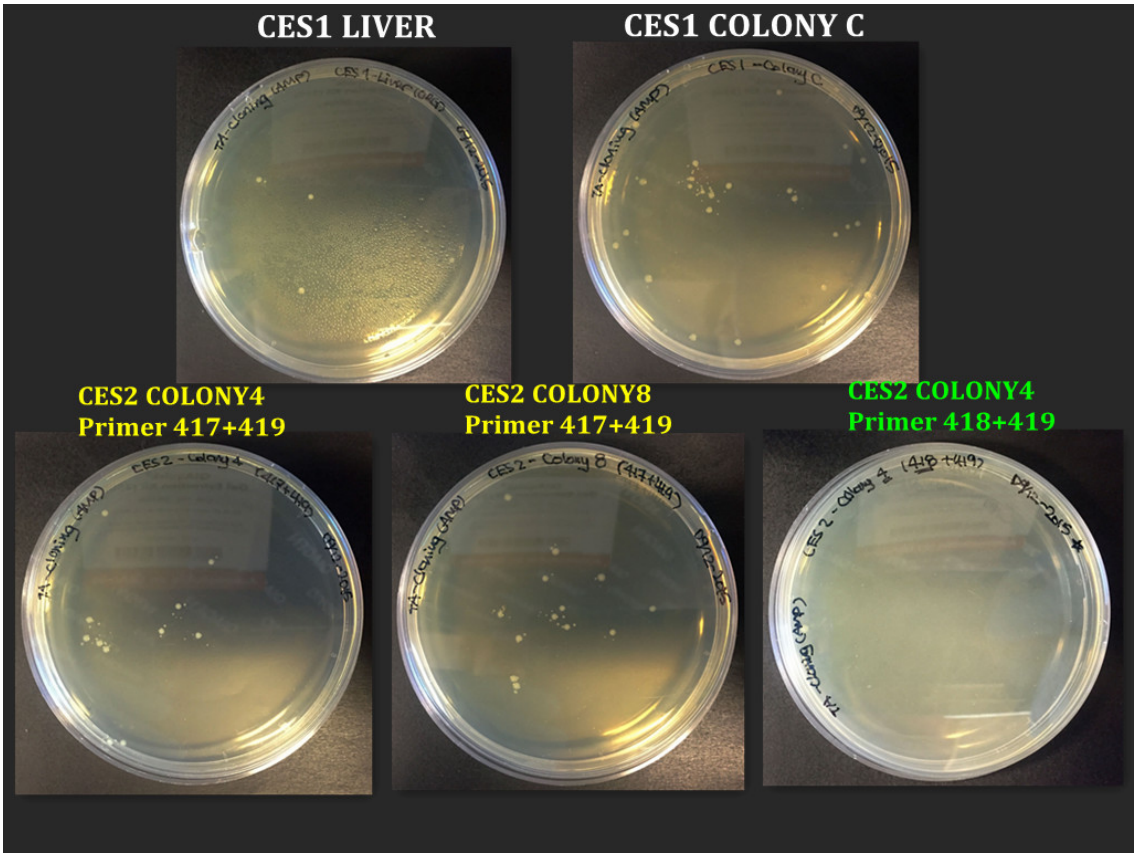


Figure 3.4.1C Overview of ampicillin agar plates with pcDNA™3.1/V5-His-TOPO® TA Expression Kit. The five purified DNA products were cloned into pCR®3.1/V5-His-TOPO® vector using TOPO TA cloning. The transformation mixture were poured onto ampicillin agar plates and incubated overnight at 37°C. The recombinant bacteria were selected by direction selection where only recombinant bacteria grow on the plates and are shown as white colonies.

For colony c of CES1 with primer pair 1, about 15 white colonies were observed. The growth of approximately 10 white colonies was observed with both colony4 and 8 of CES2 with primer pair 2. Colonies from these three agar plates were selected for further analysis. There was no growth of any colony of CES2_colony 4 with primer pair 2 (exon4-12) on agar plate, and very few colonies with liver cDNA. Therefore these were not included in further analysis.

3.4.2 Identification of correct orientation of PCR insertion – used to find correct reading frame.

Before measuring enzyme activity, it is necessary to identify the orientation of PCR insertion in order to find the correct reading frame. Several colonies were collected from each of the three plates and cut with restriction enzyme BamHI (for CES1) and enzyme XhoI (for CES2) with procedures described in chapter 2.7.3. The amount of DNA sample used was calculated based on measurement from NanoDrop (Calculation are shown in Appendix 6.2.3). The length of DNA fragment after cutting with restriction enzyme was used to determine the orientation of PCR insertion (described in details in Appendix 6.2.4). The expected lengths of DNA fragments after cutting with restriction enzymes in both orientations are listed in Table 3.4.2A.

Table 3.4.2A The expected length of DNA fragments after cutting with (BamHI/XhoI):

SAMPLE	CORRECT ORIENTATION (bp)	INCORRECT ORIENTATION (bp)
CES1_original	457 & 6786	1329 & 5914
CES1_colony c	457 & 6569	1112 & 5914
CES2_colony 8	7328 & 73	5520 & 1881
CES2_colony 4	6710 & 73	5520 & 1263

The successful insertion of PCR product in correct orientation can be confirmed by presence of two DNA fragments in expected length after gel electrophoresis. For CES1 with insertion in correct orientation, two DNA fragments (one is in length of 457bp and the other is 6786bp) would be expected. For insertion of CES2 from colony 8 (with exon 1-12) in correct orientation, we are expecting one DNA fragment of 7328bp and one fragment of 73bp. For CES2 with insertion from colony 4 (missing exon 2 and 3) in correct orientation, two DNA fragments (one is in length of 6710bp and the other is 73bp) would be expected. Fragments which are longer represent either incorrect insertion or insertion in reverse direction.

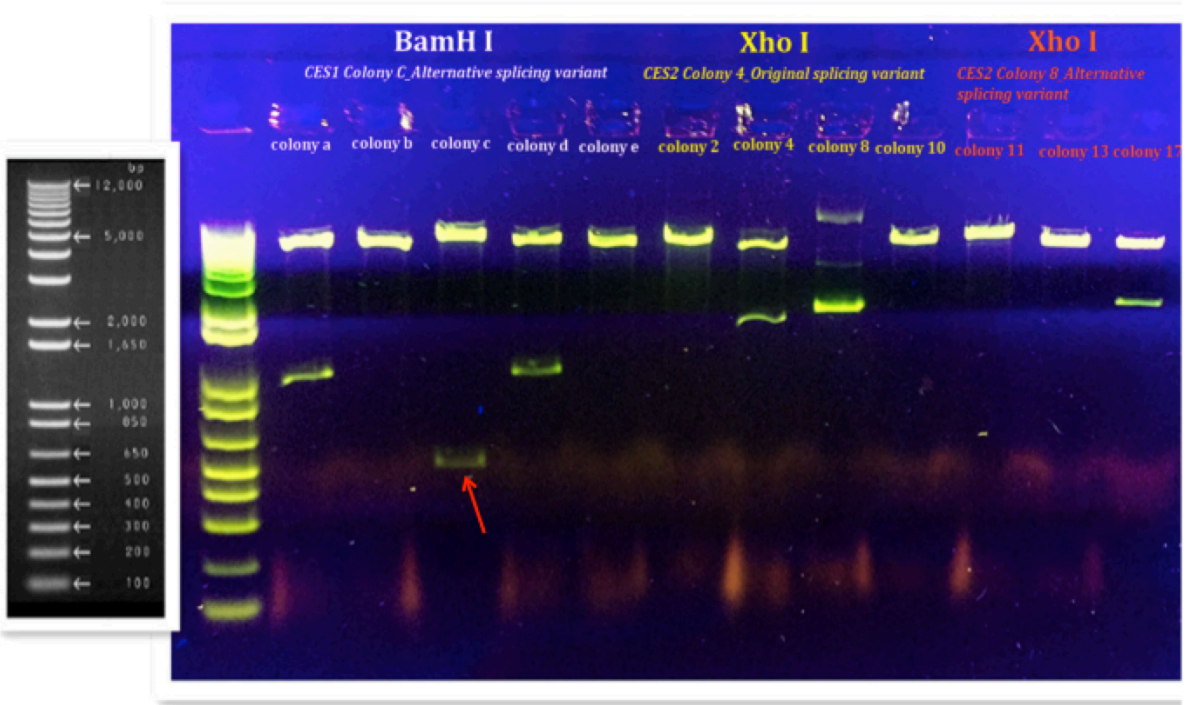


Figure 3.4.2A. Characterization of CES1 or CES2 insertion in correct orientation– part I. The PCR products were analyzed using agarose gel electrophoresis that ran for 45 minutes at 120 volts; each well was loaded with mixture of 10µl of PCR product and 1µl of loading buffer except well 1 which was loaded with ladder. Well 2-6 (labeled with white color) were loaded with PCR products of colonies from CES1_colony c which was cut with enzyme BamHI. Well 7-10 (labeled with yellow color) were loaded with PCR products of colonies from CES2_colony 4 which was cut with enzyme XhoI. Well 11-13 (labeled with red color) were loaded with PCR products of colonies from CES2_colony 4 which was cut with enzyme XhoI. The red arrow points to the product with successful insertion in correct orientation.

Table 3.4.2B Characterization of the correct insertion – part I:

WELL	SAMPLE	ENZYME	COLONY	ORIENTATION
1	Ladder			
2	CES1_colony c	BamH I	a	Reverse
3	CES1_colony c	BamH I	b	None insertion
4	CES1_colony c	BamH I	c	**Correct**
5	CES1_colony c	BamH I	d	Reverse
6	CES1_colony c	BamH I	e	None insertion
7	CES2_colony 4	Xho I	2	None insertion
8	CES2_colony 4	Xho I	4	Incorrect
9	CES2_colony 4	Xho I	8	Incorrect
10	CES2_colony 4	Xho I	10	None
11	CES2_colony 8	Xho I	11	None
12	CES2_colony 8	Xho I	13	None
13	CES2_colony 8	Xho I	17	Incorrect

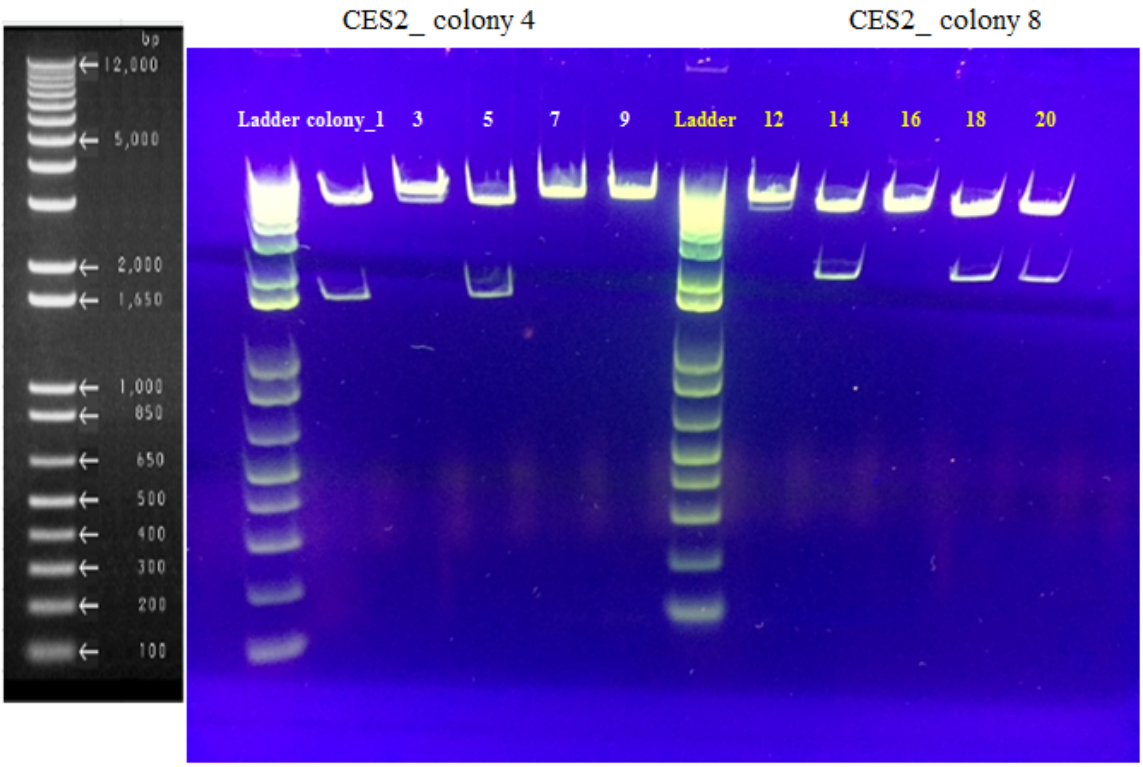


Figure 3.4.2B. Characterization of CES2 insertion in correct orientation– part II. The PCR products were analyzed using agarose gel electrophoresis that ran for 45 minutes at 120 volts; each well was loaded with mixture of 10µl of PCR product and 1µl of loading buffer except well 1 and 7 which were loaded with ladder. Well 2-6 (labeled with white color) were loaded with PCR products of colonies from CES2_colony 4 which was cut with enzyme XhoI. Well 7-12 (labeled with yellow color) were loaded with PCR products of colonies from CES2_colony 8 which was cut with enzyme XhoI.

Table 3.4.2C Characterization of the correct insertion – part II:

WELL	SAMPLE	ENZYME	COLONY	ORIENTATION
1	Ladder			
2	CES2_colony 4	Xho I	1	Incorrect
3	CES2_colony 4	Xho I	3	None insertion
4	CES2_colony 4	Xho I	5	Incorrect
5	CES2_colony 4	Xho I	7	None insertion
6	CES2_colony 4	Xho I	9	None insertion
7	Ladder			
8	CES2_colony 8	Xho I	12	None insertion
9	CES2_colony 8	Xho I	14	Incorrect
10	CES2_colony 8	Xho I	16	None insertion
11	CES2_colony 8	Xho I	18	Incorrect
12	CES2_colony 8	Xho I	20	Incorrect

As shown in Figure 3.4.2A,B and Table 3.4.2B, C, in all the colonies tested, CES1_colony c was the only one that shown to have successful insertion in correct orientation. Therefore it was not possible to measure enzyme activity of both original- and alternative splicing variants as we planned. We decided to measure and compare the enzyme activities of samples with- and without CES insertion.

3.4.3 Measure enzyme activity (Pilot study)

The enzyme activity of HEK293 cells carrying plasmid with correct insertion (CES1_colony c), one carrying plasmid without insertion (CES1_colony b) and one blank sample was measured. Procedures of cell culture and transfection are described in in chapter 2.7.5, preparation and procedures of measuring enzyme activity are described in chapter 2.7.6. Two parallel measurements were taken for each sample.

The measurements were shown in Appendix 6.2.5. The measurements after 96 hours (day5) were shown to be identical as measurements after 72 hours (day 4). It is uncertain if it was due to machine or technical problems. Thereby the measurements after 96 hours were excluded from further analysis.

As described in chapter 2.7.6, enzyme activity of both the 96-well plate where the cells were seeded (referred as “cell”) and the cell culture supernatant (referred as cell medium) were measured by machine in 50 seconds interval for 15 minutes (giving 15 measurements for each). By plotting the 15 measurements in linear graph, we have found that the measurements were quite random and varied in large range. It was difficult to define a trend for the measurements (Linear graphs are listed in Appendix 6.2.5).

Therefore we have calculated the average of the 15 measurements for each sample and plotted it against time of incubation (as shown in Figure 3.4.3). However there is still no clear trend and it is impossible to define the relation between the enzyme activity and time of incubation.

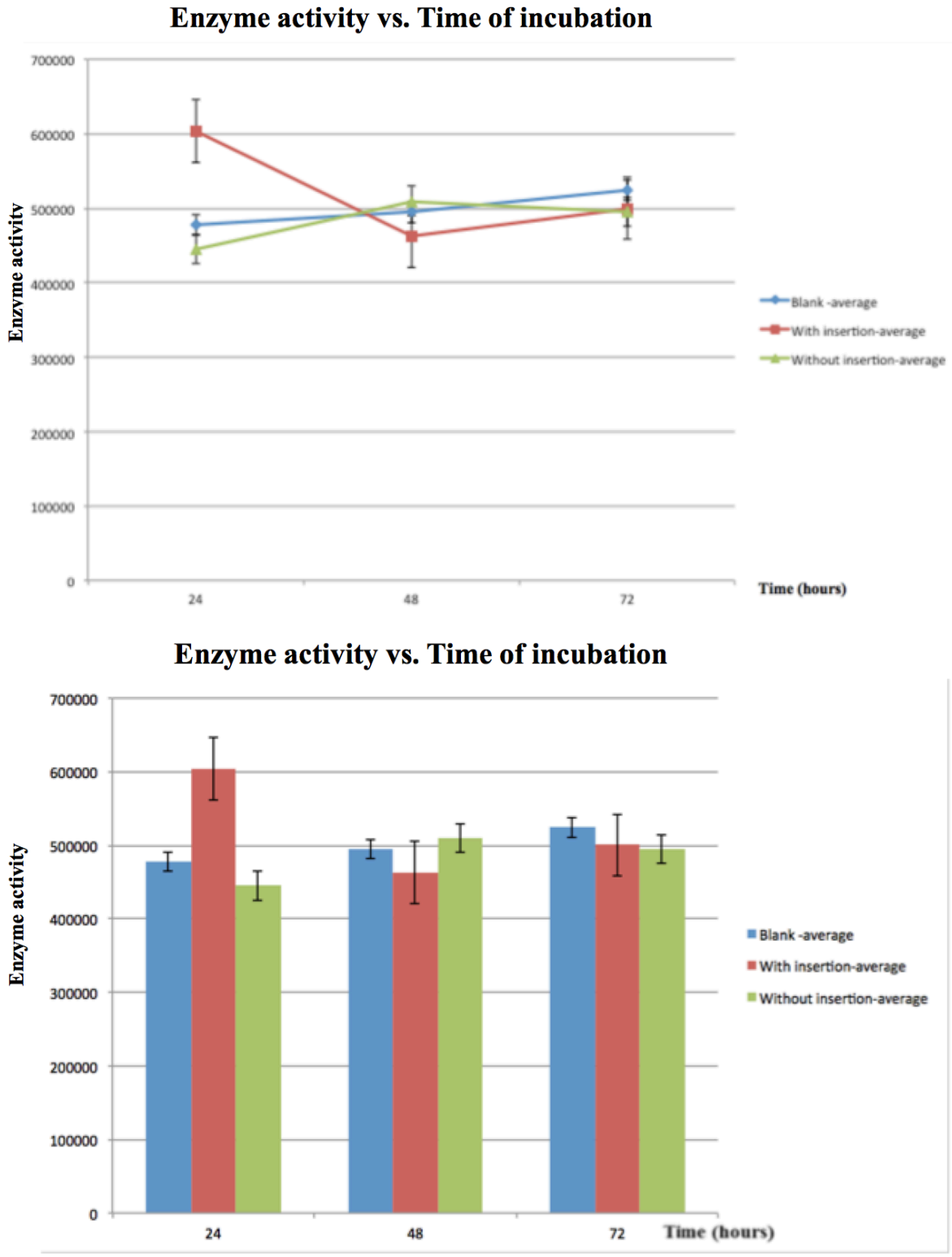


Figure 3.4.3 The enzyme activity measured after 24, 48 and 72 hours of incubation.). Average of the two parallel measurements of each sample was calculated and plotted against time of incubation using linear figure (top) and column chart (bottom).

4 Discussion

4.1 Detection of alternative splicing variants in CES1 and CES2

CES1 and CES2 are expressed in liver, heart, blood, spleen and skin tissues (39). In this study cDNA of the *CES1* and *CES2* genes from these tissues were used as templates for LR-PCR to amplify cDNA fragments with optimal primers. Multiple PCR bands that represent potential alternative splicing variants were observed in four PCR reactions. For other cDNA samples, no band or very weak bands were observed. The reasons for this could be pipetting error, insufficient template, not optimal concentration of primers or alternative splicing variants are not expressed in these tissues (12). Since we have already observed two samples for each gene that might contain alternative splicing variant, we decided to focus on them.

These PCR fragments of interest were purified, extracted and then cloned into plasmid vectors. The amount of positive colonies grew on each agar plate were quite different. White colonies from the agar plates with the most positive colonies (CES1 from blood tissue and CES2 from heart tissue) were amplified with Hot-start PCR to detect positive clones for original- and alternative splicing variants. For the *CES1* gene that was amplified with primer pair 1 (amplifies exon 5-9), one PCR fragment (from colony f) was shown to be shorter than the positive control. It could be an alternative splicing variant of CES1 with deletion of one or several exons that locate between exon 5 to 9. Other PCR fragments were shown to be in the same length as the positive control suggesting that they are either original splicing variants or have deletion of exons in locations other than exon 5 to 9. For the *CES2* gene amplified with primer pair 2 (amplifies exon 1-5), six PCR fragments (from colony 1, 2, 4, 5, 6, 7) were shown to be shorter than the positive control. These fragments could be alternative splicing variants of CES2 with deletion of one or several exons that locate between exon 1 to 5. Other PCR fragments were shown to be in the same length as the positive control suggesting that they are either original splicing variants or have deletion of exons in locations other than exon 1 to 5. For the *CES2* gene amplified with primer pair 3 (amplifies exon 7-11), all PCR fragments were shown to be in the same length as the positive control suggesting that they are either original splicing variants or have deletion of exons in locations other than exon 7 to 11. Colony -b, c, d, f of *CES1* and colony-1, 3, and 4,5,6,8 of *CES2* with primers pair 2 were sent for sequencing.

4.2 Analysis of alternative splicing variants in CES1 and CES2

Based on results of sequencing and help of several bioinformatics tools, in CES1 we found one new splicing variant and one alternative splicing variant which was also detected in a previous study(99). Based on analysis with several bioinformatics tools, we found that entire exon 12; exon 13 and most part of exon 11 (135bp) were spliced out in the new alternative-splicing variant. The missing exons could give rise to different structures of mRNA and thereby affect the function of the protein that they encoded (As described in chapter 1.2). The amino acid sequence of the new alternative splicing variant is altered as the last 136 amino acids (439-575) are missing, and is different when comparing with the reference gene. Enzyme CES1 contains three active sites; one of them is located at position 468 that is within the missing amino acid sequence. It is reasonable to suggest that the new alternative splicing variant would have lower enzyme activity than the original splicing variant. According to Uniprot, deletion of amino acids 564-567 may cause that the enzyme is not secreted. It is difficult to say if the new alternative splicing variant would encode for a functional protein. The alternative splicing variant without exon 7 was known from previous study(99). Based on analyzing with bioinformatics tools, it has been found that the deletion of exon 7 leads to missing of amino acid 267-302. Neither active site nor mutagenesis site of CES1 locate within this range, therefore it is reasonable to suggest that the alternative splicing variant could encode a functional protein.

In CES2, only known alternative splicing variant was detected. As described previously(86), one alternative splicing variant with deletion of exon 2 and 3 was detected. The alternative splicing variant lacks the first 207 amino acids relative to the original splicing variants. There are three active sites (at 228, 345 and 457) within CES2; absence of the first 207 amino acids may not affect the activity of enzyme since the missed exons do not contain the active sites. It is difficult to say whether the structure and function of the protein would be altered in the alternative splicing variant as the disulfide bond at amino acid 159-187 is missing(86). Several unmatched amino acids were observed in these DNA fragments (Table 3.2.2A and Table 3.2.3), some are silent mutations that is when change one codon for another one do not affect the coding meaning of the gene and the others are not (100). It is difficult to conclude whether these unmatched amino acids in DNA fragments are actual mutations or random PCR-based mutagenesis. Since this study is focused on naturally occurring alternative

splicing variants, the reason and consequences of unmatched amino acids would not be further studied and discussed.

4.3 Quantification of alternative splicing variants in CES2

The expression levels of CES2 in different tissues were measured using RT-qPCR with two different probes. Probe CES2_EX2_3SPL was designed to span the exon 1- exon 4 junction, and probe CES2_EX8_SPL was designed to span the exon 7 - exon 9 junction. With probe CES2_EX2_3SPL, the target gene was shown to have the highest expression level in fetal brain tissue (as shown in Figure 3.3A). The expression level is also relatively high in brain cerebellum, liver, colon, uterus, small intestine and adrenal glands tissues. With probe CES2_EX8_SPL, the target gene was shown to have the highest expression level in liver tissue. The expression level is also relatively high in small intestine tissue (as shown in Figure 3.3B). The difference between liver and small intestine tissue is not very significant. Expression level in tissues such as skin, kidney, blood, heart and fetal brain is about one-third of the expression level in liver tissues.

A previous study performed a similar qPCR using probe for the original CES2 splicing variant, where the expression level of target gene was shown to be highest in liver tissue and relatively high in small intestine and large intestine tissues (86). Comparing with this study, we observed that alternative-splicing variant of CES2 with deletion of exon 2 and 3 was expressed in different tissues. It is reasonable to suggest that alternative splicing could regulate the expression level CES2 genes and thereby determine intracellular localization of the proteins encoded by CES2.

4.4 Enzyme activity of carboxylesterase

Alternative splicing could alter the enzyme activity of proteins in different aspects (described in chapter 1.1.2). Our goal was to measure and compare the enzyme activity of both original- and alternative splicing variants of CES1 and CES2, and study whether the missing exons in alternative splicing variant would affect enzyme activity of the enzyme. For both genes, one original- and one alternative splicing variant were amplified using LR-PCR with designed primers. We observed clear PCR bands in cDNA from liver tissue (used as original splicing variant for CES1), CES1_colony c (as alternative splicing variant for CES2), CES2_colony 4 (alternative splicing variant for CES2) and CES2_colony 8 (original splicing variant for CES2) when analyzed using agarose gel electrophoresis. These PCR products were then cloned into pCR®3.1/V5-His-TOPO® expression vector for further study of gene expression in cells.

To find the correct reading frame, the orientation of PCR insertion was identified by cutting with restriction enzyme and characterized using agarose gel electrophoresis. In the first round of gel electrophoresis, we found that most PCR insertion was either unsuccessful or incorrect. There were two successful insertions in reverse orientation, and only one successful insertion in correct orientation (colony c taken from alternative splicing variant of CES1). We repeated the restriction enzyme cutting method with different colonies from original- and alternative splicing variants of CES2; the results were either incorrect insertion or non-insertion. However, among all the insertions of *CES1* or *CES2* into the expression vector that we have studied, alternative splicing variant in CES1 was the only one that found to be successfully inserted in correct orientation. We were only able to study and compare the enzyme activities of cells with- and without correct insertion of CES1.

HEK-293 cell line was used in this study as low carboxylesterase activity is present in this cell line in general (97). Significantly higher enzyme activity has been detected in whole-living HEK-293 hCES2 transfected cells than in cell extracts, where the difference is not obvious in HT-29 and Caco-2 cell lines (97). It is therefore easier to measure the enzyme activity of CES with HEK-293 cell line. In this study, the measurements of enzyme activity that were taken with 50 seconds interval are rather random and it was difficult to find a clear tendency by analyzing these results. And since we are missing measurements of original

alternative splicing variant in this study, it is impossible to predict the range for the enzyme activity and
to define whether the results were meaningful or just background noise.
We were not able to repeat this part of the study due to lack of time. Otherwise it might be helpful to repeat the experiment with a positive control involved. A positive control could either be an original splicing variant where it is possible to study the relation between enzyme activity and missing exons. And we could also use a vector with a GFR-tag which activity is detectable in the form of bioluminescent light by microscope (87).

4.5 Future studies

As only one new alternative splicing variant was found in this study, it would be interesting to test if other new alternative splicing variants can be found by designing new primers and to study the biological influences of the new alternative splicing variants.

Influence of alternative splicing on enzyme activity of CES1 and CES2 can be studied in different ways, such as including original splicing variants in the study, or use a vector with GFR-tag.

It is always interesting to study further about characterization of the alternative splicing variant with respect to enzyme activity, and the effect of alternative splicing on drug metabolism.

5 Conclusion:

In this study, one new alternative splicing variant was detected from cDNA of CES1 in blood tissue with deletion of entire exon 12, exon 13 and most part of exon 11 (135bp). The missing exons lead to difference in the last 136 amino acids (location: 439 -575) of the amino acid sequence where one active site is located. It is therefore reasonable to suggest that the new alternative splicing variant would have lower enzyme activity. One mutagenesis site also locates within the missing amino acid sequence; mutagenesis on this site could result in non-secretion of the protein. It is difficult to say if the new alternative splicing variant would encode for a functional protein.

Alternative splicing variants which were known from previous bachelor studies were also detected: one alternative splicing variant of CES1 with deletion of exon 7, and one alternative splicing variant of CES2 with deletion of exon 2 and 3.

The expression level of alternative splicing variant with deletion of exon 12, 13 and partly exon 11 is the highest in fetal brain tissue and relatively high in brain cerebellum, liver, colon, uterus, small intestine and adrenal glands tissues. The expression level of original splicing variant with full sequence is highest in liver tissue. It is believed that the alternative splicing variant has changed the expression pattern of CES2.

Study of measuring enzyme activity was a pilot study. It is difficult to make a conclusion based on the results found here. The part of study may be redesigned.

6 Appendix

6.1 Appendix for methods:

6.1.1 Reaction mixture of cDNA synthesis using qScript cDNA SuperMix:

Component	Volume (µl)
Dnase free water	15
qScript cDNA SuperMix	4
RNA (200 ng)	1
Total	20

The mixture was then incubated with following incubation parameters:

Temperature (^{o}C)	**Time (minutes)**
25	5
42	30
85	5
4	Hold

6.1.2 Dilution of cDNA

1:10 Dilution	10µl cDNA + 90µl dH_2O
1:50 Dilution	1µl cDNA + 50µl dH_2O

6.1.3 Primer dilution

1:10 Dilution = 10µl Primer stock solution + 90µl dH_2O

6.1.4 Reaction mixture of LR-PCR

Component	Volume (µl)	End concentration
H2O	17,7	
MgCl2	0,35	2,85
LongRange PCR buffer w/Mg2+, 10x	2,5	1x
dNTP mix (10mM each)	1,25	500µM each
Forward primer	0,5	0,4 µM
Reverse primer	0,5	0,4 µM
LongRange PCR Enzyme Mix (5 units/ul)	0,2	2 units/reaction
Template DNA (from diff tissues)	*2*	*ca. 200ng*
TOTAL VOLUME	25	

6.1.5 Reaction mixture of HotStar-PCR

Component	Volume (µl)	End concentration
H2O	18,75	
MgCl2	0	-
10 x buffer	2,5	1x
dNTP mix (10mM each)	0,5	500 µM
Forward primer	1	0,4 µM
Reverse primer	1	0,4 µM
HotStar *Taq*	0,25	1units/25 reaction
Template DNA (from diff. tissues)	1	ca. 200ng
TOTAL VOLUME	25	

6.1.6 Reaction mixture and thermal parameter for proofreading

Components	Volume(µl)
2mM dNTPs	0,7
10x PCR buffer	0,7
Taq polymerase	0,1
DNA template	5

PCR profile			
Step	Temperature (°C)	Time	Cycle nr.
Step 1	95	15 min	1
Step 2	72	10 min	Incubation
Step 3	4	-	Store

6.1.7 TOPO cloning:

TOPO TA Cloning:

Reagenser	**Volume (µl)**
PCR-product	2
5xT4 ligase buffer	1
Steril water	2
Topo vector (PCR vector)	1
ExpressLink T4 DNA ligase	1
Total	10

TOPO XL Cloning:

Reagenser	**Volume (µl)**
PCR-product	4
pCR-XL-TOPO® vector	1
Total	5

6.1.8 Reaction mixture of qPCR

Component	Volume (µl)
Dnase free water	7
Universal PCR Master Mix	10
FAM gene specific assay/Probe*	1
Template DNA	2
Total	20

Thermal parameters of qPCR:

Step	Temperature (°C)	Time	Number of cycle
Hold	95	10 min	1
	95	15 sec	
	60	60 sec	40

6.1.9 Reagents for cloning using pcDNA™3.1/V5-His-TOPO® expression Kit

Reagenser	Volume (µl)
PCR-product	1(*bright band) /* 2 (*weak band)*
Rnase-free water	Add to final volume 5 ul
TOPO vector	1
Salt solution	1
Total	5

Reagenser	CES1 Alternative	CES1 Original	CES2 Original	CES2 Alternative	CES2 Original (Primer: 418)
PCR-product	1	2	1	1	2
Steril water	2	1	2	2	1
TOPO vector	1	1	1	1	1
Salt solution	1	1	1	1	1
Total	5	5	5	5	5

6.1.10 Reaction mixtures for restriction enzyme BamHI/XhoI:

Reagents	Volume/Amount
Restriction enzyme: BamI/XhoI	1 ul
DNA	1 ug
10x NEB buffer	5 ul (1x)
Total Rxn volume	50 ul

6.1.11 Reaction mixture for cell transfection.

Mixture 1:

Component	**In each well (μl)**
Opti.MEM Medium	5
Lipofectamine	0,3

Mixture 2 – DNA mixture:

Component	**In each well (μl)**
Opti.MEM Medium	5
DNA	0,1**μg***
P3000 Reagent	0,2

*****Calculate the correspond volume of DNA which contains 0,1 μg of DNA.*

Add dilute DNA (Mixture 2) to Diluted Lipofectamine reagent (Mixture 1) with 1:1 ratio.

6.2 Appendix for results

6.2.1 Alignment of amino acid sequence onto human reference gene (Screen print of Clustal Omega)

A star sign underneath the two sequences means that they match with each other, otherwise it will be shown as a dash line.

2.1A CES1_colony c

CES1_Colony C

Alignment using **Clustal Omega**:

2.1B CES1_colony f

CES1_Colony F

Alignment using **Clustal Omega**:

```
CLUSTAL O(1.2.1) multiple sequence alignment

CES1_original      MWLRAFILATLSASAAWAGHPSSPPVVDTVHGKVLGKFVSLEGFAQPVAIFLGIPFAKPP
CES1_colony        MWLRAFILATLSASAAWAGHPSSPPVVDTVHGKVLGKFVSLEGFAQPVAIFLGIPFAKPP 60
                   ************************************************************

CES1_original      LGPLRFTPPQPAEPWSFVKNATSYPPMCTQDPKAGQLLSELFTNRKENIPLKLSEDCLYL
CES1_colony        LGPLRFTPPQPAEPWSFVKNATSYPPMCTQDPKAGQLLSELFTNRKENIPLKLSEDCLYL 120
                   ************************************************************

CES1_original      NIYTPADLTKKNRLPVMVWIHGGGLMVGAASTYDGLALAAHENVVVVTIQYRLGIWGFFS
CES1_colony        NIYTPADLTKKNRLPVMVWIHGGGLMVGAASTYDGLALAAHENVVVVTIQYRLGIWGFFS 180
                   ************************************************************

CES1_original      TGDEHSRGNWGHLDQVAALRWVQDNIASFGGNPGSVTIFGESAGGESVSVLVLSPLAKNL
CES1_colony        TGDEHSRGNWGHLDQVAALRWVQDNIASFGGNPGSVTIFGESAGGESVSVLVLSPLAKNL 240
                   ************************************************************

                                                    267
CES1_original      FHRAISESGVALTSVLVKKGDVKPLAEQIAITAGCKTTTSAVMVHCLRQKTEEELLETTL
CES1_colony        FHRAISESGVALTSVLVKKGDVKPLAE---------------------------------- 300
                   ****************************
                   302
CES1_original      KMKFLSLDLQGDPRESQPLLGTVIDGMLLLKTPEELQAERNFHTVPYMVGINKQEFGWLI
CES1_colony        ---KFLSLDLQGDPRESQPLLGTVIDGMLLLKTPEELQAERNFHTVPYMVGINKQEFGWLI 360
                      ************************************************************

CES1_original      PMQLMSYPLSEGQLDQKTAMSLLWKSYPLVCIAKELIPEATEKYLGGTDDTVKKKDLFLD
CES1_colony        PMQLMSYPLSEGQLDQKTAMSLLWKSYPLVCIAKELIPEATEKYLGGTDDTVKKKDLFLD 420
                   ************************************************************

CES1_original      LIADVMFGVPSVIVARNHRDAGAPTYMYEFQYRPSFSSDMKPKTVIGDHGDELFSVFGAP
CES1_colony        LIADVMFGVPSVIVARNHRDAGAPTYMYEFQYRPSFSSDMKPKTVIGDHGDELFSVFGAP 480
                   ************************************************************

CES1_original      FLKEGASEEEIRLSKMVMKFWANFARNGNPNGEGLPHWPEYNQKEGYLQIGANTQAAQKL
CES1_colony        FLKEGASEEEIRLSKMVMKFWANFARNGNPNGEGLPHWPEYNQKEGYLQIGANTQAAQKL 560
                   ************************************************************

CES1_original      KDKEVAFWTNLFAKKAVEKPPQTEHIEL
CES1_colony        KDKEVAFWTNLFAKKAVEKPPQTEHIEL
                   ****************************
```

2.1C CES2_colony 4

CES2_Colony 4

Alignment using **Clustal Omega**:

```
CLUSTAL O(1.2.1) multiple sequence alignment

CES2_original              MTAQSRSPTTPTFPGPSQRTPLTPCPVQTPRLGKALIHCWTDPGQPLGEQQRVRRQRTET   60
CES2_alternative_spliceva  ------------------------------------------------------------

CES2_original              SEPTWRLHRLRARLSAVACGLLLLLVRGQGQDSASPIRTTHTGQVLGSLVNVKGANAGVQ  120
CES2_alternative_spliceva  ------------------------------------------------------------

CES2_original              TFLGIPFAKPPLGPLRFAPPEPPESWSGVRDGTTHPAMCLQDLIAVESEFLSQFNMTFPS  180
CES2_alternative_spliceva  ------------------------------------------------------------
                                                                    207
CES2_original              DSMSEDCLYLSIYTPAHSHEGSNLPVMVWIHGGALVFGMASLYDGSMLAALENVVVVIIQ  240
CES2_alternative_spliceva  ----------------------MVWIHGGALVFGMASLYDGSMLAALENVVVVIIQ
                                                   ********************************

CES2_original              YRLGVLGFFSTGDKHATGNWGYLDQVAALRWVQQNIAHFGGNPDRVTIFGESAGGTSVSS  300
CES2_alternative_spliceva  YRLGVLGFFSTGDKHATGNWGYLDQVAALRWVQQNIAHFGGNPDRVTIFGESAGGTSVSS
                           ************************************************************

CES2_original              LVVSPISQGLFHGAIMESGVALLPGLIASSADVISTVVANLSACDQVDSEALVGCLRGKS  360
CES2_alternative_spliceva  LVVSPISQGLFHGAIMESGVALLPGLIASSADVISTVVANLSACDQVDSEALVGCLRGKS
                           ************************************************************

CES2_original              KEEILAINKPFKMIPGVVDGVFLPRHPQELLASADFQPVPSIVGVNNNEFGWLIPKVMGI  420
CES2_alternative_spliceva  KEEILAINKPFKMIPGVVDGVFLPRHPQELLASADFQPVPSIVGVNNNEFGWLIPKVMGI
                           ************************************************************

CES2_original              YDTQKEMDREASQAALQKMLTLMLPPTFGDLLREEYIGDNGDPQTLQAQFQEMGADSMF  480
CES2_alternative_spliceva  YDTQKEMDREASQAALQKMLTLMLPPTFGDLLREEYIGDNGDPQTLQAQFQEMGADSMF
                           ************************************************************

CES2_original              VIPALQVAHFQCSRAPVYFYEFQHQPSWLKNIRPPHGGADHGDELPFVFRSFFGGNYIKF  540
CES2_alternative_spliceva  VIPALQVAHFQCSRAPVYFYEFQHQPSWLKNIRPPHGGADHGDELPFVFRSFFGGNYIKF
                           ************************************************************

CES2_original              TEEEEQLSRQMMGYWANFARNGNPNGEGLPHWPLFDQEEQYLQLNLQPAVGRALKAHRLQ  600
CES2_alternative_spliceva  TEEEEQLSRQMMGYWANFARNGNPNGEGLPHWPLFDQEEQYLQLNLQPAVGRALKAHRLQ
                           ************************************************************

CES2_original              FWKGALPQKIQELEEPEERHTEL
CES2_alternative_spliceva  FWKGALPQKIQELEEPEERHTEL
                           ***********************
```

6.2.2 Measurements of RT-qPCR and calculations of target amount

Calculation of average CTMean-value for both reference genes: GAPDH and PGK1.

Exon 2_3:

Sample Name	Ct Mean_GAPDH	Ct Mean_PGK1	Average CtMean	CtMean: Exon2_3_GAPDH	CtMean: Exon2_3_PGK1	Average CtMeanExon2_3
Adipose	25,493	27,426	26,459	Invalid	Invalid	Invalid
Adrenal glands	22,327	24,076	23,201	32,947	32,947	32,947
Blood B	24,066	25,983	25,024	36,778	36,778	36,778
Brain cerebellum	Invalid	22,249	22,249	Invalid	30,822	30,822
Brain Whole	21,207	23,561	22,384	32,115	32,115	32,115
Colon	Invalid	25,057	25,057	Invalid	33,832	33,832
Fetal brain	21,448	24,033	22,740	30,653	30,653	30,653
Heart	20,885	23,423	22,154	34,708	34,708	34,708
Kidney	21,684	22,933	22,308	33,387	33,387	33,387
Liver A	22,871	25,138	24,004	32,747	32,747	32,747
Liver B	23,895	25,419	24,657	34,429	34,429	34,429
Lung	23,100	24,538	23,819	34,332	34,332	34,332
Placenta	22,496	23,110	22,803	34,306	34,306	34,306
Prostate	22,070	23,497	22,784	34,061	34,061	34,061
Salivary glands	21,645	24,636	23,141	35,488	35,488	35,488
Skeletal muscle	18,108	23,039	20,574	35,209	35,209	35,209
Skin	23,469	24,106	23,787	34,929	34,929	34,929
Small intestine	21,829	24,498	23,164	32,723	32,723	32,723
Spleen	23,521	25,333	24,427	34,651	34,651	34,651
Stomach	21,621	Invalid	21,621	33,265	Invalid	33,265
Thymus	20,615	23,072	21,843	32,261	32,261	32,261
Thyroid	21,687	23,383	22,535	32,522	32,522	32,522
Trachea	22,076	24,494	23,285	34,187	34,187	34,187
Uterus	23,239	25,047	24,143	33,493	33,493	33,493

Exon 8:

Sample Name	CtMean_GAPDH	CtMean_PGK1	Average CtMean	CtMeanExon8_GAPDH	CtMeanExon8_PGK1	Average CtMeanExon8
Adipose	25,493	27,426	26,459	37,097	37,097	37,097
Adrenal glands	22,327	24,076	23,201	33,810	33,810	33,810
Blood B	24,066	25,983	25,024	34,944	32,614	33,779
Brain cerebellum	Invalid	22,249	22,249	Invalid	32,189	32,189
Brain Whole	21,207	23,561	22,384	33,552	33,552	33,552
Fetal brain	21,448	24,033	22,740	31,717	31,717	31,717
Heart	20,885	23,423	22,154	31,094	31,094	31,094
Kidney	21,684	22,933	22,308	31,041	31,041	31,041
Liver A	22,871	25,138	24,004	31,634	31,634	31,634
Liver B	23,895	25,419	24,657	31,720	31,720	31,720
Lung	23,100	24,538	23,819	33,697	33,697	33,697
Placenta	22,496	23,110	22,803	35,814	35,814	35,814
Prostate	22,070	23,497	22,784	33,756	33,756	33,756
Salivary glands	21,645	24,636	23,141	35,457	35,457	35,457
Skeletal muscle	18,108	23,039	20,574	33,922	33,922	33,922
Skin	23,469	24,106	23,787	30,558	34,145	32,352
Small intestine	21,829	24,498	23,164	30,488	30,488	30,488
Spleen	23,521	25,333	24,427	35,263	36,303	35,783
Stomach	21,621	Invalid	21,621	32,987	Invalid	32,987
Thymus	20,615	23,072	21,843	35,338	35,338	35,338
Thyroid	21,687	23,383	22,535	36,172	36,172	36,172
Trachea	22,076	24,494	23,285	33,338	33,338	33,338
Uterus	23,239	25,047	24,143	34,182	34,182	34,182

Calculation 2^ΔΔCt.

ΔCt = Average CtMean Exon2_3 – Average CtMean

Select the tissue sample with the smallest ΔCt as reference

ΔΔCt = ΔCt_Reference - ΔCt

6.2.3 Calculation of amount of DNA samples used for restriction enzyme cutting

Giving protocol:

Restriction enzyme: XhoI/BamH I	1 µl
DNA	1 µg
10x NEB buffer	5 µl (1x)
Total Rxn volume	50 µl

Total final volume is 50 µl. As every mixture should contain 1 µl enzyme + 5 µl buffer = 6 µl. Therefore the total amount of water and DNA would be 50µl – 6 µl = 44µl.

The volume of DNA needed to obtain 1µg of DNA= 1 µg/concentration measured by Nanometer = 1000ng / (ng/µl).

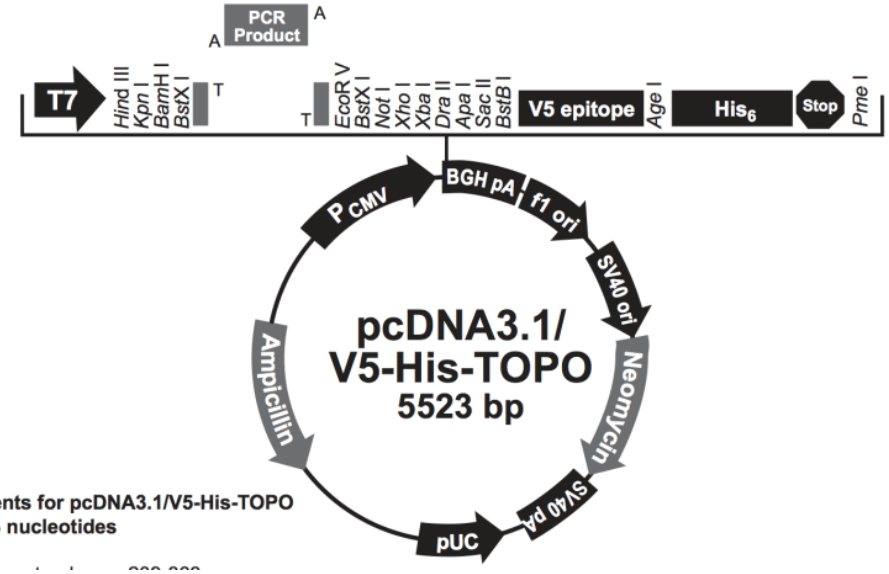
ENZYME	DNA	ng/ul	For 1ug (ul)	Round up (ul)	Half (=0,5ng)	H2O(ul)
BamHI	CES 1 COLONY C_ Colony a	92,89	10,77	11,00	5,50	38,50
	CES 1 COLONY C_ Colony b	69,90	14,31	14,00	7,00	37,00
	CES 1 COLONY C_ Colony c	87,11	11,48	11,00	5,50	38,50
	CES 1 COLONY C_ Colony d	87,40	11,44	11,00	5,50	38,50
	CES 1 COLONY C_ Colony e	96,88	10,32	10,00	5,00	39,00
XhoII	CES 2 COLONY4_ colony 2	99,44	10,06	10,00	5,00	39,00
	CES 2 COLONY4_ colony 4	37,73	26,50	26,00	13,00	31,00
	CES 2 COLONY4_ colony 8	44,73	22,36	22,00	11,00	33,00
	CES 2 COLONY4_ colony 10	34,22	29,22	29,00	14,50	29,50
XhoII	CES 2 COLONY4_ colony 11	63,00	15,87	16,00	8,00	36,00
	CES 2 COLONY4_ colony 13	50,62	19,76	20,00	10,00	34,00
	CES 2 COLONY4_ colony 17	49,34	20,27	20,00	10,00	34,00

6.2.4 Calculation of expected length of DNA fragments after cutting with restriction enzyme (BamHI/XhoI) in both orientations:

The total length of pcDNA 3.1 vector is 5523bp (Ref: TA cloning 3.1 Protocol)

Map

The figure below summarizes the features of the pcDNA™3.1/V5-His-TOPO® vector. The vector is supplied linearized between base pairs 953 and 954. This is the TOPO® Cloning site. **The complete nucleotide sequence is available for downloading from www.invitrogen.com or from Technical Support (page 24).**

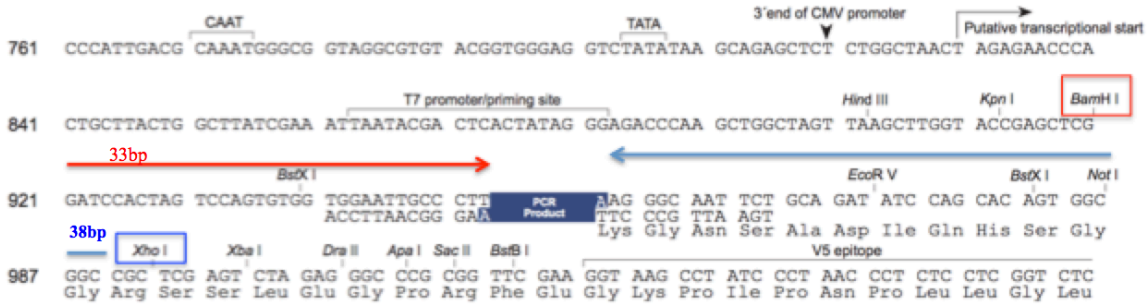


Comments for pcDNA3.1/V5-His-TOPO
5523 nucleotides

Enzyme BamI cuts at 33bp from PCR insertion (shown as red)

Enzyme XhoI cuts at 38bp fafter PCR insertion (blue)

-



CES1:

Sequence of original alternative splicing: 1720bp

Total vector: 5523 bp

Cutting site of BamI:

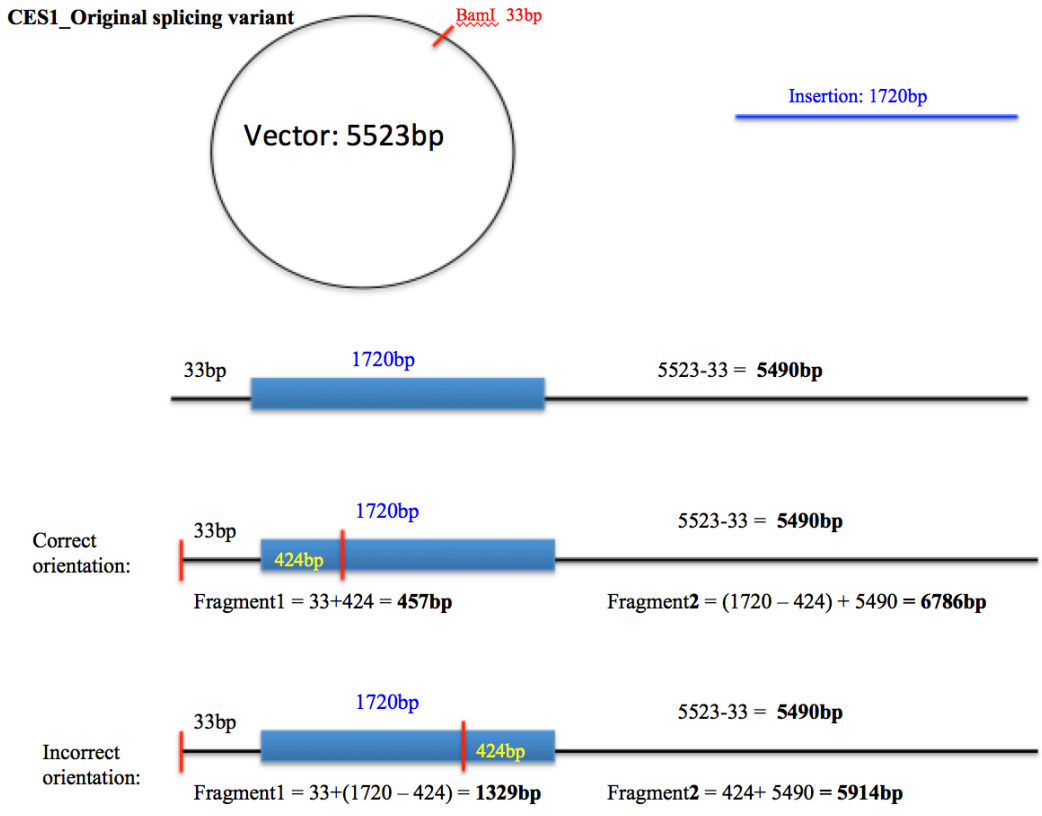
Protocol: 33bp after insertion and

NEBcutter: 424/428bp inside insertion (Paste in sequence and select “1 cutter”)

Number of cuts = 1 OK Sort order: Alphabetical Save as text file

#	Enzyme	Specificity	Sites & flanks	Cut positions (blunt - 5' ext. - 3' ext.)
1	AflIII	A^CRYG_T	list	1345/1349
2	AleI	CACNN^NNGTG	list	1154
3	ApaI	G_GGCC^C	list	1445/1441
4	ApaLI	G^TGCA_C	list	269/273
5	BamHI	G^GATC_C	list	424/428

By using restriction enzyme BamI for CES1_original, the possible fragments after cutting are:



Correct orientation: 457bp and 6786bp

Incorrect orientation: 1329bp and 5914bp.

CES1:

Sequence of original alternative splicing: 1503bp

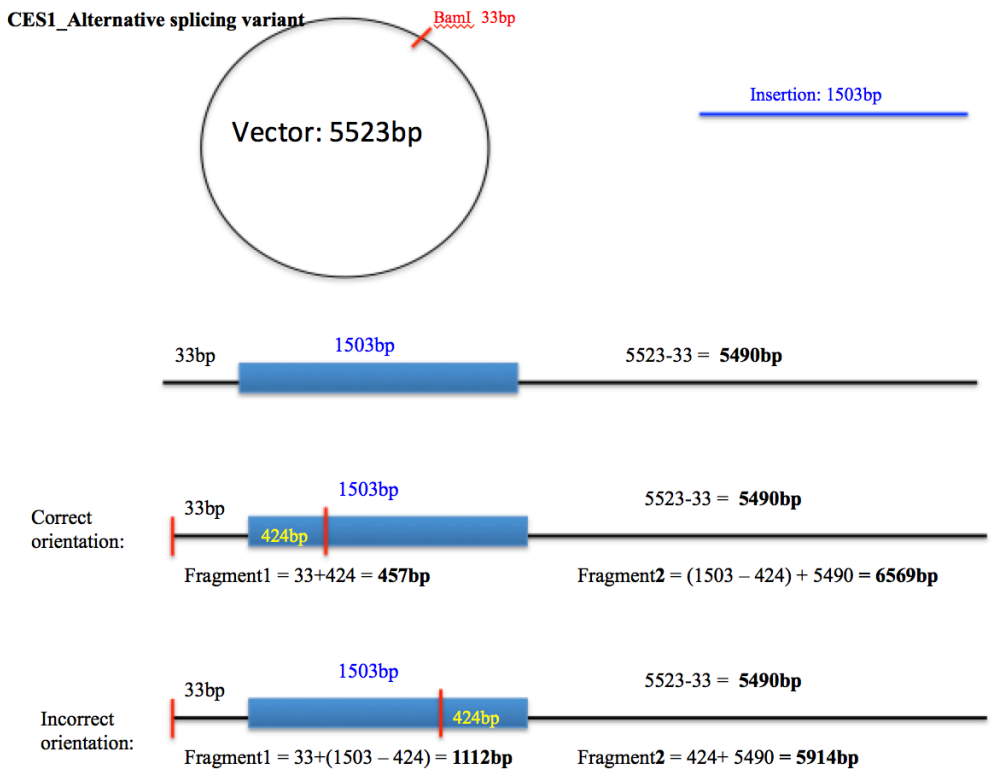
Total vector: 5523 bp

Cutting site of BamI:

Protocol: 33bp after insertion and

NEBcutter: 424/428bp inside insertion (Paste in sequence and select “1 cutter”)

By using restriction enzyme BamI for CES1_alternative splicing variant, the possible fragments after cutting are:



Correct orientation: 457bp and 6569bp

Incorrect orientation: 1112bp and 5914bp.

CES2:

Sequence of original alternative splicing (colony 8): 1878bp

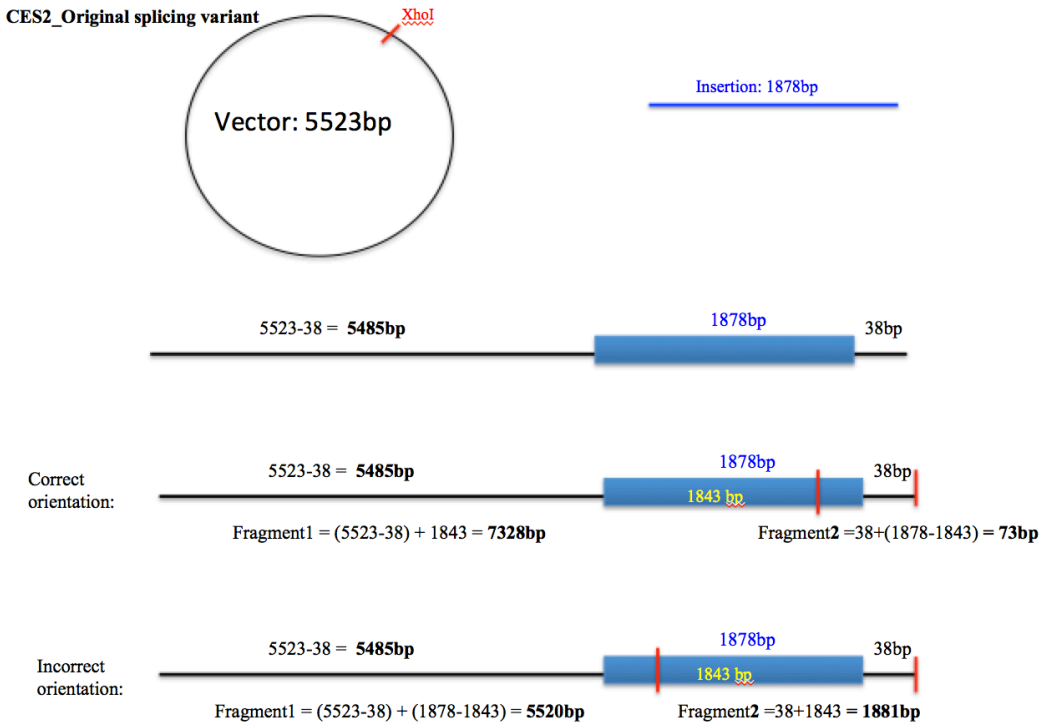
Total vector: 5523 bp

Cutting site of XhoI:

Protocol: 38bp after insertion and

NEBcutter: 1843/1847bp inside insertion (Paste in sequence and select “1 cutter”)

By using restriction enzyme XhoI for CES2_colony 8, the possible fragments after cutting are:



Correct orientation: 7328bp and 73bp

Incorrect orientation: 5520bp and 1881bp.

CES2:

Sequence of original alternative splicing (colony 8): 1260bp

Total vector: 5523 bp

Cutting site of XhoI:

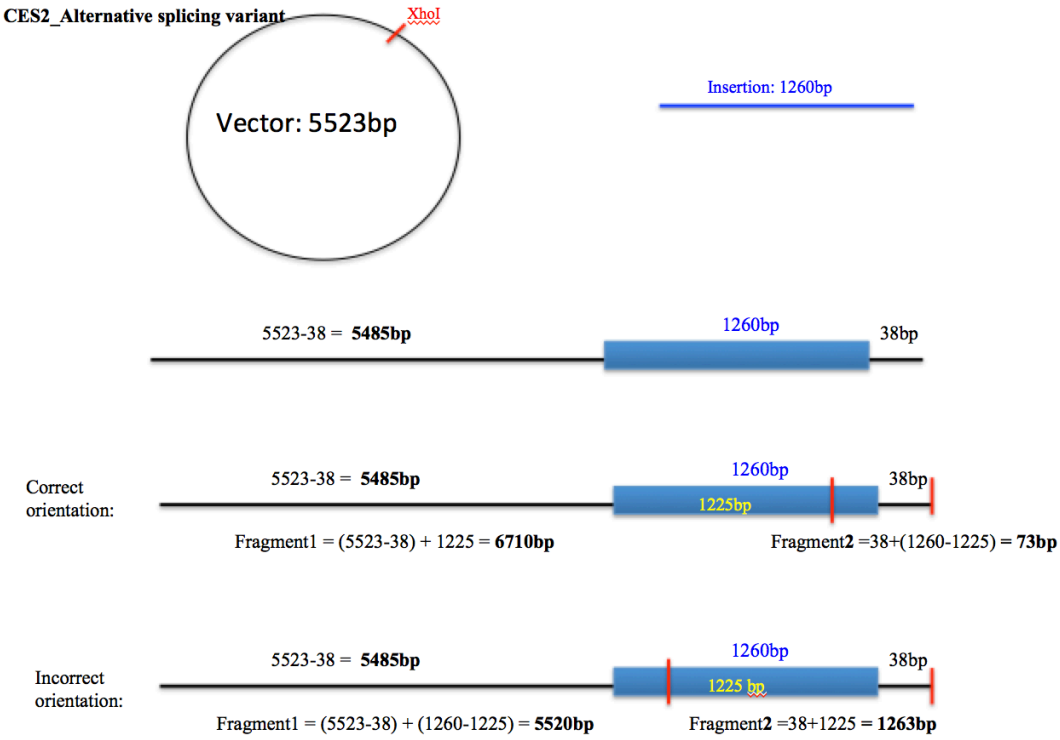
Protocol: 38bp after insertion and

NEBcutter: 1225/1229bp inside insertion (Paste in sequence and select “1 cutter”)

1.

36	Tth111I	GACN^N_NGTC	list	241/242
37	XhoI	C^TCGA_G	list	*1225/1229
38	XmaI	C^CCGG_G	list	*866/870

By using restriction enzyme XhoI for CES2_original splicing variant, the possible fragments after cutting are:



Correct orientation: 6710bp and 73bp

Incorrect orientation: 5520bp and 1263bp.

6.2.5 Measurements of enzyme activity after 24,48,72 and 96 hours

24 hours (Day2):

SAMPLE	REPEAT														
CELL MEDIUM	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Blank-1	432291	586228	734696	480542	431959	381231	741796	891262	844612	745694	836273	70396	122635	209921	632394
Blank-2	79671	199437	411535	131789	113631	848510	571605	37191	630491	320925	710918	808175	459967	699256	168589
With Insertion-1	966946	186113	286784	374130	949845	323704	204029	994682	661036	941658	604235	854099	754698	297449	509093
With Insertion-2	686355	819022	573055	836334	922170	461538	763701	576862	205872	293219	749743	218471	689286	442322	963411
Without Insertion-1	161701	480512	685418	44745	554051	279170	841047	60003	843736	342950	260196	124448	548008	240104	698803
Without Insertion-2	878482	163997	758293	521843	352528	983140	403679	536013	132092	148860	153029	557676	83146	582089	925554

48 hours (Day3):

SAMPLE	REPEAT														
CELL MEDIUM	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Blank-1	510000	691280	787509	65360	75290	153362	119523	237143	133844	696597	891322	856547	869992	373859	974651
Blank-2	234817	536829	557284	642818	177230	633874	809958	257054	932322	403709	17100	74203	648075	697594	778113
With Insertion-1	297389	610217	363526	140310	627862	408392	143361	546981	89309	266571	941567	812949	709498	226448	871684
With Insertion-2	920780	401232	956160	848601	347573	261163	291406	984983	56619	14290	605534	665447	94597	164359	218532
Without Insertion-1	555652	15831	634539	865671	326212	145174	790228	63266	68130	530333	788295	694573	556468	248141	462051
Without Insertion-2	666172	710795	184814	600912	373889	856637	896277	90609	343041	955254	580910	804670	948425	526224	13716
CELLS															
Blank-1	956553	497612	626835	140310	855429	804368	433530	746359	432533	404616	248685	230104	688772	282222	115353
Blank-2	81545	385340	864614	745845	24532	808810	577617	619070	103721	438455	589703	389177	151337	153724	903619
With Insertion-1	876880	501842	496585	952202	135204	531270	309262	367514	266208	946703	262583	14229	692609	981962	447307
With Insertion-2	657320	795425	749863	615867	710828	614991	552570	858088	892621	838811	687171	423741	730617	623783	89309
Without Insertion-1	328901	514169	501933	37554	620973	800199	233306	236207	240237	538884	945827	91424	779956	299322	784276
Without Insertion-2	113752	388542	386306	336545	225390	901596	318236	864946	155084	843676	441144	663332	40757	923258	180070

72 hours (Day4):

SAMPLE	REPEAT														
CELL MEDIUM	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Blank-1	897002	988639	358601	467459	33505	490089	367725	702761	878482	685418	922170	204029	630491	836273	24109
Blank-2	824611	451235	66468	242974	382107	213305	160039	509758	823463	758293	554051	763701	661036	710918	122635
With Insertion-1	749138	127892	611607	863617	101697	746298	232853	156020	586228	724696	352528	841047	205872	604235	459967
With Insertion-2	182367	14622	785485	217505	680433	473774	430901	950087	199437	480542	477007	403679	843736	749743	754698
Without Insertion-1	860354	18822	804580	781859	65652	463381	804852	150159	186113	131789	381231	505891	132092	260196	689286
Without Insertion-2	713396	384101	524683	845217	18701	949664	521934	906066	819022	374130	848510	891262	118828	153029	548008
CELLS															
Blank-1	567949	310954	575472	877213	999697	283128	117196	754033	310018	163997	44745	461538	994682	320925	70396
Blank-2	201371	24834	605746	729409	615384	280167	603057	352105	432291	508882	521843	279170	576862	941658	808175
With Insertion-1	810653	100488	611094	956341	398513	592029	391382	177297	79671	734696	666354	983140	60003	293219	854099
With Insertion-2	589159	370807	174571	926460	273550	694271	737657	660734	966946	411535	431959	304821	536013	342950	218471
Without Insertion-1	485074	155839	666414	543960	304307	839234	612876	496978	686335	286784	113631	741796	202126	148860	124448
Without Insertion-2	356788	174239	456281	150914	841742	729228	576681	72904	161701	573055	949845	571605	844612	735754	557676

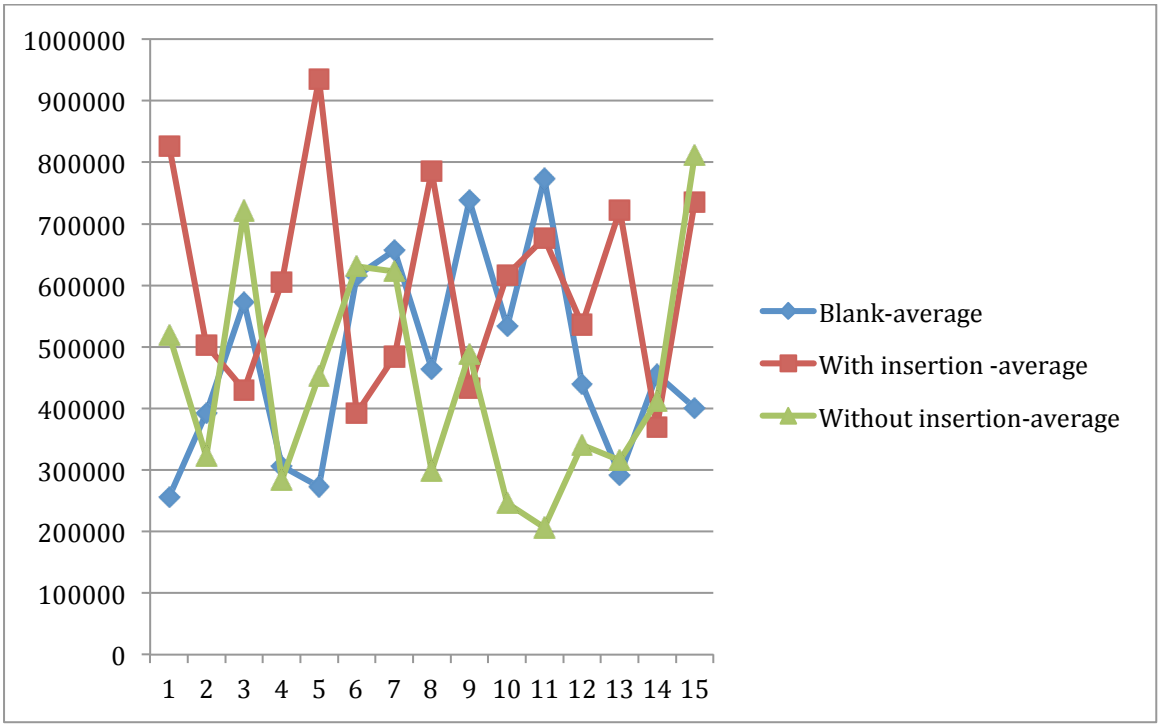
96 hours (Day5):

SAMPLE	REPEAT														
CELL MEDIUM	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Blank-1	897002	988639	358601	467459	33505	490089	367725	702761	878482	685418	922170	204029	630491	836273	24109
Blank-2	824611	451235	66468	242974	382107	213305	160039	509758	823463	758293	554051	763701	661036	710918	122635
With Insertion-1	749138	127892	611607	863617	101697	746298	232853	156020	586228	724696	352528	841047	205872	604235	459967
With Insertion-2	182367	14622	785485	217505	680433	473774	430901	950087	199437	480542	477007	403679	843736	749743	754698
Without Insertion-1	860354	18822	804580	781859	65652	463381	804852	150159	186113	131789	381231	505891	132092	260196	689286
Without Insertion-2	713396	384101	524683	845217	18701	949664	521934	906066	819022	374130	848510	891262	118828	153029	548008
CELLS															
Blank-1	567949	310954	575472	877213	999697	283128	117196	754033	310018	163997	44745	461538	994682	320925	70396
Blank-2	201371	24834	605746	729409	615384	280167	603057	352105	432291	508882	521843	279170	576862	941658	808175
With Insertion-1	810653	100488	611094	956341	398513	592029	391382	177291	79671	734696	666354	983140	60003	293219	854099
With Insertion-2	589159	370807	174571	926460	273550	694271	737657	660734	966946	411535	431959	304821	536013	342950	218471
Without Insertion-1	485074	155839	666414	543960	304307	839234	612876	496978	686355	286784	113631	741796	202126	148860	124448
Without Insertion-2	356788	174239	456281	150914	841742	729228	576681	72904	161701	573055	949845	571605	844612	735754	557676

6.2.6 Linear graph of enzyme activity after 24,48,72 and 96 hours of incubation.

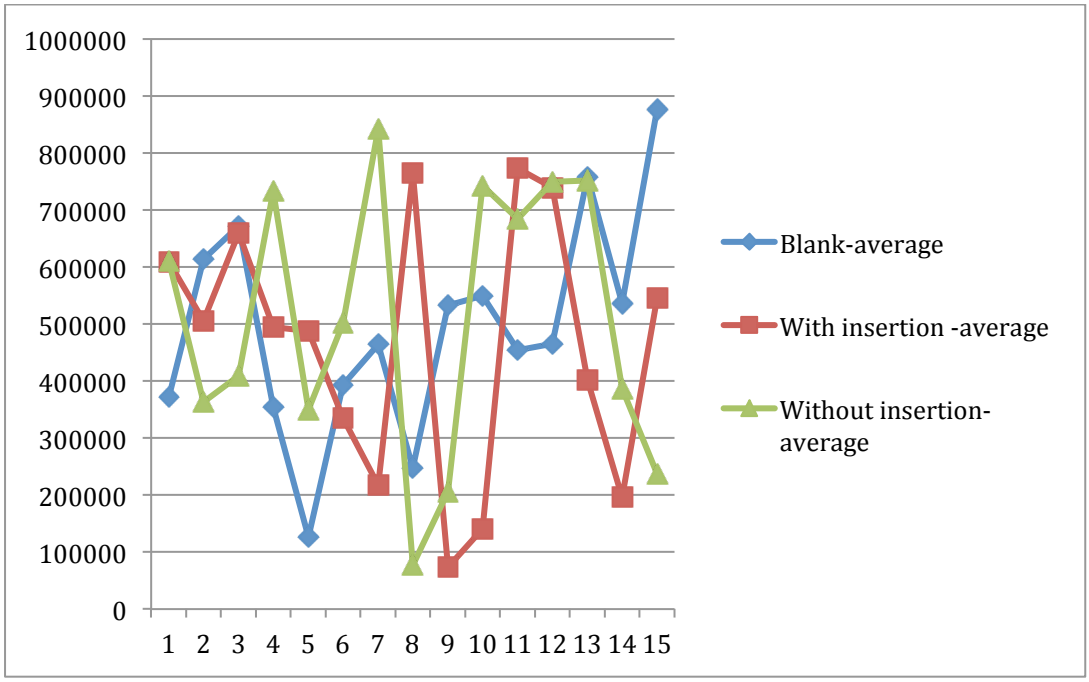
The enzyme activity for each sample is measured at for 15 times with 50 seconds between each measurement. The X-axis represents the number of measurements and y-axis represent the measurement of enzyme activity.

24 hours – cell medium

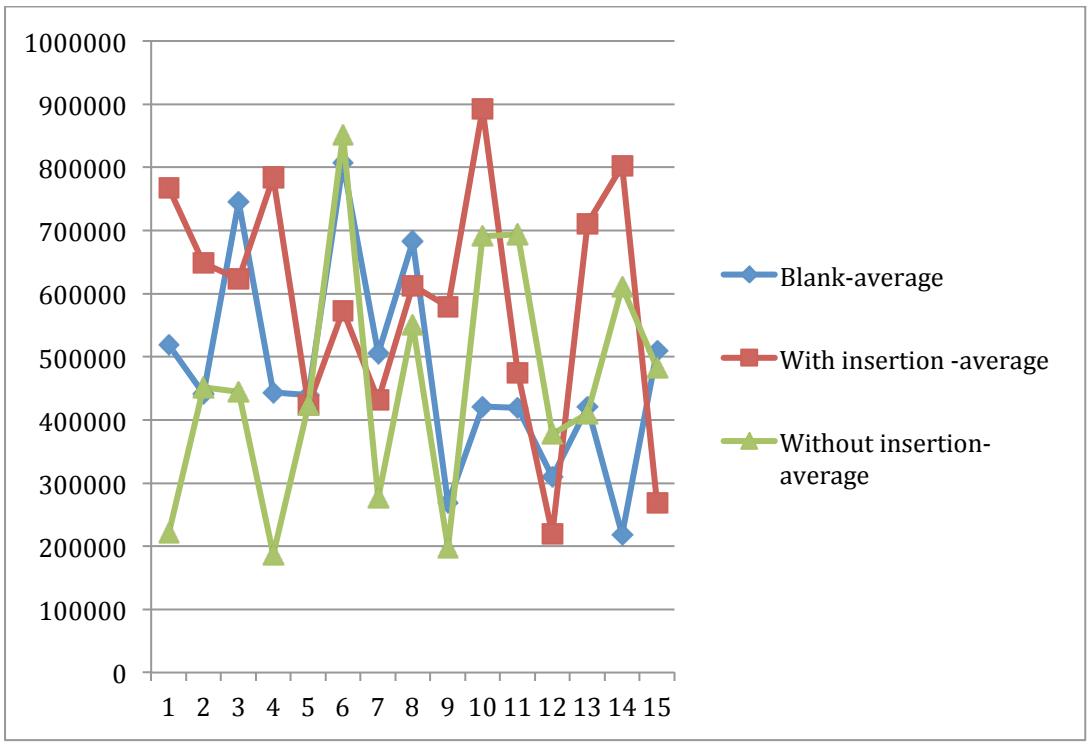


48 hours:

Cell medium:

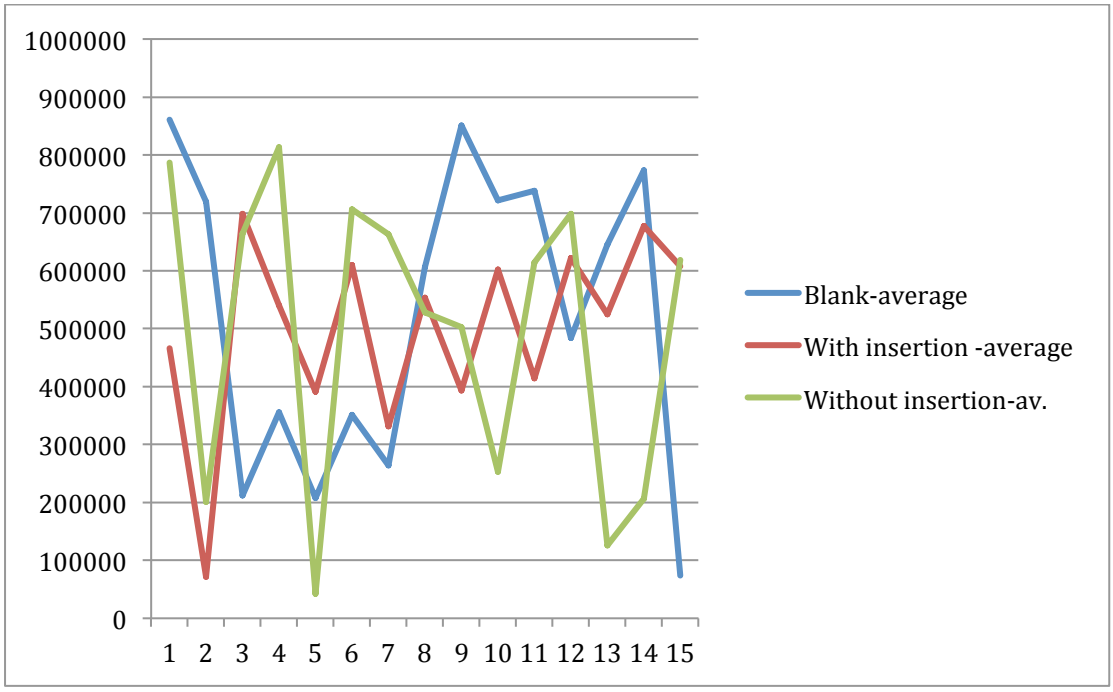


Cells:

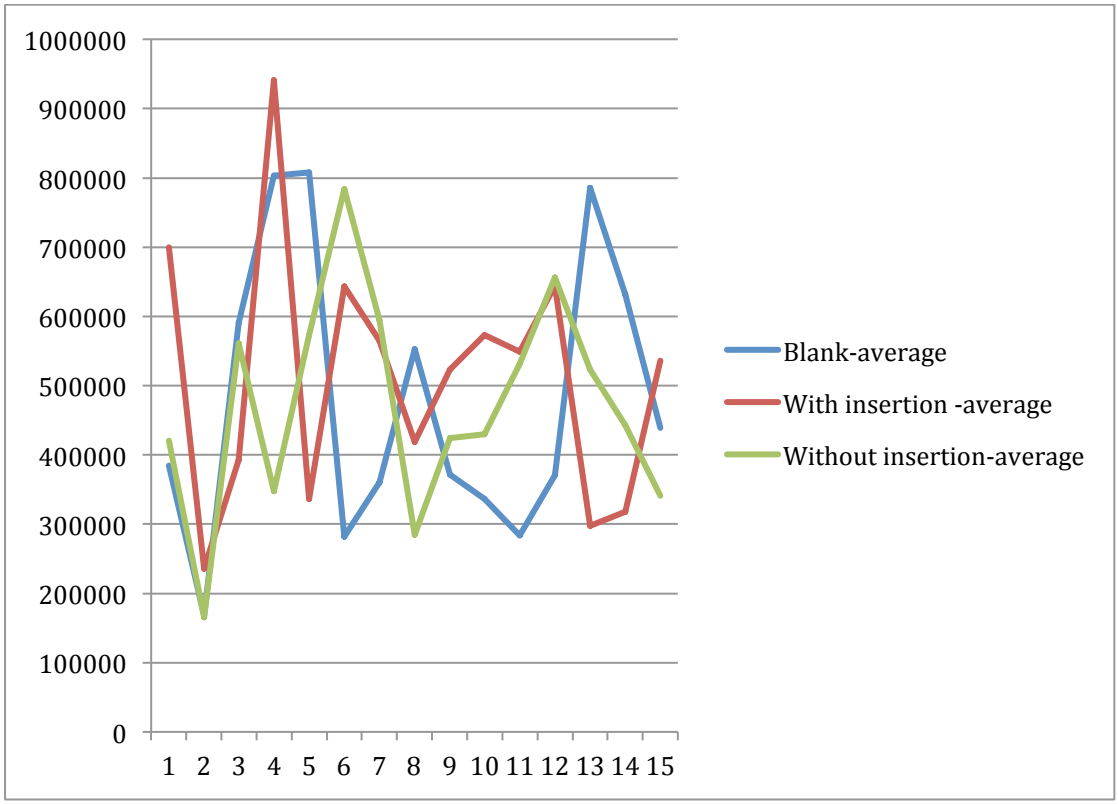


72 hours:

Cell medium:



Cells:



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