MUTATIONAL ANALYSIS OF A SOUTH AFRICAN HAEMOPHILIA B POPULATION

By

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Dissertation submitted in fulfilment of the requirements for the degree

Master of Medical Science (MMedSc)

with specialisation in Human Molecular Biology

in the

Human Molecular Biology Unit

School of Biomedical Sciences

Faculty of Health Sciences

University of the Free State

Bloemfontein, South Africa

July 2023

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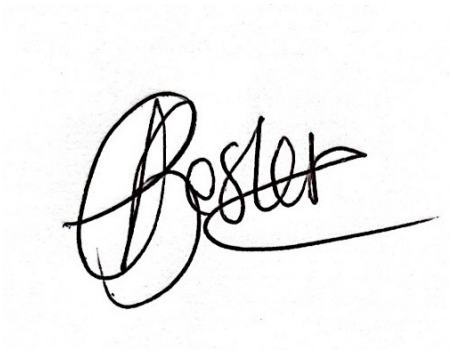

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ACKNOWLEDGEMENTS

I would like to express my heartfelt acknowledgments to the following individuals:

- Prof Wattie Janse van Rensburg, for being an exceptional supervisor. Your valuable insights, motivation, and guidance have been instrumental in completing my study. I am immensely grateful for your mentorship, as I could not have accomplished this on my own.
- Mr Jean Kloppers, for generously sharing your profound knowledge of molecular biology. Thank you for your advice, assistance, and those impromptu dilution calculations that have undoubtedly enriched my understanding. Your contributions will benefit me for years to come.
- Dr Lariska Haupt, for your expertise in haemophilia B and for assisting me with patient recruitment at Universitas Academic Hospital.
- The Human Molecular Biology Unit, for providing me with the necessary facilities and resources that were crucial for conducting my research.
- Dr Liezl du Plessis, for your assistance with patient recruitment at the Robert Mangaliso Sobukwe Hospital in Kimberley.
- The National Health Laboratory Service, for conducting the one-stage FIX assays as part of my functional analysis.
- My parents and my incredible sisters, Anandie and Danielle, for their moral support, motivation, and unconditional love that sustained me through challenging times.
- My fellow minions: Lomari Geertsema, thank you for your endless motivation, laughter, and friendship. Kirsten Lange, your support and radiant positivity have been uplifting throughout.
- My husband, Jean Coetser, thank you for your abundant love, unwavering support, and motivation throughout this journey. It wasn't always easy, but I am forever grateful for your presence. I love you deeply.
- Last but most importantly, I would like to extend my utmost gratitude to God. His steadfast presence has been a constant source of strength and encouragement during the rewarding and challenging moments of completing this dissertation. I am truly grateful for His boundless love, mercy, and grace, which have sustained me throughout this journey.

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CLARIFYING NOTE ON VARIANT VS MUTATION NOMENCLATURE

The American College of Medical Genetics and Genomics (ACMG) and the Association of Molecular Pathology (AMP) variant classification were confronted with two crucial terminology points. Firstly, the ACMG/AMP alluded to the use of the word “mutation”, which initially referred to any deviation from the original deoxyribonucleic acid (DNA) sequence, irrespective of its pathogenicity. However, the improving clarification of variants in patients, ranging from no phenotypic impact to pathogenic, has complicated the use of the term “mutation”. Therefore, the ACMG/AMP advocate using more accurate terminology, such as a “pathogenic variant”, “risk variant”, “disease-causing variant”, or a (new) “novel variant” (Jarvik and Evans, 2017). Throughout this dissertation, it was attempted to refer to the more accurate terminology of “variant” where results were discussed. However, the word “mutation” was not completely removed from the dissertation, especially when it was used in the context of historical literature.

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LIST OF ABBREVIATIONS AND ACRONYMS

α	Alpha
β	Beta
γ	Gamma
®	Registered
™	Trademark
°C	Degrees Celsius
µL	Microlitre(s)
µM	Micromolar(s)
%	Percentage
©	Conservative amino acid change
®	Radical amino acid change
<	Less than
=	Equal
>	Greater than
~	Approximately
∞	Infinity
3'	Three prime
3D	Three dimensional
5'	Five prime
aa	Amino acids
AAV	Adeno-associated virus
AAV5	Adeno-associated virus serotype 5
ACMG	American College of Molecular Genomics

AIDS	Acquired Immunodeficiency Syndrome
Ala	Alanine
AMP	Association of Molecular Pathology
AP	Activation peptide
aPTT	Activated Partial Thromboplastin Time
ASA	Accessible Surface Area
Asn	Asparagine
Asp	Aspartic Acid
BLAST	Basic Alignment Search Tool
bp	Base pair(s)
BT	Bleeding Time
BU	Bethesda Units
C	Carboxy terminal
c.	Coding reference sequence
Ca^{2+}	Calcium ions
cDNA	Complimentary Deoxyribonucleic acid
CFVD	Coagulation Factor Variant Databases
CFC	Clotting factor concentrate
CI	Confidence Interval
CMA	Conditional Marketing Authorisation
COVID	Coronavirus Disease
CSGE	Confirmation Sensitive Gel Electrophoresis
ddH_2O	Double Distilled Water
del	Deletion
DHPLC	Denaturing High-Performance Liquid Chromatography
dL	Decilitre

DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphates
EAHAD	European Association for Haemophilia and Allied Disorders
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
EGF1	Epidermal Growth Factor like 1
EGF2	Epidermal Growth Factor like 2
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicines Agency
ER	Endoplasmic reticulum
EU	European Union
F8	Factor VIII gene
F9	Factor IX gene
FDA	The Food and Drug Administration
FI	Factor I (Fibrinogen)
FIa	Activated Factor I (Fibrin)
FII	Factor II (Prothrombin)
FIIa	Activated Factor II (Thrombin)
FIX	Factor IX
FIXa	Activated Factor IX
FP	Fusion protein
FS	Free State province
fs	Frame shift
FSDOH	Free State Department of Health
FV	Factor V
FVa	Activated Factor V

FVII	Factor VII
FVIIa	Activated Factor VII
FVIII	Factor VIII
FVIIIa	Activated Factor VIII
FX	Factor X
FXa	Activated Factor X
FXI	Factor XI
FXIa	Activated Factor XI
FXII	Factor XII
FXIIa	Activated Factor XII
FXIII	Factor XIII
FXIIIa	Activated Factor XIIIa
g	Centrifugal force
GGCX	Gamma-glutamyl carboxylation enzyme
GLA	Gamma-carboxyglutamic Acid
Glu	Glutamic acid
h	Hour(s)
H_2O	Water
HA	Haemophilia A
HB	Haemophilia B
HGMD	Human Gene Mutation Database
His	Histidine
HIV	Human Immunodeficiency Virus
HMWK	High Molecular Weight Kininogen
HOD	Head of Department
HRM	High-Resolution Melting

HSREC	Health Science Research Ethics Committee
HTC	Health Treating Centre
HU	Haemophilia of uncertain type
IgG	Immunoglobulin G
IL-10	Interleukin-10
ISTH	International Society on Thrombosis and Haemostasis
ITI	Immune Tolerance Induction
IU	International Unit
kbp	Kilo base pair(s)
kDa	Kilodaltons
Leu	Leucine
LOD	Limit of detection
LOF	Loss of Function
Lys	Lysine
M	Molar
MAF	Minor allele frequency
Met	Methionine
MHC	Major Histocompatibility Complex
min	Minute(s)
mL	Millilitre(s)
mM	Millimolar(s)
MMedSc	Masters of Medical Science
Mr	Molecular mass
mRNA	Messenger RNA
n	Total amount/Number of cases
N	Amino acid terminal

N/A	Not Applicable
N9-GP	Nonacog beta pegol
NAb	Neutralising Antibodies
NaCl	Sodium Chloride
NBA	Nijmegen-Bethesda Assay
NBU	Nijmegen-Bethesda Units
NC	Northern Cape province
NG_	Accession number for genomic sequences on NCBI
NCBI	National Centre of Biotechnology Information
NCDOF	Northern Cape Department of Health
NEB	New England Biolabs
NG_	Accession number for genomic sequences on NCBI
NGS	Next generation sequencing
NHLS	National Health Laboratory Service
nm	Nanometres
NM_	Accession number for nucleotide (mRNA) reference sequences on NCBI
nM	Nanomolars
NP_	Accession number for protein reference sequences on NCBI
nt	Nucleotide(s)
NTC	Non-Template Control
NY	New York
OD	Optical Density
OMIM	Online Mendelian Inheritance in Man
OR	Odds Ratio
ORF	Open Reading Frame
p	p-value

p-value	Calculated probability
p.	Protein reference sequence
PCR	Polymerase Chain Reaction
pd	Plasma-derived
PDB	Protein Data Bank
pdFIX	Plasma-derived Factor IX
pH	Potential of Hydrogen
Phe	Phenylalanine
PKK	Prekallikrein
PND	Prenatal diagnosis
PP	Propeptide
PT	Prothrombin Time
PTM	Post-translational Modification
PTMs	Post-translational modifications
q	Long arm of chromosome
QoL	Quality of Life
r	Recombinant
RBDs	Rare bleeding disorders
rFIX	Recombinant Factor IX
rFIX	Recombinant Factor IX
RIN	Residue Interaction Network
RNA	Ribonucleic Acid
rpm	Revolutions per minute
RXN	Reaction
sec	Seconds
Ser	Serine

SNPs	Single Nucleotide Polymorphism(s)
SOP	Standard Operating Procedure
SP	Serine protease
ss-DNA	Single-stranded DNA
SSC	Scientific and Standardization Committee
$t_{1/2}$	Half-life
TAE	Tris-Acetate-EDTA
TE	Tris-EDTA
Ter	Termination
TF	Tissue Factor
Thr	Threonine
TT	Thrombin Time
U	Units
UCLA	University of California, Los Angeles
USA	United States of America
UTR	Untranslated Region
UV	Ultraviolet
V	Volts
Val	Valine
vCJD	Variant Creutzveldt Jacob Disease
WFH	World Federation of Hemophilia
WHO	World Health Organisation
ZR	Zymo Research

SUMMARY

KEYWORDS: Haemophilia B • Factor IX (FIX) • FIX gene (*F9*) • Mutational analysis • *F9* conventional PCR assay • One-stage FIX assay • FIX ELISA assay • Pathogenic variant •

Introduction and Aim: Haemophilia B is an X-linked recessive bleeding disorder characterised by a deficiency of coagulation factor IX (FIX), due to a wide spectrum of causative mutations in the FIX encoding gene (*F9*). Based on the plasma concentration of normal FIX coagulant activity (FIX:C), haemophilia B can be classified as mild (>5 – <40 international units per decilitre (IU/dL)), moderate (1 – 5 IU/dL) or severe (<1 IU/dL). Genetic testing is an important aspect in the haemophilia B diagnostic approach and appropriate patient management. Given the low prevalence, haemophilia A and B are considered orphan diseases; however, because haemophilia B is much rarer than haemophilia A, it is a disorder that is often neglected in terms of basic research, resulting in suboptimal disorder management. The aim of this study was to screen haemophilia B patients in our region for known and novel *F9* causative gene variants, as well as determine the genotype/phenotype relationship for each study participant.

Methods: A total of 21 participants were enrolled in this study. All the participants were screened using conventional PCR assays to amplify *F9* exon 1 – 8, followed by direct Sanger sequencing to analyse and confirm causative *F9* gene variations. The *F9* variants identified were compared to various *F9* gene variants databases to confirm known and novel variants. Furthermore, for the functional analysis a FIX one-stage and an enzyme-linked immunosorbent assay (ELISA) assay were done to measure the FIX:C. Discrepancies between the ELISA and one-stage assay were determined by calculating the *p*-value. The genotype/phenotype relationship was determined for the study participants and subsequently compared to previously published data.

Results and Discussion: A total of eight pathogenic *F9* variations were identified throughout the FIX protein domains: p.Ser^{406}Leu, p.Glu^{241}*, p.Asn^{393}del, p.Asn^{104}Metfs*31, p.Phe^{121}Leufs*3, p.Lys^{459}Serfs*24, p.Val^{243}Phefs*2, and Thr^{84}Glufs*20. Approximately 63% of the variants detected were novel. The previously published variants identified in our study did correlate with previously published data.

Conclusion: The discovery of novel *F9* pathogenic variations in our South African population is an exciting finding, proving that genetic analysis of people with Haemophilia B is an important undertaking to better understand the pathogenesis of Haemophilia B in our population, and the wider sub-Saharan African population.

CHAPTER 1: INTRODUCTION

Haemostasis is a complex process that, under normal physiological conditions, maintains blood flow. A normal haemostatic balance depends on the delicate equilibrium of coagulation and anticoagulation. The disruption of this haemostatic balance can lead to either uncontrolled thrombosis or bleeding (Bonar et al., 2017; Mohammed et al., 2018; Peters and Harris, 2018; Zaidi and Green, 2022). Rare bleeding disorders (RBDs) are known to represent only 3 – 5% of all coagulation factor deficiencies and haemophilia is one of the most common hereditary RBDs (Gupta et al., 2019b; Mahmood et al., 2020; Palla et al., 2015).

Haemophilia A and B are congenital X-linked coagulopathies, characterised by a deficiency of the coagulation factor VIII (FVIII) and vitamin K-dependent factor IX (FIX) proteins, respectively (Bowen, 2002; Castaman and Matino, 2019; Hazendonk et al., 2018; Tjärnlund-Wolf and Lassila, 2019). Haemophilia A (FVIII deficiency), with an incidence rate of 1 in every 5,000 male births, represents 80 - 85% of all haemophilias. Haemophilia B is rarer, with an incidence rate of 1 in every 30,000 male births (Anson et al., 1984; Santagostino and Fasulo, 2013; Srivastava et al., 2013; Thorat et al., 2018).

According to the 2020 global survey by the World Federation of Hemophilia (WFH), there are 2,365 individuals diagnosed with haemophilia in South Africa. Of the 2,365 haemophilia patients, 1,986 (~84%) were identified as haemophilia A and 379 (~16%) as haemophilia B cases, respectively (World Federation of Haemophilia, 2021) (Table 1.1).

Table 1.1. Summary of the demographics for individuals identified with haemophilia globally and in South Africa (World Federation of Haemophilia, 2021).

	Population	Haemophilia (total)	HA	HB	HU
Globally[#]	5,537,527,603	195,263	157,517	31,997	5,749
South Africa	58,558,270	2,345	1,967	378	0

[#]: World population covered by the countries in the survey report; HA Haemophilia A; HB: Haemophilia B; HU: Haemophilia of unknown type.

Haemophilia B is considered an orphan disease, which by definition affects very few individuals in a specific region. The definition of an orphan disease varies in different countries, depending on the country's prevalence criteria and the population size. In the United States of America (USA), a disease is considered rare when fewer than 200,000 people are affected (Aronson, 2006; Thorat et al., 2018). The prevalence-based definitions in different countries range from 1 in 2,000 to 1 in 500,000 (Dharsi et al., 2017). Unfortunately, according to the South African Non-Communicable Diseases Alliance annual report of 2017 on rare diseases, there is no decisive definition of a rare disease in South Africa (Rare Diseases South Africa – Annual Report, 2017). With orphan diseases often being neglected in academic and industry-driven research and development, we feel it is imperative to investigate these disorders to contribute towards improved quality of life (QoL) for people affected by these rare diseases or disorders.

Haemophilia B is caused by a wide spectrum of mutations in the FIX gene (*F9*), which is located on the X chromosome and comprises eight exons (Álvarez Román et al., 2021; Bowen, 2002; Burke et al., 2021; Lv et al., 2019; Lyu et al., 2016; Rocino et al., 2017; Soucie et al., 2018; Stark, 2020). Based on the data published by the European Association for Haemophilia and Allied Disorders Coagulation Factor Variant Databases (EAHAD-CFVD) 1,244 unique *F9* genetic variants corresponding to 4,713 individual cases have been reported (available at: https://*F9*-db.eahad.org/). These *F9* variants are distributed throughout the coding as well as the non-coding regions of the *F9* gene. Point mutations were found to be the most prominent, representing 71.9% of all *F9* variants, followed by deletions (17.1%), polymorphisms (4.1%), insertions (1.1%), insertion/deletions (indels) (1.4%), and duplications (3.9%) (Shen et al., 2022).

The detection of existing and novel variants plays an important role in improved diagnosis of different haemophilia B variants, improved treatment models, and improved risk prediction of FIX inhibitor development (Salviato et al., 2019). Furthermore, mutational analysis to identify pathogenetic *F9* variants can also assist in prenatal diagnosis and detection of carriers of haemophilia B variants or women/girls with haemophilia B (van Galen et al., 2021). Therefore, mutational analysis of the South African haemophilia B population may not only improve patient management and care but may also assist extended families in management of the potential disorder, and add to the current understanding of haemophilia B in our region.

CHAPTER 2: LITERATURE REVIEW

INTRODUCTION

This chapter is a comprehensive expansion of the introductory chapter, and provides an overview of the available literature regarding haemophilia B. A brief history of haemophilia B is given, followed by the discussion of the inheritance pattern, classification, and the genetic aspects of this disorder. Furthermore, the current diagnostic methods and various treatment options for haemophilia B are also discussed briefly, as causative genetic variants may influence these entities.

2.1 Background of haemophilia B

Haemophilia B, also known as FIX deficiency or Christmas Disease, is a congenital rare bleeding disorder characterised by a deficiency of coagulation FIX caused by mutations in the *F9* gene (Álvarez Román et al., 2021; Burke et al., 2021; Lyu et al., 2016; Soucie et al., 2018; Stark, 2020). Haemophilia B (Christmas Disease) was first described in 1952 when a patient, Stephan Christmas from the Western Cape in South Africa, presented with deficient plasma levels of the FIX protein (Biggs et al., 1952). Thus, the South African haemophilia B population has been at the forefront of haemophilia B research since the beginning of the specific classification. Unfortunately, due to the rarity of haemophilia B compared to haemophilia A in the country, the majority of South African haemophilia studies focus more on haemophilia A. As a consequence, limited data exists regarding haemophilia B in the country.

2.2 Inheritance pattern of haemophilia B

Haemophilia B follows an X-linked recessive inheritance pattern, predominantly affecting males. A carrier mother has a 25% chance of having an affected boy and a 25% chance of a carrier girl (Figure 2.1). However, in the case of an affected father, none of his boys will be affected, whereas 100% of his daughters will be haemophilia B carriers or women with haemophilia B, depending on their FIX levels (Radic et al., 2013).

It has been reported that the term “haemophilia carrier” and its corresponding connotations, such as potential or possible carrier, have not only restricted research but also appropriate treatment and patient management in these cases (van Balen et al., 2021). In rare cases, a woman may inherit the mutated X chromosome from both parents, resulting in either the complete expression of the variant’s severity or, due to skewed lyonisation, present with moderate to severe haemophilia B (van Balen et al., 2021). Therefore, it is important to not only look at the genetic variants in females, but also to evaluate their phenotypic information, in order to provide an accurate diagnosis and effective patient management.

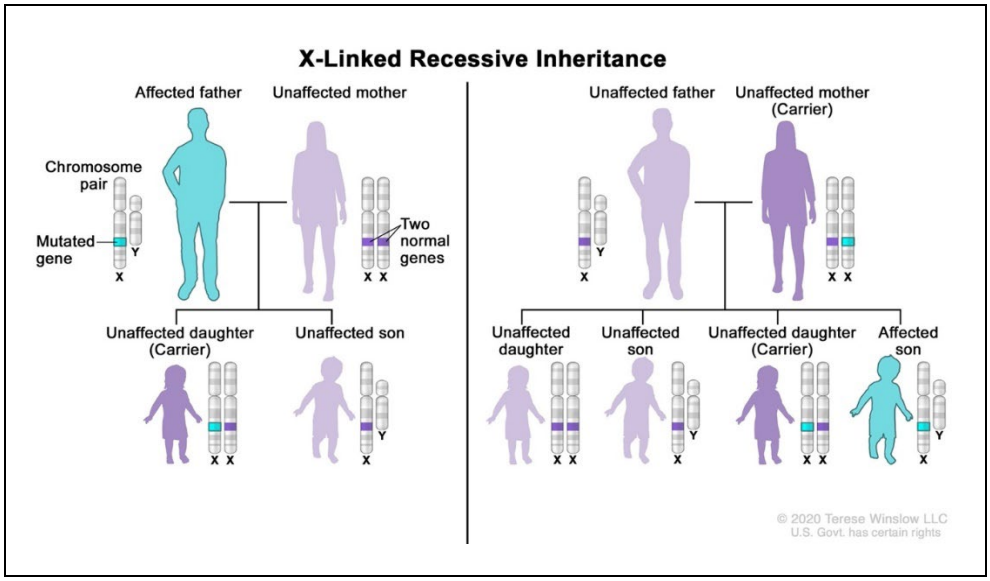

Figure 2.1. Illustration of the X-linked recessive inheritance pattern of haemophilia B. *(Available at and copied from https://www.cancer.gov/publications/dictionaries/genetics-dictionary/def/x-linked-recessive-inheritance Accessed: 10 July 2022).*

2.3 Haemostasis and the role of FIX in coagulation

Multiple haemostatic mechanisms are involved in maintaining a normal blood flow and tissue perfusion by preventing sudden changes in blood volume and pressure (Mohammed et al., 2018). In the case of a vascular injury, the formation of a fibrin plug relies on a series of sequential events, known as the coagulation cascade (Peters and Harris, 2018; Zaidi and Green, 2022). The traditional coagulation cascade (Figure 2.2) is defined as the initiation of coagulation proteins through the extrinsic or intrinsic pathways, which ultimately intersect at the common pathway (Ho and Pavey, 2017).

The initial trigger of the traditional coagulation cascade is the activation of the extrinsic pathway (Bowen, 2002; Ho and Pavey, 2017).

The extrinsic pathway is activated by releasing tissue factor (TF) at the site of vessel injury. Tissue factor has a high affinity for its cofactor, activated factor VII (FVIIa), thus, resulting in the formation of the TF/FVIIa complex. The TF/FVIIa complex catalyses the conversion of factor X (FX) to activated factor X (FXa) and FIX to activated FIX (FIXa) (Grover and Mackman, 2019; Palta et al., 2014). FIXa, together with its cofactor, activated factor VIII (FVIIIa), forms the tenase complex and promotes further FX activation. FXa will propagate the common pathway by forming the prothrombinase complex with its cofactor, activated factor V (FVa) (Bowen 2002; Chaudhury et al., 2021).

The intrinsic pathway is initiated in the presence of prekallikrein (PKK), high molecular weight kininogen (HMWK), and factor XII (FXII), leading to the activation of FXII to activated FXII (FXIIa). FXIIa converts factor XI (FXI) to activated factor XI (FXIa), which can also activate FIX, and ultimately the activation of FX in the common pathway (Lowe, 2001; Rallapalli et al., 2013; Tjärnlund-Wolf and Lassila, 2019; Top et al., 2019; Zaidi and Green, 2022). Consequently, the prothrombinase complex converts prothrombin (FII) to thrombin (FIIa) (Rallapalli et al., 2013; Tjärnlund-Wolf and Lassila, 2019). Thrombin will cleave fibrinogen (FI) to insoluble fibrin (FIa) and induce the activation factor XIII (FXIII), which will stabilise the fibrin clot by creating a fibrin network, through the incorporation of crosslinked fibrin polymers (Top et al., 2019; Zaidi and Green, 2022).

The traditional coagulation model suggests two separate, redundant pathways that operate independently, with limited interaction between them. The traditional model, however, explains mechanisms involved in the maintenance of haemostasis *in vivo* inadequately. In addition, clinical observations in hereditary bleeding disorders, due to coagulation factor deficiencies, do not support the independent working of the extrinsic and intrinsic pathways depicted by the traditional coagulation cascade (Ho and Pavey,

2017). Therefore, an alternative cell-based model was developed to replace the traditional coagulation cascade (Velou and Ahila, 2020).

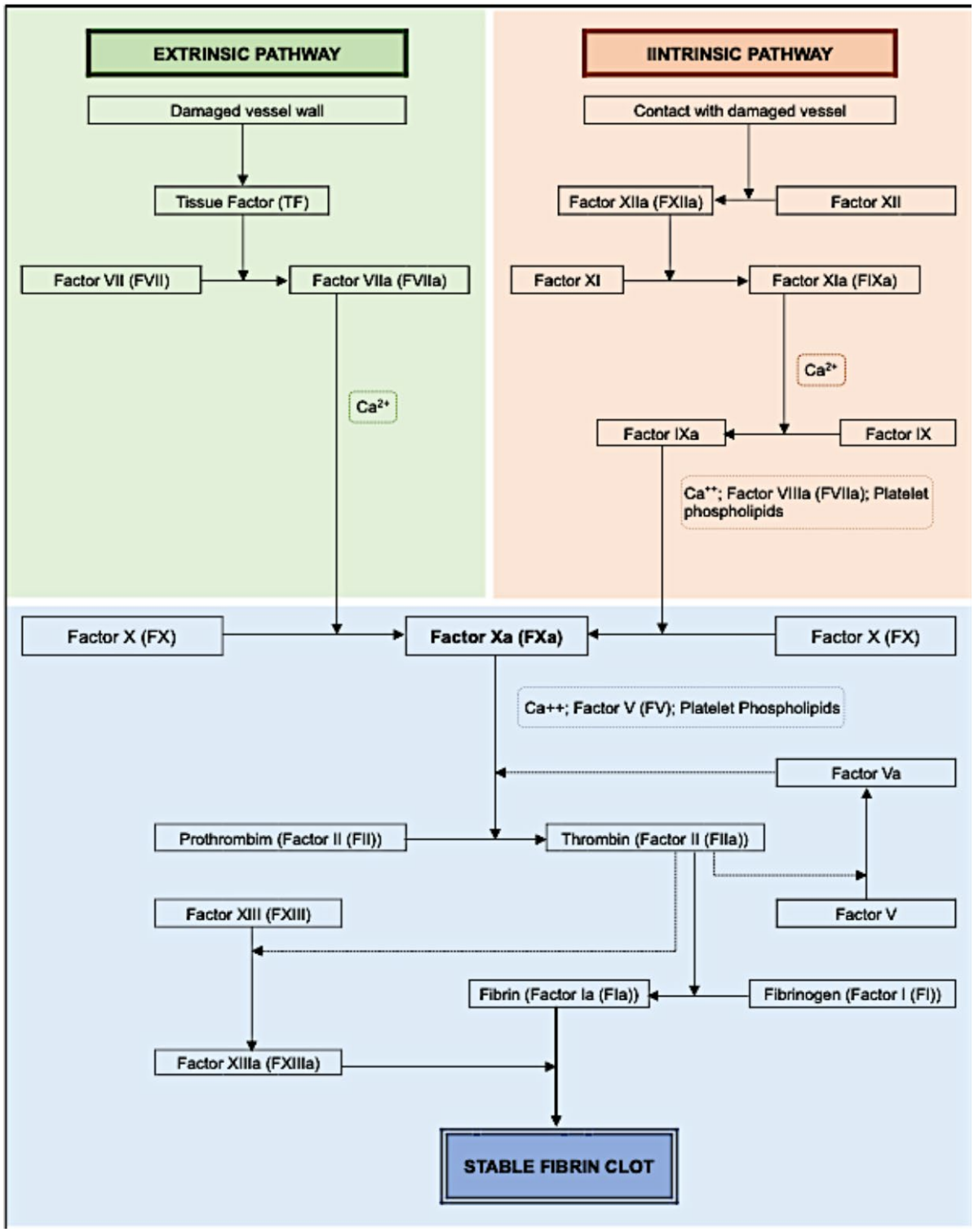

Figure 2.2. Depiction of the traditional coagulation cascade.

The cell-based model, highlighting the interaction of coagulation factor proteins with respective cell surfaces, sheds light on the constraints demonstrated by the hypothesis of the traditional coagulation models. This model is portrayed as three overlapping phases: initiation, amplification, and propagation (Ho and Pavey, 2017; Velou and Ahila, 2020). In the cell-based model (Figure 2.3) the two pivotal cellular components are TF and platelets, whereas thrombin and fibrinogen are the main clotting factors that interact with the cell-based components to maintain a haemostatic balance (Velou and Ahila, 2020).

The initiation phase is activated when blood cells are exposed to TF at the site of injury. The TF, together with its cofactor, FVIIa, forms the TF/FVIIa complex to activate small amounts of FIX and FX. FXa subsequently forms the prothrombinase complex with its cofactor, FVa. Subsequently, the formation of FXa cleave prothrombin to produce thrombin. The little thrombin formed is important for the activation of the cofactors, FV and FVIII, located on the surface of activated platelets. Furthermore, FIXa diffuses to the activated platelets and attaches to the surface receptor, followed by the interaction with its cofactor (FVIIIa) to form the tenase complex (FIXa/FVIIIa). However, the amount of thrombin produced during the initiation phase is too small to produce a fibrin clot but is crucial for the amplification phase (Ferreira et al., 2010; Ho and Pavey, 2017; Velou and Ahila, 2020).

The amplification phase involves additional platelets, activated by the thrombin, to be recruited to the site of injury. In addition, during amplification, the tenase complex is effective in producing the appropriate amount of FXa to maintain haemostasis. Furthermore, platelets can bind to the tenase and prothrombinase complex, which enhance an increased thrombin production. The positive feedback loop of the activated coagulation factors and the cooperative working of the tenase and prothrombinase complexes rapidly starts the propagation phase (Ferreira et al., 2010; Ho and Pavey, 2017; Velou and Ahila, 2020).

The propagation phase involves the continued activation of the coagulation cascade, leading to the formation of a fibrin mesh that strengthens the clot. This phase is

initiated when, in the presence of calcium and phospholipid membrane over activated platelets, both the tenase and prothrombinase complexes are available at the same time and site. The thrombin burst allows for high volumes of fibrinogen conversion to fibrin. FXIIIa, activated by thrombin, links the fibrin polymers to form a stable fibrin clot (Ferreira et al., 2010; Ho and Pavey, 2017; Velou and Ahila, 2020). When evaluating both the traditional and the contemporary cell-based models of coagulation, it is clear that FIX has a central role in coagulation. Therefore, any change in function or quantity of FIX may compromise the body's ability to form a stable blood clot, resulting in excessive bleeding.

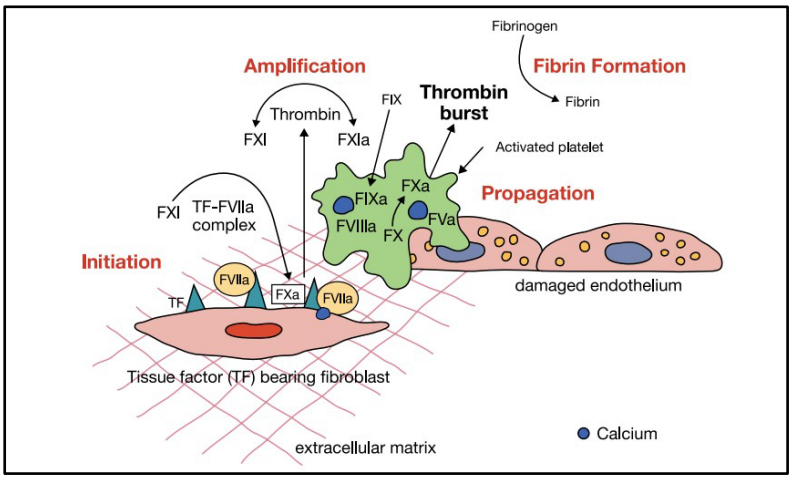

Figure 2.3. Depiction of the cell-based coagulation cascade *(Copied from Ho and Pavey, 2017).*

2.4 Molecular biology of the factor IX gene (*F9*)

The understanding behind the molecular basis of haemophilia B began with the characterisation of the FIX gene (*F9*) during the 1980s. The complete nucleotide sequence for *F9* was published in 1985 (Goodeve, 2015; Yoshitake et al., 1985). The *F9* gene (OMIM 306900) is located on the long arm (q) of chromosome X, at position 27.1 (Xq27.1) and spans roughly 34 kilobase pairs (kbp) (Bowen, 2002; Gomez, 2010; Perez Botero et al., 2018; Yi et al., 2020). *F9* comprises eight exons, transcribed to a messenger ribonucleic acid (mRNA) transcript of 2,803 base pairs (bp), which contain a five prime (5') untranslated region of 29 bp, an open reading frame (ORF) of 1,380 bp, and a three prime (3') untranslated region of 1,390 bp in length (Bowen, 2002).

The ~1.4 kbp ORF *F9* mRNA molecule translates into an immature FIX glycoprotein (Bowen, 2002; Sutherland et al., 2020).

FIX, a highly post-translationally modified glycoprotein, is the largest vitamin K-dependent coagulation factor protein synthesised by hepatocytes. The immature FIX polypeptide (461 amino acids (aa)) comprises six extensive protein domains: a signal peptide (28 aa residues), an N-terminus propeptide (18 aa residues), the primary mature peptide (415 aa residues) comprising a gamma-carboxyglutamic acid (GLA) domain, two consecutive epidermal growth factor (EGF) like domains (EGF1 and EGF2), a short linker domain, the activation peptide (AP) domain, and the C-terminus serine protease (SP) domain (Figure 2.4) (Goodeve, 2015; Radic et al., 2013; Rallapalli et al., 2013; Zacchi et al., 2021).

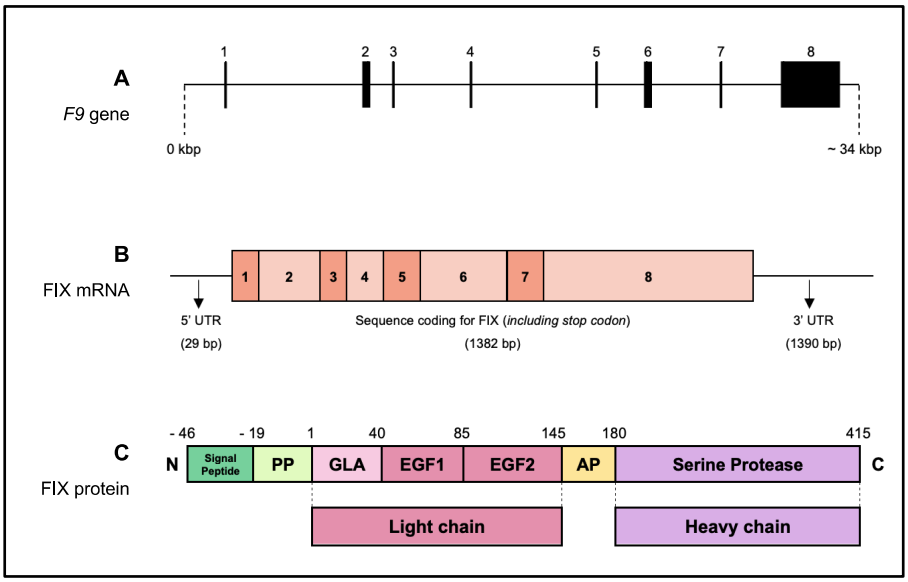

Figure 2.4. Schematic presentation of the relationship between F9 and the domain structure of the FIX protein. *(A) Structure of the human F9 gene, with the vertical bars (1-8) depicting the eight exons. The thickness of each bar is approximate to the size of the specific exon. (B) The primary FIX mRNA transcript demonstrates the relative size and location of the coding sequences transcribed from each exon, depicted by the numbered blocks. (C) The FIX protein structure, which includes propeptide domains and mature poly-peptide domains. (Copied and modified from Rallapalli et al., 2013 and Shen et al., 2022). *AP: Activation Peptide; bp: base pairs; C: Carboxy terminal; EGF: Epidermal growth factor; FIX: Coagulation factor IX; GLA: Gamma-carboxyglutamic acid; N: Amino terminal; PP: Propeptide; UTR: Untranslated region.*

The mature FIX protein structure (Figure 2.5) consists of an N-terminal propeptide, the GLA domain (residues 1 – 40), a six—residue hydrophobic stack (residues 41 – 46), the two consecutive EGF-like domains (EGF1, spanning from residue 47 – 83; EGF2, spanning from residue 88 – 127), which is connected by linker residues 84 – 87, the AP domain (residues 146 – 180), and a C-terminal protease domain (residue 181 - 415) (Zacchi et al., 2021). The binding of Calcium ions (Ca^{2+}) to the different FIX protein domains imparts specific properties to the FIX structure, which confer specific biological function (Liu et al., 1997; Schmidt and Bajaj, 2003). The GLA domain possesses multiple low- and intermediate-affinity Ca^{2+} binding sites, whereas both the EGF1 and protease domain possess a single high-affinity Ca^{2+} binding site (Bajaj et al., 1992, Rao et al., 1995; Schmidt and Bajaj, 2003). The Ca^{2+} binding sites are essential for FIXa to achieve complete enzymatic activity through the formation of the Ca^{2+}-dependent tenase complex with its cofactor (FVIIIa) (Shen et al., 2022).

The complex process of FIX biosynthesis involves several post-translational modifications (PTMs) (Figure 2.5). The majority of the PTMs occur close to or on the GLA domain, EGF-like 1 domain and the AP domain (Zacchi et al., 2021). These modifications include gamma (*y*)-carboxylation of the first 12 Glutamate (Glu) residues ($Glu^{7,8,15,17,20,21,26,27,30,33,36,40}$) of the GLA domain; *O*-glycosylation of Serine (Ser)^{53}, Ser^{61}, Threonine (Thr)^{159}, Thr^{169}, Thr^{172}, and Thr^{179}; β-hydroxylation of Aspartic acid (Asp)^{64}; *N*-glycosylation of Asparagine (Asn)^{157} and Asn^{167}; sulfation of Tyrosine (Tyr)^{155}; and phosphorylation of Ser^{158} (Schmidt and Bajaj, 2003). Additionally, the N-terminus propeptide and AP domain are removed by means of proteolytic events, which is vital for FIX (Zacchi et al., 2021). Consequently, the mature FIX protein molecule comprises a 415 single aa chain (Mr 57,000), containing roughly 17% carbohydrate by weight (Di Scipio et al., 1977; Schmidt and Bajaj, 2003).

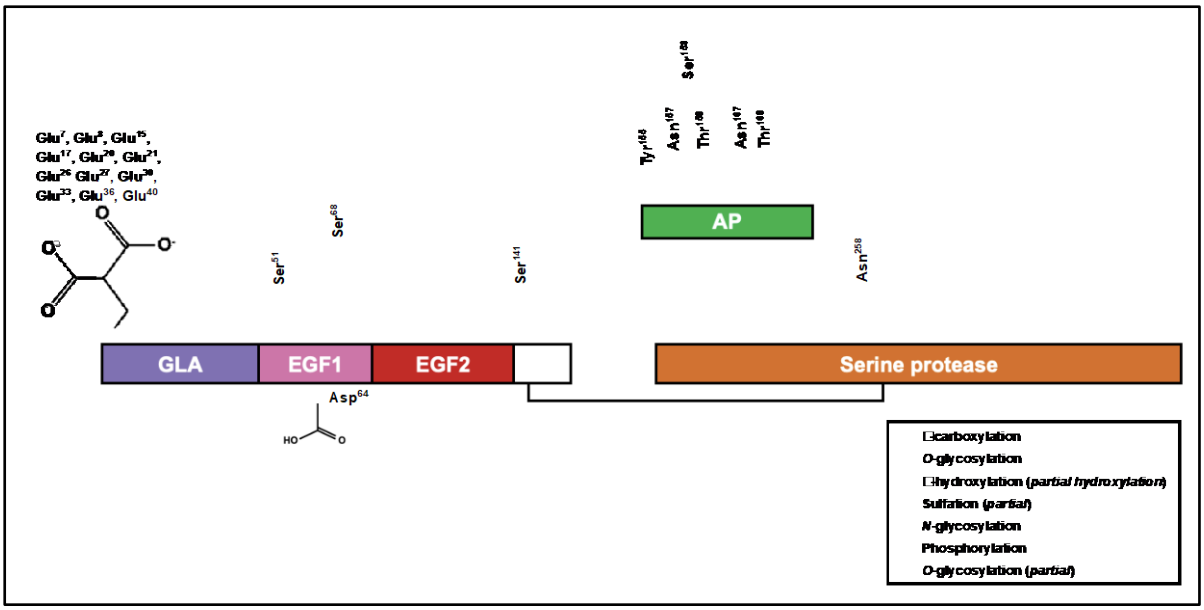

Figure 2.5. The coagulation FIX structural protein domains and post-translational modifications (PTMs). *Illustration of the previously discussed PTMs in FIX (plasma derived or recombinant). The GLA domain (purple), undergoes* γ*-carboxylation at 12 potential Glu residues (*$Glu^{7,8,15,17,20,21,26,27,30,33,36,40}$*). The EGF1 domain undergoes O-glycosylation of* Ser^{51} *and* Ser^{61}*,* β*-hydroxylation of* Asp^{64}*, and phosphorylation of the* Ser^{68} *residue. The EGF2 domain (red) does not contain any potential sites for PMTs. The EGF2 domain is linked to the AP domain (green) by the short linker domain (white), which contains one potential site for O-glycosylation on* Ser^{141}*. The AP domain (green) contains two potential sites for N-glycosylation at* Asn^{157} *and* Asn^{167}*; four sites of O-glycosylation (partial) at* Thr^{159}*,* Thr^{169}*,* Thr^{172}*, and* Thr^{179}*, sulfation of* Tyr^{155}*, and phosphorylation of* Ser^{158}*. Finally, the Serine protease domain (orange) contains one potential site for N-glycosylation at residue* Asn^{258}*. **γ*: Gamma;* β*: Beta; AP: Activation peptide; Asn: Asparagine; Asp: Aspartic Acid; EGF: Epidermal growth factor; GLA: Gamma-carboxyglutamic acid domain; Glu: Glutamic acid; Ser: Serine; Thr: Threonine; Tyr: Tyrosine (Copied and modified from Zacchi et al., 2021).*

Subsequently, FIX will be secreted into the bloodstream as a zymogen, with a molecular mass of approximately 57 kilodaltons (kDa) (Giannelli et al., 1998; Orlova et al., 2012; Radic et al., 2013; Top et al., 2019). In the case of injury to a vessel wall, in terms of the traditional coagulation cascade, FIX will be cleaved by FXIa (intrinsic pathway) or by the TF/FVIIa complex (extrinsic pathway), to produce circulating activated FIX (FIXa) (Figure 2.6) (Bowen, 2002; Orlova et al., 2012; Top et al., 2019).

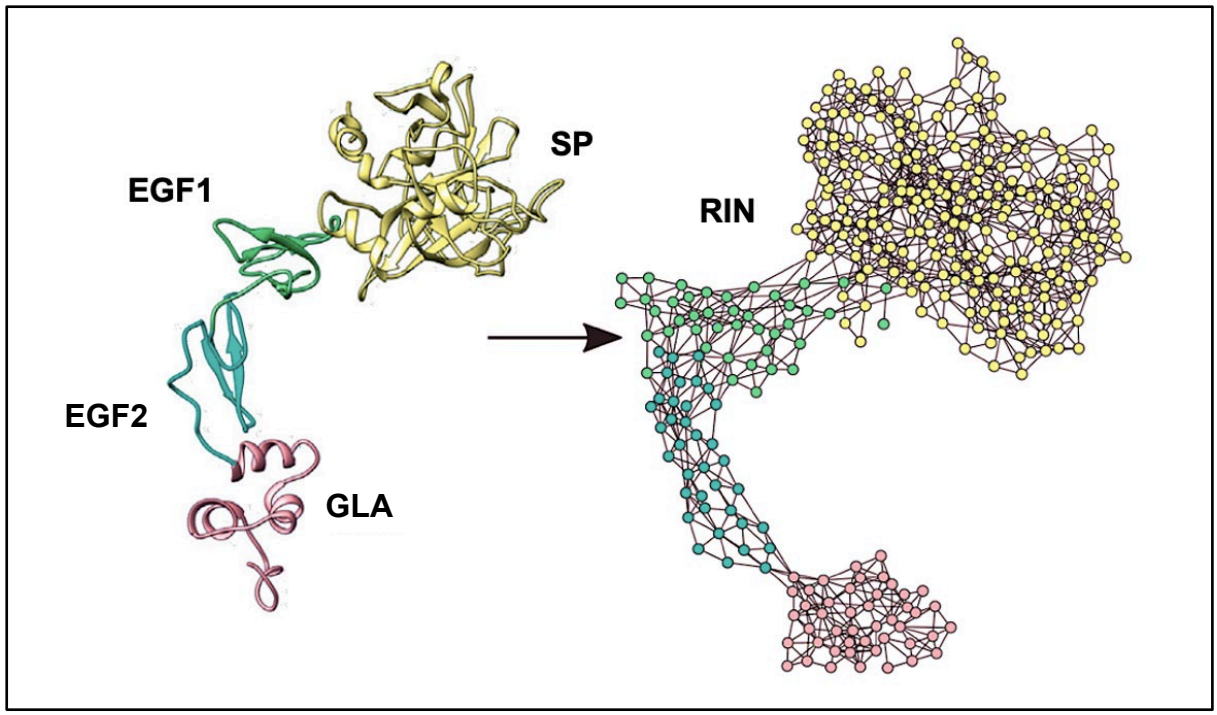

Figure 2.6. Depiction of the activated coagulation FIX (FIXa) protein structure as a residue interaction network. *Illustrated on the three-dimensional (3D) ribbon FIXa structure (left), is the GLA domain (pink), two consecutive EGF-like (EGF1 (turquoise); EGF2 (green)) domains and the SP domain. In the FIXa residue interaction network (RIN) structure, each amino acid is represented by a node, which are connected by an edge if their atoms are in close proximity (~5 Å). *3D: three dimensional; EGF1: Epidermal growth factor like 1 domain; EGF2: Epidermal growth factor like 2 domain; GLA: Gamma-carboxyglutamic acid; RIN: Residue interaction network; SP: Serine protease (Copied and modified from Lopez et al., 2022).*

2.5 Distribution of *F9* variants

Knowledge of genetic variants within the *F9* gene contributed significantly to the understanding of haemophilia B. Currently, the European Association for Haemophilia and Allied Disorders (EAHAD) Coagulation Factor Variant Databases (CFVD) (available at: https://*F9*-db.eahad.org/index.php, November 2022) have compiled a total of 1,244 unique *F9* variants, corresponding to 4,713 individual haemophilia B patients. This heterogeneous spectrum of variants occurs throughout the coding and non-coding regions of the *F9* gene, including the promoter region, introns, as well as in the 3' untranslated region (UTR) (McVey et al., 2020). Of the 1,244 reported *F9* variants, 895 (71.9%) are point mutations, 213 (17.1%) deletions, 51 (4.1%) polymorphisms, 48 (3.9%) duplications, 18 (1.4%) indels, 14 (1.1%) insertions, and 5 (0.4%) complex variants.

2.6 Classification and clinical aspects of haemophilia B

Taking into account the crucial role of circulating FIX in coagulation, deficiencies in both quality and quantity, due to variants in *F9*, are connected to bleeding phenotypes (Figure 2.7). Haemophilia B, as reported by the WFH and the International Society on Thrombosis and Haemostasis (ISTH), can be classified into three levels of severity based on the residual FIX activity in the plasma (Srivastava et al., 2013). The severity of haemophilia B generally correlates with the plasma level or residual clotting activity of FIX. It can be classified as mild (FIX:C >5 – <40 IU/dL), moderate (FIX:C 1 – 5 IU/dL) or severe (FIX:C <1 IU/dL) (Table 2.1) (Nigam et al., 2014; Radic et al., 2013; Santroro et al., 2018; Srivastava et al., 2020).

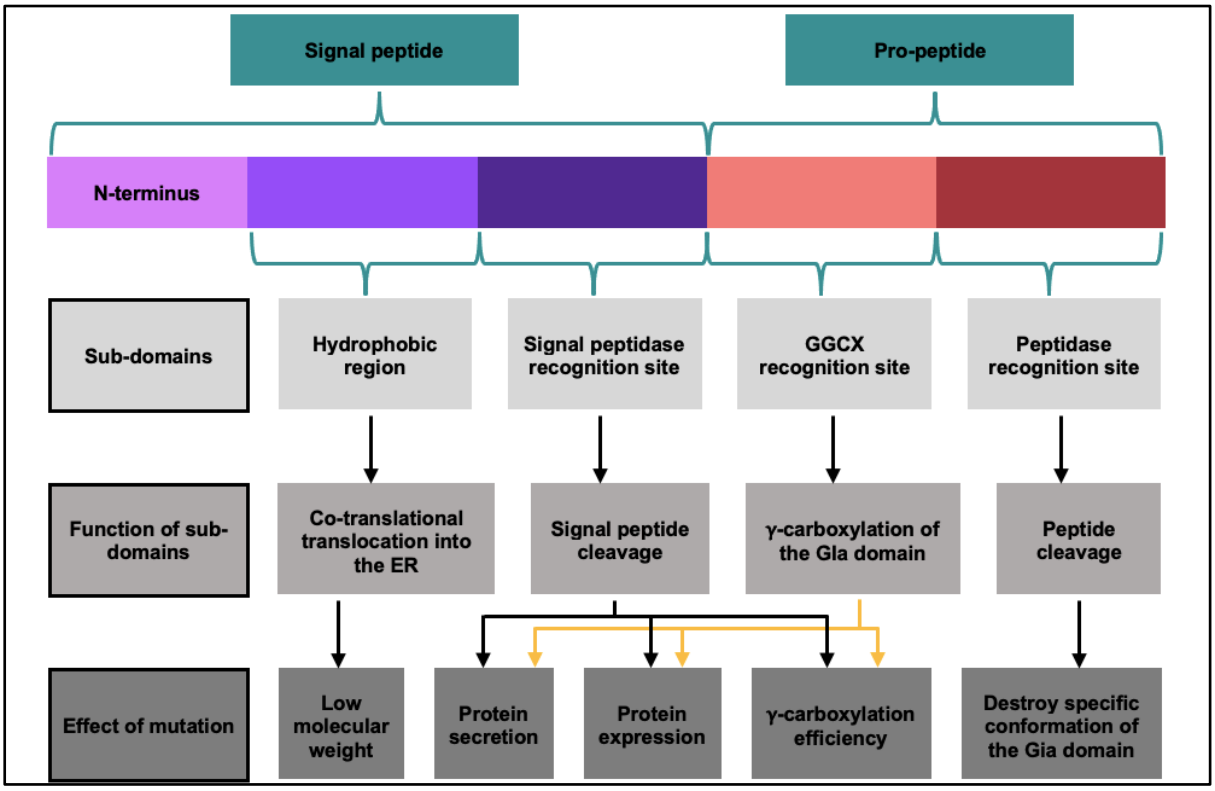

Figure 2.7. Hierarchical demonstration of the mutational effect on the functional sub-domains of the FIX signal- and propeptide. *The signal peptide comprises two subdomains, namely the hydrophobic region and the signal peptidase recognition site. The hydrophobic region is recognised by signal recognition particles, which assist in co-translational translocation of FIX into the Endoplasmic Reticulum (ER). Furthermore, mutations in this region will result in the protein molecule having a lower molecular weight. Mutations within the signal peptidase recognition site will alter the cleavage of the signal peptide, compromise the process of the protein secretion, expression, as well as the γ-carboxylation efficiency. *γ: Gamma; ER: Endoplasmic Reticulum; GGCX: Gamma-glutamyl carboxylation enzyme (Copied and modified from Gao et al., 2020).*

Table 2.1. Classification of the haemophilia B severity, based on the plasma concentration of functional FIX and correlation to clinical features.

Severity	Plasma FIX	Clinical features	Reference(s)
Mild	>5 – <40 IU/dL (5 – 40% FIX)	Spontaneous bleeding is rare. Bleeding events may occur after moderate to severe trauma, surgery, dental extractions and accidents.	Bolton-Maggs and Pasi, 2003 Franchini et al., 2013 Srivastava et al., 2013
Moderate	1 – 5 IU/dL (1 – 5% FIX)	Seldom spontaneous bleeds into joints (hemarthrosis) and muscles. Usually prolonged bleeding following mild trauma or surgery.	Bolton-Maggs and Pasi, 2003 Franchini et al., 2013 Srivastava et al., 2013
Severe	<1 IU/dL (<1% FIX)	Spontaneous and recurrent bleeding events into joint and muscle. Excessive haemorrhage may occur after surgery, accident related injuries and dental extractions.	Bolton-Maggs and Pasi, 2003 Booth et al., 2018 Salviato et al., 2019

**** dL: Decilitre, FIX: Factor IX, IU: Infusion units***

A new nomenclature for haemophilia B carriers has been clinically defined into five distinct categories (Figure 2.8). For the international standardisation of terminology and classification of haemophilia B carriers, based on the plasma level of FIX, haemophilia B carriers can be clinically classified as mild (FIX:C >5 – <40 IU/dL), moderate (FIX:C 1 – 5 IU/dL), severe (FIX:C <1 IU/dL), symptomatic haemophilia B carrier (FIX:C <40 IU/dL; bleeding phenotype), or asymptomatic haemophilia B carrier (FIX:C >40 IU/dL; woman or girl without a bleeding phenotype) (van Galen et al., 2021).

Patients with mild haemophilia usually present with excessive bleeding only after major injuries or surgical trauma. In contrast, in moderate haemophilia B patients, excessive bleeding events may occur due to minor traumatic injuries (Santagostino and Fasulo, 2013). Furthermore, spontaneous, and frequent bleeding events may be experienced by patients diagnosed with severe haemophilia B (Nigam et al., 2014; Santagostino and Fasulo, 2013).

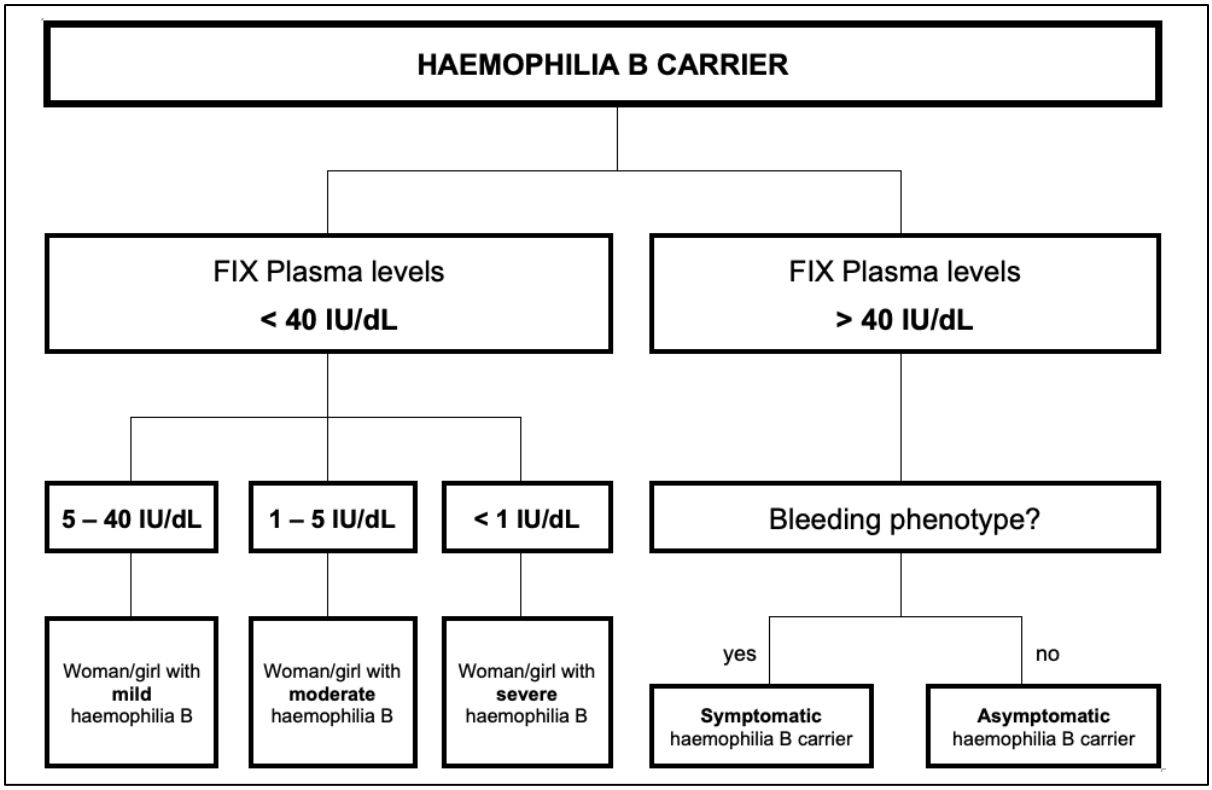

Figure 2.8. The new nomenclature for haemophilia B carriers and women and/or girls with haemophilia B *(Copied and modified from van Galen et al., 2021).*

2.7 Genotype-phenotype relationship for haemophilia B

The activity level of endogenous FIX dictates the severity of haemophilia B, however, the absence of FIX alone does not always correlate with the bleeding phenotype. Therefore, related patients with the same *F9* pathogenic variant might present with different bleeding tendencies, due to the potential change in other related genotypes, which consequently tilt the haemostatic balance more to either the procoagulant or anti-coagulant state (Tjärnjund-Wolf and Lassila, 2019).

Also, knowledge of specific pathogenic variants in haemophilia B is vital in understanding the genotype-phenotype relationship. The severity of clinical and biochemical haemophilia B is in close relation to the specific pathogenic variants, distributed throughout the protein domains and controlling regions of *F9*, except the poly-A site (Figure 2.4) (Gao et al., 2020; Giannelli et al., 1994; Radic et al., 2013). Patients with severe haemophilia B reportedly have large or small deletions and nonsense mutations in *F9*. In contrast, missense mutations are more likely to be found

in patients diagnosed with moderate and mild forms of haemophilia B (Oldenburg and Pavlova, 2006). Additionally, there are a few databases that document *F9* mutations, such as the EAHAD-CFVD (https://*F9*-db.eahad.org/), the Human Gene Mutation Database HGMD (www.hgmd.cf.ac.uk/), FIX mutation database (http://www.factorix.org/), and UniProt (http://www.uniprot.org/).

The aims of these databases are (Li et al., 2013):

1. To confirm whether *F9* variants detected in a study population were previously described,
2. To identify and report novel variants, and
3. To predict the pathogenicity of a variant.

At the time of writing this dissertation, South Africa had not contributed genetic data to any of these databases. Therefore, limited genotype-phenotype data exist for our population. Thus, it is vital to perform genetic studies in people with haemophilia B to better understand the molecular contributions to phenotypes in this population.

2.8 Treatment of haemophilia B

Knowledge of how a certain pathogenic variant may influence the possible treatment options is vital to ensure appropriate patient management. Different treatment options, together with their advantages and disadvantages, will be discussed in the following paragraphs. The initial haemophilia treatment protocol included direct blood transfusions in the year 1840, followed by fresh frozen plasma as a treatment for bleeding episodes in the 1950s and most of the 1960s. In 1965, modern treatment was initiated when Judith Pool identified the cryoprecipitate fraction of fresh frozen plasma (Key and Negrier, 2007; Peyvandi et al., 2016). Currently, replacement therapy of deficient coagulation FIX is the predominant treatment choice for haemophilia B (Weynand and Pipe, 2019). FIX replacement therapy can either be administered at the time of a bleeding event (episodic) or at regular intervals with the aim of maintaining haemostasis to prevent bleeding and the development of joint damage (prophylaxis) (Franchini et al., 2013; Martinowitz and Lubetsky, 2013; Srivastava et al., 2020; Weynand and Pipe, 2019). Plasma-derived FIX (pdFIX), as well as recombinant FIX (rFIX) coagulation concentrates, are standard treatment

methods for haemophilia B (Franchini et al., 2013). An article published by Potgieter et al. (2017) highlights that FIX replacement therapy remains the dominant treatment approach for haemophilia B in South Africa. However, it is important to note that the presence of large molecular defects in the *F9* gene, carries an increased risk for adverse reactions against this common treatment approach. Therefore, determining the causative mutation in a person with haemophilia B may potentially influence the chosen treatment plan of the clinician.

2.8.1 Plasma-derived FIX (pdFIX) concentrates

In the 1970s, plasma-derived FIX concentrates became commercially available, resulting in effective treatment for bleeding events, as well as the emergence of home and self-infusion replacement therapies (Bolton-Maggs and Pasi, 2003). The pdFIX concentrates available include the following: intermediate-purity pdFIX (Konyne-80 and Bebulin®), high-purity pdFIX (AlphaNine® SD), and ultra-high-purity monoclonal-purified pdFIX (Mononine®, Nonafact®). However, none of these products are commercially available in South Africa. Patients receive these therapies through intravenous infusions (Gater et al., 2011). The transmission of viral and blood-borne diseases is a notable safety concern associated with the conventional FIX concentrate derived from human plasma (Shapiro et al., 2005).

The utilisation of contaminated pdFIX concentrates caused the widespread transmission of blood-borne infections such as the human immunodeficiency virus (HIV) in patients with haemophilia in the early 1980s. It increased mortality rates from the acquired immune deficiency syndrome (AIDS) in this population (Gater et al., 2011; Mannucci, 2003). The safety of modern-day plasma-derived products has advanced significantly due to the adoption of multiple viral inactivation and purification steps for FIX products (Franchini et al., 2013). However, the risk of disease transmission through plasma-derived products, such as the variant Creutzfeldt Jacob disease (vCJD), and non-capsulated viruses, still exists (Gater et al., 2011; Ludlam and Turner, 2006; Ungchusak et al., 2005). Although FIX replacement therapy is widely available, effective, and a safe treatment option for haemophilia B, patients are still experiencing unfortunate bleeding episodes and recurrent joint problems.

2.8.2 Recombinant FIX (rFIX) concentrates

The introduction of rFIX replacement therapy overcame some of the disadvantages of the pdFIX concentrates, such as viral transmissions (Weynand and Pipe, 2019). Recombinant FIX is manufactured without any proteins derived from humans or animals, which makes it free from the risk of infection and furthermore deemed to be more effective and safer than pdFIX (Franchini et al., 2013; Weynand and Pipe, 2019). The introduction of the human FIX into Chinese hamster ovary cells led to the production of genetically engineered rFIX, which became commercially available in 1998 (Franchini et al., 2013; Hoots and Shapiro, 2018; Shapiro et al., 2005). Currently, the licenced standard half-life rFIX products available for haemophilia B treatment include, BeneFIX®, Ixinity and Rixubis (Franchini et al., 2013; Hoots and Shapiro, 2018). Another study demonstrated that BAX 326, a novel rFIX with two viral inactivation steps, is a safe and effective treatment option, with a pharmacokinetic profile similar to the commercially available rFIX products (Windyga et al., 2014).

While recombinant FIX products closely resemble the natural protein, none of them are entirely identical. The primary reason for these variations lies in the various PTMs. Additionally, an interesting factor to consider is a polymorphism present in plasma-derived FIX, specifically at residue 148. Two variants have been identified for this polymorphism: Thr^{148} and Ala^{148}. The prevalence of this polymorphism varies across different populations, with Thr^{148} consistently being the predominant variant. Up until now, all developed rFIX products have been designed to match the Ala^{148} variant. However, a newly approved FDA-licensed recombinant human FIX called IXINITY (trenonacog alfa) differs by having the primary amino acid sequence aligned with the prevalent Thr^{148} polymorphism (Monroe et al., 2016). Currently available rFIX products, as well as pdFIX, have a relatively short half-life. Therefore, patients require frequent intravenous infusions (2 – 3 times per week) in order to achieve adequate prophylaxis (Martinowitz and Lubetsky, 2013). The half-life ($t_{1/2}$) of all these rFIX products is between 16 and 17 hours. Extended half-life rFIX products are available which could translate into reduced infusion frequencies (Dolan et al., 2018; Hoots and

Shapiro, 2018). There are currently three licenced extended half-life rFIX products available; Alprolix, Idelvion and Refixia (Table 2.2) (Hart et al., 2022).

Table 2.2. Currently licenced rFIX products with extended half-lives.

rFIX product	Strategies and properties	Reference(s)
rFIX-Fc	rFIX-Fc (Alprolix) is a fusion protein with a single FIX molecule fused to the Fc-portion of human IgG1, developed to address the short half-life ($t_{1/2}$) of current available FIX products. rFIX-Fc demonstrates a 2- to 3-fold longer $t_{1/2}$ (approximately 84 hours) than rFIX. Moreover, the rFIX-Fc product has a greater effect regarding the treatment of episodic bleeds, due to the potential reduction in the number of follow-up treatments.	Peters et al., 2010 Shapiro et al., 2012 Franchini et al., 2013 Jiménez-Yuste et al., 2014 Hart et al., 2022
rFIX-FP	The rFIX-albumin (Idelvion) protein composed of the FIX gene fused with the albumin gene via a cleavable linker sequence. This product was approved by the Food and Drug Administration (FDA) in 2016 as prophylaxis and/or treatment for bleeding episodes in patients with haemophilia B. This rFIX-FP product has demonstrated a 5- to 6-fold prolongation in $t_{1/2}$ (approximately 102 hours) than standard rFIX.	Nolte et al., 2012 Santagostino et al., 2016 Hoots and Shapiro, 2018 Hart et al., 2022
N9-GP	Nonacog beta pegol is a site specific glycoPEGylation product (Refixia) composed of FIX gene fused with a PEG moiety, which is attached to an activation peptide of the FIX protein, by site-directed glycoPEGylation. Pharmacokinetic data demonstrate a 5-fold increased $t_{1/2}$ (approximately 93 hours), compared to standard rFIX.	Negrier et al., 2011 Collins et al., 2014 Hart et al., 2022

FIX: Factor IX; IgG1: Immunoglobulin G; N9-GP: GlycoPEGylated rFIX; PEG: Polyethylene glycol; rFIX: Recombinant FIX; $t_{1/2}$: Half-life

2.8.3 Episodic versus prophylaxis

The main objectives for episodic treatment include the immediate and short-term compensation of FIX, in order to halt ongoing haemorrhage, as well as to decrease the impact of a bleeding episode on survival and long-term culminating damage (Gater et al., 2011). Prophylaxis for haemophilia was first described during the 1940s and 1950s. The rationale for prophylactic FIX replacement therapy is based on an observation made during a Swedish study in 1965, which demonstrated the association between arthropathy and the baseline levels of clotting factor (Ahlberg et al., 1965; Castaman, 2018; Fischer, 2012). Many observational studies regarding the impact of prophylaxis were reported in a haemophilia A and B combination group (Ahlberg et al., 1965; Castaman, 2018). Contrary to episodic treatment, the principle of prophylaxis is to prevent the occurrence of future bleeding events using FIX replacement therapy timeously (Coppola et al., 2009; Gater et al., 2011).

Prophylaxis is a long-term treatment approach that necessitates regular adherence. Its primary objective is to prevent joint bleeds and mitigate the long-term complications that arise from such bleeds, including the development of arthropathy, in individuals with haemophilia. (Coppola et al., 2009; Hoots and Shapiro, 2018; Oldenburg, 2015). Terminology to clarify whether prophylaxis is primary, secondary or tertiary, depending on the time of treatment initiation, previous joint bleeds and age, was updated in the 2020 WFH Guidelines for the Management of Hemophilia (Table 2.3) (Srivastava et al., 2020). Prophylaxis can be characterised based on the time of initiation, the number of previous joint bleeds and age. Primary prophylaxis is defined as regular prophylaxis initiated in absence of joint disease previously documented, prior to a second joint bleed and usually before or at the age of three. Secondary prophylaxis is when the regular intervals of prophylaxis are started after the occurrence of two or more joint bleeds, but before the development of joint disease, and normally at the age of three years or older. Tertiary prophylaxis is characterised as the initiation of regular continuous prophylaxis at the commencement of documented joint disease and normally pertains to prophylaxis started during adulthood (Srivastava et al., 2020).

Table 2.3. Different regimens for replacement therapy in haemophilia B.

Regime	Definitions	Reference(s)
Episodic	FIX replacement therapy administered at the time of a clinically evident bleeding event.	Berntorp et al., 2021 Castaman, 2018 Srivastava et al., 2020
Continuous prophylaxis	Regular replacement of clotting factor concentrate (CFC), FIX, to prevent bleeding events.	Berntorp et al., 2021 Castaman, 2018 Srivastava et al., 2020
Primary	*Regular, continuous prophylaxis in absence of a first or second joint bleed, no clinical documentation of joint disease and therapy and is initiated prior to the age of three years.*	
Secondary	*Regular, continuous prophylaxis started after the occurrence of two or more joint bleeding events but before the onset of chronic joint disease and is usually initiated at the age of three years or older.*	
Tertiary	*Regular, continuous prophylaxis initiated after the development of clinically evident joint disease to prevent future arthropathy and usually pertains to the start of prophylactic treatment started during adulthood.*	
Intermittent Prophylaxis	Administration of FIX replacement therapy in order to prevent short term bleeding events (e.g. during or after a surgical procedure), not more than 45 of 52 weeks (85%) of the year.	Berntorp et al., 2021 Castaman, 2018

CFC: Clotting factor concentrate; FIX: Factor IX

The introduction of prophylaxis has changed the lives of many patients with haemophilia remarkably (Hazendonk et al., 2018). On that account, compared to episodic treatment, prophylactic regimens demonstrated fewer bleeding episodes, notably less joint arthropathy and a tolerable quality of life, including reduced physical and psychological restrictions (Castaman, 2018; Coppola et al., 2009; Hazendonk et al., 2018). Even though prophylactic FIX replacement treatment is an effective option to prevent the occurrence of spontaneous bleeding events, it is not a cure for haemophilia. Further disadvantages include the cost and inconvenience for the patient who needs regular FIX infusions (Nathwani, et al., 2014; Pipe, 2021). These disadvantages are especially relevant in our resource-restricted setting.

2.9 Gene therapy

Gene therapy is ideal for treating haemophilia B, because only a minor increase of factor FIX concentration to 1–2% of normal is required to attain successful prophylaxis, but without the need for regular infusions (Bolton-Maggs and Pasi, 2003). Therefore, the aim of gene therapy is to alleviate all the complications encountered with other forms of haemophilia treatment and provide patients with a life-long haemostatic balance after gene transduction using a suitable vector (Samelson-Jones and Arruda, 2019). Adeno-associated virus (AAV) vectors are being used in the majority of all published and currently ongoing studies (Doshi and Arruda, 2018; Pipe 2021).

Manno et al. (2006) demonstrated the first gene transfer with compelling evidence of success in a liver-directed AAV gene therapy in a haemophilia B trial (Manno et al., 2006; Samelson-Jones and Arruda, 2019). Additionally, a study published by O'Hara and Neumann (2022) suggests that a vector-based delivery of the *F9* gene provides a long-term approach as treatment for patients diagnosed with haemophilia B (O'Hara and Neumann, 2022). Published data suggests that the first liver-directed AAV gene therapy for haemophilia B served as a breakthrough towards current successful strategies, by demonstrating achievement of a therapeutic functional FIX level in a dose-dependent manner. Pre-existing neutralising antibodies (Nab) to the AAV capsid can prevent liver transduction (Arruda et al., 2018; Dolan et al., 2018; Manno et al.,

2006). After decades of clinical research, phase 3 gene therapy trials for haemophilia B are finally underway (O'Hara and Neumann, 2022).

The clinical potential of gene therapy licencing of a gene therapy product for haemophilia B is a very realistic expectation. Etranacogene dezaparvovec (HEMGENIX®), a gene therapy under investigation, is an AAV serotype 5 (AAV5) vector, which contains a FIXa Padua R338L transgene controlled by a liver-specific promoter. The phase 3 HOPE-B clinical trial of HEMGENIX® reached its point of primary efficacy by providing haemostatic balance, surpassing the standard FIX prophylactic care, with a follow-up period of 52 weeks after the expression of stable FIX Padua (Pipe et al., 2022). In December 2022, the European Medicines Agency (EMA) recommended that conditional marketing authorisation (CMA) should be granted in the European Union (EU) for the first haemophilia B gene therapy, HEMGENIX®. In February 2023, the Global Biotechnology leader, CSL, announced that the European Commission has granted CMA for HEMGENIX® for treatment of adults diagnosed with moderately severe or severe haemophilia B (CSL, 2023). The current approach to haemophilia B gene therapy does not take causative mutations into account, but rather focuses on replacement of the whole gene segment (O'Hara and Neumann, 2022). Subsequently, we hypothesise that a more focused approach, focusing on the correction of the causative defect, may in future result in a more personalised treatment option, with fewer potential adverse reactions. These adverse reactions may manifest as immediate or delayed responses to the treatment. Some possible adverse reactions include: Hepatotoxicity, which refers to liver damage, is a frequently observed side effect associated with AAV-based gene therapies for haemophilia B. This adverse reaction occurs due to an immune response triggered by the therapy, leading to an elevation in liver enzyme levels known as transaminases. Fortunately, this condition can be effectively managed through the administration of corticosteroids. Additionally, it is essential to closely monitor patients for any infusion-related reactions. Headache and flu-like symptoms are also commonly reported as side effects of this treatment (EMA, 2022). It is important to note that adverse reactions can vary in severity and occurrence rate among individuals undergoing gene therapy for hemophilia B. Thorough monitoring, close follow-up, and ongoing research are essential to understand and mitigate these potential risks.

2.10 Development of inhibitors against FIX in patients with haemophilia B

Inhibitors (alloantibodies) against coagulation FIX concentrate is a significant complication in haemophilia B care. FIX antibodies can contribute to the ineffectiveness of classic substitution therapy, limited access to safe, effective treatment, as well as an increase in morbidity and mortality (Dolan et al., 2018; Oldenburg and Pavlova, 2006; Thorat et al., 2018).

Previous studies reported an inhibitor incidence rate of 1 – 5% of haemophilia B cases, occurring almost exclusively in patients diagnosed with severe haemophilia B. Given the small number of haemophilia B patients, large enough cohorts of previously untreated patients are not sufficient and not easy to obtain. Therefore, the FIX inhibitor incidence rate in haemophilia B is inadequately defined. To date, the majority of studies had a very small population group and included patients with different levels of severity and also excluded prospective follow-up visits for inhibitor incidence (Male et al., 2021). The presence of FIX alloantibodies is typically detected as a high titre and presents shortly after (approximately nine to eleven days after treatment exposure) the initiation of FIX replacement therapy (Giangrande, 2005). Furthermore, the development of inhibitors reflects a multifactorial process that involves genetic and non-genetic risk factors (Bolton-Maggs and Pasi, 2003; Oldenburg and Pavlova, 2006).

2.10.1 Genetic risk factors for inhibitor formation

The genetic predisposition for the development of inhibitors can be linked to the type of *F9* gene mutation (Abla et al., 2018; Dolan et al., 2018). In the majority of patients, inhibitor development is commonly associated with the complete absence of FIX antigen as a result of large deletions or other major changes, including nonsense mutations, small deletions, and splice mutations of *F9* (Table 2.4) (Male et al., 2021; Warrier and Lusher, 1998). However, small deletions and splice mutations display a lower risk for inhibitor development than large deletions and nonsense mutations (Abla et al., 2018; Goodeve, 2015).

It has been described that other risk factors, such as ethnicity and age, also influence inhibitor formation (Dolan et al., 2018; Puetz et al., 2014). In the study conducted by Puetz et al. (2014), using bivariate statistical analysis, the authors found a higher prevalence of FIX inhibitor formation in non-white ethnic populations (black, Hispanic, etc.). However, for the multivariate study results, only individuals of black ethnic background demonstrated a higher risk for inhibitor development than white individuals, with an odds ratio of 2.2 (95% confidence interval (CI) 1.2 – 4.1). Additionally, children under the age of 11 demonstrated an odds ratio of 2.5 (95% CI 1.5 – 4.0) for inhibitor development. Finally, with an odds ratio of 13.2 (95% CI 6.2 – 28.7), individuals with severe haemophilia B were found to have a higher risk of FIX inhibitor development than those diagnosed with a mild to moderate level of severity (Puetz et al., 2014). Irrespective of the product, the risk of antibody development is the highest within the first 20–100 treatments, therefore, increasingly evident in young children (Bolton-Maggs and Pasi, 2003).

Table 2.4. *F9* variants associated with inhibitor development in patients diagnosed with haemophilia B.

Series	Inhibitor cases/ total cases	Inhibitor cases/ severe cases	*F9* abnormalities associated with inhibitor development (n)	Reference(s)
1	(11/77) 14.3%	(11/38) 29%	Complete *F9* deletion (3) Partial *F9* deletion (1) Small *F9* deletion (3) Nonsense (4)	Ljung et al., 2001
2	(8/236) 3.4%	(8/152) 5.3%	Large *F9* deletion (3) Nonsense (5)	Belvini et al., 2005
3	(8/302) 2.6%	(8/211) 4%	Complete *F9* deletion (2) Large *F9* deletion (1) Nonsense (5)	Tagariello et al., 2007
4	(1/33) 3%	(1/27) 3.7%	Large *F9* deletion (1)	Kwon et al., 2008
5	(2/144) 1.4%	(2/47) 4.2%	Large *F9* deletion (2)	Miller et al., 2012
6	(5/55) 3.8%	(5/43) 5%	Large *F9* deletion (2) Nonsense (3)	Radic et al., 2013

F9: Factor IX gene; n: number of cases

2.10.2 Non-genetic risk factors

The low prevalence rate and inconsistent methodology to define the incidence of inhibitor development in current literature cause major hindrances in addressing the risks of inhibitor development. Thus, more extensive studies with larger cohorts are

required for a more comprehensive investigation into the epidemiology and immunological aspects of FIX inhibitor development (Chitlur et al., 2009; Dolan et al., 2018). FIX neutralising antibodies might be polyclonal (DiMichele, 2007). Available data suggest that allergic reactions might be associated with transient IgG1-subclass antibody production (Christophe et al., 2001; DiMichele, 2007; Sawamoto et al., 1996). Even though the current understanding with regards to the immunological aspects of inhibitor development against FIX is still rudimentary, a FIX antibody response has previously been observed and confirmed, with FIX epitopes reported to be recognised predominantly by the IgG1 and IgG4 subclass antibodies (Christophe et al., 2001). Furthermore, the FIXa/FVIIIa complex, responsible for the activation of FX, is inhibited by antibodies through at least two mechanisms, including interference with binding of FIX to the phospholipids and phospholipid-independent FIX binding to the FVIII light chain (Christophe et al., 2001). Nevertheless, additional *in vivo* and *in vitro* studies need to be conducted to confirm and define the immunological and biochemical nature of FIX inhibitory antibody response (DiMichele, 2007).

2.10.3 Complications due to FIX inhibitor development

Apart from a reduced clinical response to FIX therapy, FIX inhibitors can also be associated with an elevated risk of an allergic reaction, often accompanied by severe anaphylactic shock (Dolan et al., 2018; Sawamoto et al., 1996). The exact cause of the allergic responses is unclear due to a lack of data regarding patients with FIX inhibitor development (DiMichele, 2007; Male et al., 2021; Sawamoto et al., 1996). However, studies based on genetic linkage in multiple recombinant inbred strains of mice with haemophilia B shows that multiple gene loci could be connected to inhibitor immune response (DiMichele, 2007; Lozier et al., 2005). These studies revealed that, in mice, the loci of the major histocompatibility complex (MHC) class II (H-2) and/or K class I-a (IaK) were critical to this response. Furthermore, other genes were also shown to contribute to FIX inhibitor development, and the observed linkages include polymorphic markers from chromosomes 1 and 10, with proximity between the immunoregulatory genes, Interleukin-10 (IL-10) and interferon-γ (logarithm of the odds score of 2.3 – 3.6) (DiMichele, 2007).

2.11 Inhibitor eradication

Given the low incidence rate of haemophilia B, research with regards to diagnosis and treatment is limited (Rivas-Pollmar et al., 2022). Additionally, there is a lack of evidence-based data to support clinical decisions regarding inhibitor eradication, given the low risk of FIX inhibitor development (DiMichele, 2007; Rivas-Pollmar, et al., 2022; Santoro et al., 2018). In addition to the lack of experience with immune tolerance induction (ITI) in patients diagnosed with haemophilia B, the very limited data published demonstrates a poor success rate (Rivas-Pollmar et al., 2022). However, the current treatment guidelines suggest that ITI treatment initiation may be justified in haemophilia B patients with high-titre FIX inhibitors, with frequent bleeding events, and who are unresponsive to bypass agents, and should be closely monitored and evaluated (Rivas-Pollmar et al., 2022).

Currently, prolonged exposure to FIX by frequent administration of high dosage FIX concentrates is the only way to eradicate inhibitors. ITI, when successful, suppresses the immune response and subsequently restores FIX tolerance, thus, enabling treatment with the FIX concentrates (Giangrande et al., 2018). Based on data from the International Society on Thrombosis and Haemostasis, Scientific and Standardization Committee (ISTH-SSC) registry, there is a noteworthy indication that patients with severe haemophilia B, treated with ITI therapy, demonstrate a poor response to these ITI regimens. Furthermore, the occurrence of nephrotic syndrome (in patients with a history of previous anaphylaxis or allergies) was a unique association observed during ITI treatment with FIX-containing products (Chitlur et al., 2009; DiMichele, 2007; Santoro et al., 2018).

2.12 Current diagnostic tests

The diagnostic approach to haemophilia comprises a family bleeding history interrogation (seen in two-thirds of patients) and coagulation screening tests to determine bleeding tendency (Konkle et al., 2018; Srivastava et al., 2013; Salviato et al., 2019). Furthermore, genetic testing can assist in diagnosis, and serve as a predictor for severity and FIX inhibitor development, as well as carrier detection. The diagnosis of haemophilia is usually established shortly after birth when any

spontaneous or mild post-trauma haemorrhage events occur (Fijnvandraat et al., 2012).

2.12.1 Diagnosis based on FIX levels

Accurate diagnosis is critical to ensure appropriate management and treatment of haemophilia B and carrier patients (Srivastava et al., 2013). In the laboratory measurement to quantify the level of FIX in the plasma, FIX:C, usually expressed as a percentage, refers to the activity level of FIX. The measurement of FIX:C evaluates the levels of FIX by comparing the activity level of functional FIX in the blood plasma to a reference standard. Functional testing for FIX quantification is conducted for the clinical classification of haemophilia B (Bhatnagar and Hall, 2018; Sørensen and Young, 2010). Functional FIX quantification tests are based on the general principles of parallel line bioassays. For FIX, this test is based on the APTT. The test sample and a control sample (with known FIX levels) are mixed FIX-deficient plasma, and a range of serial dilutions are made. APTT clotting times are then determined and plotted on a graph against the plasma concentrations. Subsequently, the levels of functional FIX can be determined from the graph (Lewis et al., 2006). In our setting, this process is automatically performed by the automated coagulation instrument.

2.12.2 Molecular genetic testing

Over the past decades, genetic testing has been an extremely beneficial aid in comprehensive management models and genetic counselling (Salviato et al., 2019). Molecular genetic testing is conducted to confirm known family mutations or identify novel mutations in new (de novo) haemophilia B cases. Furthermore, genetic testing is a significant part of haemophilia care and management (Bhatnagar and Hall, 2018). The WFH recommends that genetic testing should be performed for all patients with haemophilia, as mutational analysis is vital to assist in the diagnostic confirmation of haemophilia B, potentially predict the risk of inhibitor development against FIX, to identify carriers and/or women/girls with haemophilia B, as well as to offer the opportunity for prenatal diagnosis (PND), should that be desired (Sutherland et al., 2020). Genetic testing is essential for mutation detection in both possible carriers and in prenatal diagnosis of haemophilia B to aid genetic counselling. Polymerase Chain

Reaction (PCR) deoxyribonucleic acid (DNA) amplification is generally used for mutation detection, followed by Sanger sequencing of the eight exons, as well as the 5' and 3' untranslated regions and splice boundaries of *F9* (Goodeve, 2015).

There are different *F9* pre-sequencing screening methods available for the detection of possible *F9* variants. These include single-strand conformation analysis, high resolution melting (HRM) analysis, denaturing high-performance liquid chromatography (DHPLC), denaturing gradient gel electrophoresis, and conformation sensitive gel electrophoresis (CSGE). These pre-sequencing methods enable the visualisation of a change in fragment size, therefore, identifying the region of interest, which needs to be confirmed. Furthermore, each method differs in applicability and efficacy of variant detection (Peake et al., 1993; Hinks et al., 1999; Lin et al., 2014; Salviato et al., 2019). Pre-sequencing tools are beneficial in decreasing analytic time and cost. However, they cannot be used as standalone diagnostic tests, and the genetic variants detected by one of these pre-sequencing screening methods require confirmation with sequencing analysis.

Depending on the resource availability, full *F9* gene screening should be performed with Sanger sequencing or next-generation sequencing (NGS) for the detection and confirmation of genetic variants. In clinical practice, the most frequent molecular diagnostic screening method for the confirmation of haemophilia B *F9* variants is Sanger sequencing. NGS is an alternative molecular screening method, which allows for the simultaneous screening of multiple genes at a reasonable cost while additionally overcoming the limitations of Sanger sequencing (Li et al., 2020). However, in a setting with limited resources, a more cost-effective screening approach might be the method of choice (Sutherland et al., 2020). Even though NGS demonstrates a high level of sensitivity and sample throughput, with a low limit of detection (LOD), it is not cost-effective as well as being very time-consuming to sequence small numbers of DNA samples. Sanger sequencing has proved to be less time-consuming and more cost-effective for the sequencing of low numbers of targets (roughly 1 – 20 samples) (Lin et al., 2014; Sutherland et al., 2020). Therefore, given the small haemophilia B population in the central South African region, Sanger

sequencing remains the method of choice in our setting. Finally, the NGS method should only be conducted after it is confirmed that specific structural variants can be detected using this screening technique. Furthermore, all genetic testing results should be confirmed by independent testing of any DNA sample. This may be achieved by repeating the original assay or using a different methodology, such as performing Sanger sequencing analysis to confirm the NGS result obtained (Srivastava et al., 2020). Thus, further confirming the sustained use of Sanger sequencing as a screening method.

2.13 Current mutational status of haemophilia B patients in the central South African region

According to the HGMD, as well as the Factor IX Gene Variant Database, over a thousand *F9* variants have been described globally. The extensive heterogeneity of haemophilia B has been demonstrated by the mutational analysis of different cohorts, identifying different *F9* variants among thousands of people diagnosed with haemophilia B (Bors et al., 2015; Goodeve, 2015). However, in the central South African region, there was at the time of writing no routine genetic testing performed to identify the causative mutations in patients with haemophilia B.

A previous publication described a patient who presented with FIX deficiency at our local Haemophilia Treatment Centre (HTC) at Universitas Hospital in Bloemfontein, South Africa. The patient had FIX inhibitors and experienced anaphylaxis after FIX replacement therapy. This was the first such case reported in South Africa (Stones and McGill, 2000). Unfortunately, variant detection was not available for this patient. This case illustrated the importance of genetic testing in this patient cohort, as a complex *F9* variant may have been a positive risk predictor for the adverse reaction.

Therefore, it is vital to determine whether there is a link between certain *F9* variants and inhibitor development and consequently anaphylaxis. Furthermore, molecular genetic testing is also a very important tool for prenatal diagnosis as well as carrier detection. Given the importance of haemophilia B genetic testing, the issue of limited genetic testing done in South Africa needs to be addressed. This is illustrated by the

fact that, at the time of writing, no South African laboratory or HTC has contributed mutational data towards the Factor IX Gene Variant Database (http://www.factorix.org/), EAHAD-CFVB (https://*F9*-db.eahad.org/), or the HGMD (www.hgmd.cf.ac.uk/).

CHAPTER 3: METHODOLOGY

INTRODUCTION

This chapter presents the rationale, aim and objectives for the study. The study design, study population and the ethical considerations that were adhered to, are also discussed. In addition, a complete outline of the study procedure and the various methods used to conduct this study are highlighted.

3.1 Rationale, aim and objectives

3.1.1 Rationale

There is a heterogeneous mutational spectrum for all types of haemophilia B, with no common prevalent variants identified. The mode of pathogenicity has been explored through in vivo expression for a minority of mutations. However, very limited molecular data exist in the Southern African haemophilia B population. Therefore, knowledge of the specific causative variants can assist in more accurate diagnosis, inhibitor risk prediction, improved treatment models, carrier detection, as well as prenatal diagnosis in affected families.

3.1.2 Aim

The aim of this study was to analyse and confirm the mutational status of a haemophilia B population in South Africa.

3.1.3 Objectives

1. The first objective was to optimise a Sanger Sequencing method for *F9* mutational analysis.
2. The second objective was to determine the mutational status of people with haemophilia B in central South Africa.
3. The third objective was to determine the genotype-phenotype relationships in our confirmed haemophilia B population.

3.2 Study design

A laboratory-based analytical study was conducted in the Human Molecular Biology Unit of the Department of Haematology and Cell Biology at the Faculty of Health Sciences, University of the Free State, South Africa.

3.3 Study procedure

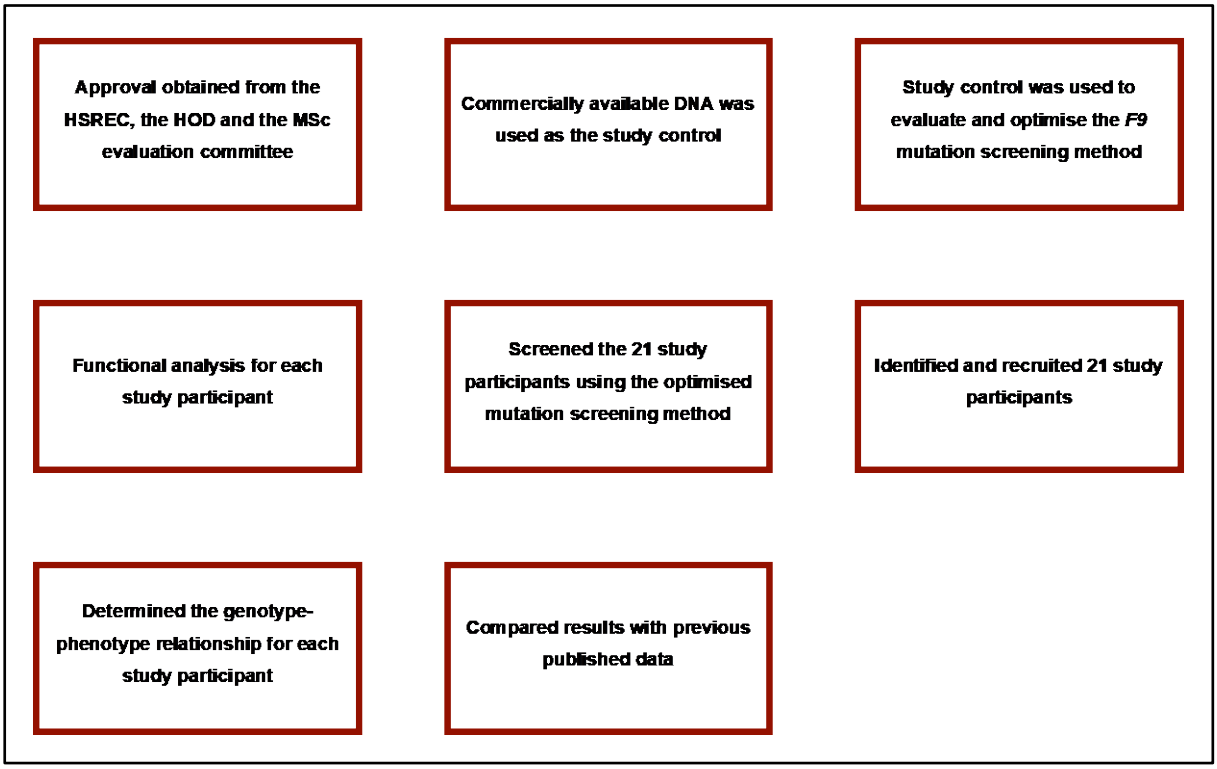

Figure 3.1. A schematic flow diagram, representing the outline of the study procedure.

**DNA: deoxyribonucleic acid; F9: Factor IX gene; HOD: Head of department; HSREC: Health Science Research Ethics Committee.*

3.4 Optimisation of the *F9* conventional PCR assay

3.4.1 Normal control sample

Commercially available DNA (University of California, Los Angeles (UCLA), USA), with a known concentration of 100 ng/µL, was used as the normal study control to optimise the variant detection method.

3.4.2 Primer selection

Previously published primers by Abla et al. (2017) (Table 3.1) were evaluated to optimise the cycling conditions of the *F9* conventional PCR assay. The primers were evaluated using the Basic Alignment Search Tool (BLAST) to ensure that each primer pair is specific to their *F9* target sequences (available at: https://www.ncbi.nlm.nib.gov/BLAST).

Table 3.1. Eight *F9* primer pairs for the mutational analysis of the *F9* gene (Abla et al., 2017).

F9 exon	Primer sequence	Primer name	Expected fragment size
Exon 1	5'-AGTCCAAAGACCCATTGAGG-3' 5'-GACTCTTCAATATTGCTGTCAAATC-3'	HEMBex1-M13	321 bp
Exon 2,3	5'-TGCCCTAAAGAGAAATTGGC-3' 5'-TGGGTTAGAGGGTTGGACTG-3'	HEMBex2-3M13	627 bp
Exon 4	5'-GAGGACCGGGCATTCTAAG-3' 5'-CCAGTTTCAACTTGTTTCAGAGG-3'	HEMBex4-M13	236 bp
Exon 5	5'-CAAAATTTCTCTCCCCAACG-3' 5'-GGTCTAATTCAAGCTACTGATATTTTC-3'	HEMBex5-M13	411 bp
Exon 6	5'-TGTAATACATGTTCCATTTGCC-3' 5'-TAGCCTCAGTCTCCCACCTG-3'	HEMBex6-M13	342 bp
Exon 7	5'-TTTCTGCCAGCACCTAGAAG-3' 5'-ACCCTTCTGCCTTTAGCCC-3'	HEMBex7-M13	353 bp
Exon 8a	5'-GCCAATTAGGTCAGTGGTCC-3' 5'-CTTCATGGAAGCCAGCAC-3'	HEMBex8a-M13	458 bp
Exon 8b	5'- TGTAAGTGGCTGGGGAAGAG-3' 5'-TGAGAGGCCCTGTTAATTTTC-3'	HEMBex8b-M13	388 bp

3' – Three prime end; 5' – Five prime end; bp: Base pairs

3.4.3 *F9* conventional PCR optimisation

3.4.3.1 Optimisation of PCR annealing temperature

To determine the optimal annealing temperature of each *F9* primer pair, a temperature gradient experiment was conducted, with the different annealing temperatures ranging from 54°C to 62°C, with an increment rate of 2°C. Using the commercial DNA, three reactions were prepared for each *F9* PCR fragment, which were subsequently conducted at five different temperatures (54°C, 56°C, 58°C, 60°C, 62°C). A seventh reaction that contained no input DNA, served as a no-template control (NTC) for quality control, and was conducted at the lowest temperature. The PCR reactions were prepared using the Q5 High-Fidelity 2X Master Mix (New England Biolabs (NEB), USA) (Table 3.2). The temperature gradient of each *F9* PCR assay (Table 3.3) was performed on the Veriti thermocycler (Applied Biosystems, USA) in the Human Molecular Biology Unit.

Table 3.2. Standard PCR reaction protocol (According to the NEB Q5 High-Fidelity 2X Master mix package insert)

Components	25 μL RXN	50 μL RXN	Final concentration
10 μM Forward Primer	1.25 μL	2.5 μL	0.5 μM
10 μM Reverse Primer	1.25 μL	2.5 μL	0.5 μM
Template DNA	variable	variable	<1,000 ng
Q5 High-Fidelity 2X Master Mix	12.5 μL	25 μL	1X
Nuclease-Free Water	to 25 μL	to 50 μL	

DNA: Deoxyribonucleic acid; RXN: Reaction

Table 3.3. PCR cycling conditions.

PCR cycle steps		Temperature	Time	Number of cycles
Phase 1	Initial denaturation	94°C	5 min	1
	Denaturation	94°C	30 sec	
Phase 2	Annealing	54-58°C	1 min	35
	Propagation/Extension	72°C	1 min	
Phase 3	Final	4°C	∞	1

The PCR products were analysed using agarose gel electrophoresis. The PCR products of each reaction were resolved on a 2% agarose gel in 1X TAE buffer (2 M Tris-acetate and 0.5 M Ethylenediaminetetraacetic acid (EDTA) pH 8.0), stained with 5 µL of EZ-Vision® Bluelight DNA dye. The gel was electrophorised at 150 V for 40 minutes (min). A molecular weight marker (100 bp DNA ladder) (NEB, USA) was also resolved on the gel to analyse and confirm the specific PCR fragment sizes obtained. Finally, the gel images were visualised under ultraviolet (UV) light on the Kodak Gel Logic documental system (Carestream Molecular Imaging, Rochester, New York (NY), USA), in order to determine if the PCR assay was successful and consequently analyse the DNA fragment sizes obtained. However, in some cases even optimised PCR conditions yielded more than one fragment on visualisation. In these cases, the fragment corresponding to the expected fragment size was excised from the gel and incubated in double-distilled water (ddH_2O) overnight. This process allows the elution of the PCR product from the gel. Subsequently, we used the elution as the input DNA in a follow-up PCR reaction. The corresponding PCR reaction was repeated, and agarose gel electrophoresis was performed as described previously to determine if the non-specific fragments were successfully removed.

3.4.3.2 Determination of the limit of detection (LOD) for each F9 PCR assay

The lower limit of detection (LOD) for input DNA for each of the *F9* conventional PCR assays was determined. The control sample with a known starting DNA concentration of 100 ng/µL was diluted with nuclease-free H_2O to 50 ng/µL, 25 ng/µL, 12.5 ng/µL, 6.25 ng/µL, and 3.125 ng/µL, respectively. Subsequently, for each of the eight *F9* conventional PCR assays, six PCR reactions, using the control sample (100 ng/µL) and the five different diluted samples, were performed at the respective optimal annealing temperatures, as determined in section 3.4.3.1. For each PCR assay a NTC reaction was included to serve as quality control. Finally, the PCR results were analysed using gel electrophoresis, as described in section 3.4.3.1.

3.4.4 PCR product purification

The PCR products that consequently yielded the correct size single DNA fragments, were purified using the ExoSAP-IT® Express PCR product clean-up kit (Applied

Biosystems, USA), as highlighted in Table 3.4. PCR product purification was performed in order to eliminate contaminants such as enzymes, salts, primers, buffer components or any other additional impurities that could compromise the downstream analysis.

Table 3.4. PCR product purification protocol (According to the Applied Biosystems ExoSAP-IT® Express PCR product clean-up kit package insert).

Cycle components	Cycle 1	Cycle 2
5 µL PCR product 2 µL ExoZAP-IT® Express	Incubation 37°C 4 min	Inactivation of ExoZAP-IT® enzyme 80°C 1 min

3.4.5 Sanger sequencing to confirm the PCR products obtained

The purified product produced in 3.4.4 above was used to perform a Sanger sequencing reaction. Sanger sequencing analysis was used to confirm the PCR results obtained with the commercial DNA. All the *F9* PCR assays were conducted using optimal cycling conditions, determined in sections 3.4.3.1. and 3.4.3.2. The analysis was based on whether each of the optimised *F9* assays amplified the desired *F9* exon sequences.

The purified PCR product of each *F9* fragments produced with the conventional PCR assays, which yielded fragments detectable by gel electrophoretic analysis, were used to perform a Sanger sequencing reaction using the BigDye™ Terminator V3.1 Kit (Applied Biosystems, USA). The Sanger sequencing reactions were prepared as tabulated in Table 3.5. The Sanger sequencing cycling conditions are summarised in Table 3.6.

Table 3.5. Sanger sequencing reaction.

Components	Reaction volume			
	Standard reaction quantity [20 µL]	Final reaction quantity [10 µL]	Forward reaction	Reverse reaction
BigDye™ Terminator V3.1 Ready Reaction Mix	8 µL	4 µL	4 µL	4 µL
Forward primer [3.2 µM] Reverse primer [3.2 µM]	3.2 pmol	3.2 µM	1 µL -	- 1 µL
Deionized H_2O (RNase/DNase-free]	Varies based on primer and template volume	4 µL	4 µL	4 µL
PCR template	Based on template quantity	1 µL	1 µL	1 µL
Total volume	**20 µL**	**10 µL**	**10 µL**	**10 µL**

**** PCR: Polymerase chain reaction***

Table 3.6. Sanger sequence cycling conditions.

Steps	Temperature	Time	Remarks
Denaturation	96°C	2 min	1 cycle
Denaturation **Annealing** **Extension**	96°C 50°C 60°C	10 sec 5 sec 4 min	25 cycles
Final	4°C	∞	Hold

3.4.5.1 Sequencing product purification

The sequencing products were purified using the ZR DNA Sequencing Clean-Up Kit™ (Zymo Research, USA) according to the manufacturer's instructions. Firstly, 240 µL Sequencing Binding Buffer was added to each 10 µL sequencing reaction and the mixture was transferred to the Zymo-Spin™ IB Column. The column was placed in a collection tube provided and centrifuged for 30 seconds (sec) at 13,000 x centrifugal force (*g*). For the wash step, 300 µL Sequencing Wash Buffer was added to each column and again centrifuged for 30 sec at 13,000 x *g*. Finally, to elute the DNA, 10 µL ddH_2O was added directly to the matrix of the column. The column was transferred to 1.5 mL microcentrifuge tube and centrifuged at 13,000 x *g* for 15 sec. The eluted DNA for each sequencing reaction was used for the Sanger sequencing analysis.

3.4.5.2 Sequence analysis

The raw sequence reads were loaded onto the ABI Prism 3500 Genetic Analyser (Applied Biosystems, USA) and through the process of sequence reads, each sequence was constructed into an electropherogram using the Sequencing Analysis Program V5.3.1. All the sequencing data was evaluated using the Chromas-lite version 2.6 software (available at: https://chromas-lite.software.informer.com/2.6/) and converted to simplified nucleotide text using the online Sequence Massager version 1.0 software (available at: https://biomodel.uah.es/en/lab/cybertory/analysis/massager.htm). Online Pairwise Sequence alignment version 7.13.8 software (available at: https://www.ebi.ac.uk/Tools/psa/emboss_needle/#) was used to align each nucleotide sequence to its known *F9* reference sequence (NM_000133.4; Appendix G), retrieved from the NCBI online database (available at: https://www.ncbi.nlm.nih.gov/gene/2158). Subsequently, each nucleotide sequence was translated to an amino acid sequence using the online ExPasy translation software tool (available at: http://web.expasy.org/translate). The amino acid sequences were compared to the reference *F9* sequence (NP_000124.1), obtained from NCBI, using the Online Pairwise Sequence alignment version 7.13.8 software (available at: https://www.ebi.ac.uk/Tools/psa/emboss_needle/#).

3.5 Variant detection in people with haemophilia B in Central South Africa

3.5.1 Study participants

Individuals were enrolled only after written informed consent was obtained to participate in the study. According to the latest available clinic data in our setting, there were nine adults, and sixteen (16) minors diagnosed with haemophilia B, who regularly attended the haematology clinics at the Universitas Academic Hospital in Bloemfontein, as well as at the Robert Mangaliso Sobukwe Hospital in Kimberley (previously known as Kimberley Hospital Complex). The recruitment was done by the primary investigator when patients came for their routine follow-up at the respective clinics. Unfortunately, not all people with haemophilia B in our region regularly attended the clinic during the time we performed volunteer recruitment, possibly resulting in a discrepancy in the estimated and real recruitment numbers.

Considering that haemophilia B is an orphan disease, a small population for this study was predicted. As stated earlier, an orphan disease is defined as a rare disease by the fact that it affects only a small number of people in a specific region. The definition of an orphan disease varies in different countries, depending on the country's prevalence criteria and the size of their population (Thorat et al., 2018). We aimed to recruit at least twenty (20) participants. We believe that a population size of twenty (20) participants is a mid-range sample size when compared with other haemophilia B published studies which ranged from seven to sixty-one (61) participants (Mukherjee et al., 2004; Nigam et al., 2014; Abla et al., 2017; Pasi et al., 2017; Zahari et al., 2018; Salviato et al., 2019).

3.5.1.1 Inclusion criteria

The study included minor (<18 years) and adult (≥18 years) participants if they met the following criteria:

(1) Participants were included if they were diagnosed with haemophilia B or identified as a potential carrier according to the guidelines of the International Society on Thrombosis and Haemostasis (ISTH) (Borhany et al., 2018);

(2) All participants had to voluntarily give written informed consent or assent if old enough (minors over the age of five years);

(3) The parents or legal guardians of minors also had to give written consent.

3.5.1.2 Exclusion criteria

Participants were excluded from the study for the following reasons:

(1) The participants were diagnosed with acquired haemophilia B;

(2) The participant did not/was not able to give informed consent;

(3) The participant was pregnant;

(4) Parents/legal guardians of minors refused to give written consent for minors;

(5) Minors were unwilling to complete an assent form.

3.6 Ethics considerations

For this study, ethics approval was obtained from the Health Sciences Research Ethics Committee (HSREC) (UFS-HSD2019/1569/2611) (Appendix A) in the Faculty of Health Sciences at the University of the Free State, Bloemfontein, South Africa. This study was also approved by the Free State Department of Health (FSDOH) (FS_201910_009) and the Northern Cape Department of Health (NCDOH) (NC_201910_001), South Africa (Appendix B). Each participant had to give written informed consent (Appendix C) and was given an information document for genetic research (Appendix D). The parents or legal guardians had to sign an informed consent form in case of the participant being a minor younger than 18 years old (Appendix E). All minors over the age of five years, had to complete an assent form to participate in the study (Appendix F). All consent forms were available in English, Afrikaans and Southern Sotho. Furthermore, approval for the study was obtained from the relevant University of the Free State authorities, as well as from the business managers of the different National Health Laboratory Service (NHLS) laboratories at the respective hospitals.

3.7 Sample collection

A total of approximately fifteen millilitres (15 mL) of venous blood was collected from each study participant in one EDTA containing tube and two sodium citrate containing tubes (BD Vacutainer, Becton Dickinson, South Africa). DNA extraction was performed from the blood-containing EDTA tube and subsequently used to screen each participant for *F9* mutations. The sodium citrate tubes were used for functional analysis to measure the FIX plasma levels and screen for the presence of FIX inhibitors. Blood collections took place during the routine follow-up clinic visits. Therefore, research-specific samples could be obtained by the phlebotomist or treating clinician while collecting the routine samples. This limited the discomfort of the participant by reducing the number of venepunctures.

3.7.1 DNA extraction

The MACHEREY-NAGEL™Nucleospin® Blood kit (Separations, South Africa) was used to isolate the DNA from the twenty-one (21) study volunteers. During the step of cell lysis, 25 μL Proteinase K, which is used to digested proteins and additional contaminants, and 200 μL blood were pipetted into a 1.5 mL microcentrifuge collection tube. Thereafter, 200 μL of Buffer B3 was added, and the samples were vortexed vigorously for 10 – 20 sec. The samples were incubated at 70°C for 10 – 15 min. Next, 210 μL of a 98% ethanol solution was added to the samples, which were vortexed to adjust the DNA binding conditions. The purpose for washing the DNA with ethanol, is to increase the level of purity, while keeping the DNA bounded to a silica membrane, by removing excess contaminating proteins, ribonucleic acid (RNA), and lipopolysaccharides. Subsequently, the sample lysate was transferred into a NucleoSpin® Blood Column, placed in a collection tube. The samples were centrifuged for 1 min at 11,000 *x g* and the collection tube containing the flow-through was discarded. The silica membrane for each sample was washed in two separate steps. During the first wash step, the NucleoSpin® Blood Column was placed into a new 2 μL collection tube and 500 μL Buffer BW was added. The sample was centrifuged for 1 min at 11,000 x *g* and again, the collection tube with the flow-through was discarded. For the second wash step, the NucleoSpin® Blood Column was placed into a new 2 μL collection tube, followed by the addition of 600 μL Buffer B5 to the NucleoSpin® Blood Column. The samples were centrifuged for 1 min at 11,000 x *g*. Subsequently, only the flow-through was discarded and the collection tube was reused. The NucleoSpin® Blood Column was placed back into the collection tube and centrifuged for 1 min at 11,000 *x g*, in order to dry the silica membrane. The last step aimed to elute highly pure DNA by placing the NucleoSpin® Blood Column in a 1.5 mL microcentrifuge tube and adding 100 μL preheated Buffer BE (70°C), followed by incubation at room temperature for 1 min. Lastly, the sample was centrifuged for 1 min at 11,000 x *g* and the elution containing the highly pure DNA was collected in the 1.5 mL microcentrifuge collection tube.

3.7.2 Determination of DNA concentration

The DNA concentrations (ng/μL) of the study samples were measured using the BioDrop μLITE UV/VIS Spectrophotometer (BioDrop, United Kingdom). Firstly, 2 μL of nuclease-free water was pipetted onto the measurement pedestal for cleaning purposes. The instrument was then blanked by pipetting 2 mL of 0.1X TE (Lonza Rockland, ME, USA) buffer onto the measurement pedestal. The DNA concentration was measured by pipetting 2 μL of the eluted DNA from each sample onto the pedestal. The DNA concentration measurement step was performed in duplicate, followed by the determination of the mean DNA concentration value, which was documented. Finally, the extracted DNA was stored at minus 20°C (-20°C) in a temperature-controlled freezer in the Human Molecular Biology Unit, until needed for further analysis.

3.7.3 *F9* conventional PCR assay to screen study participants

The mutational screening for the study participants was conducted using the optimised conventional *F9* PCR assays (optimised conditions are discussed in the results section to follow). For each study sample, eight PCR reactions were conducted, which included seven *F9* PCR assays, as well as an NTC reaction for quality control purposes.

3.7.4 Sanger sequencing analysis

For each study sample, the PCR products of all the *F9* PCR assays, which yielded DNA fragments detected by gel electrophoretic analysis, were purified, as explained in section 3.4.5. The purified DNA samples for each study participant were subjected to Sanger sequencing for mutational analysis. The sequencing reaction conditions, the purification of sequencing products, as well as sequence analysis were conducted as described in section 3.7.4. The alignment results obtained for each study participant, using Online Pairwise Sequence alignment software (Appendix M) were analysed to confirm *F9* variants. The nucleotide sequence was then translated to the amino acid sequence using the online translation software, Expasy (available at: https://web.expasy.org/translate/). Subsequently, the altered amino acid sequence of

each genetic variant for each study sample was compared to the reference amino acid sequence using the online sequence alignment software (available at: https://www.ebi.ac.uk/Tools/psa/emboss_needle/#). The pathogenicity of the *F9* variants was predicted using the VarSome online software (available at: https://varsome.com/), which is based on the American College of Medical Genetics and Genomics (ACMG) criteria. Finally, our results were evaluated against previously published data and databases in order to identify whether the variant has been described previously or whether it is a novel variant.

3.7.5 Mutational analysis of each *F9* study participant

The mutational analysis for each sample was done by comparing the DNA sequences obtained for each study participant to the *F9* reference sequence (NM_000133.4), obtained from NCBI. The different *F9* variants observed for each sample were documented, interpreted, and described according to the ACMG and the Association for Molecular Pathology guidelines (Richards et al., 2015). The ACMG criteria for the classification of benign and/or pathogenic variants was used to categorise the *F9* variants identified in this study. The criteria for the classification of benign *F9* variants (Table 3.7), was scrutinised as stand-alone (BA1), strong (BS1 – BS4), or supporting (BP1 – BP7). Furthermore, the criteria for the classification of pathogenicity (Table 3.8) of a *F9* variant was weighed as very strong (PVS1), strong (PS1 - PS4), moderate (PM1 – PM6), or supporting (PP1 – PP5). Ultimately, for each *F9* variant detected, the selected criteria were combined based on the ACMG scoring guidelines (Table 3.9) to classify the *F9* variant pathogenicity from the five-tier system (Richards et al., 2015).

Table 3.7. The ACMG criteria for classifying benign variants (Copied and modified from Richards et al., (2015)).

Evidence of benign impact	Category	
Stand-alone	**BA1**	Allele frequency is above 5% in Exome Sequencing Project, 1000 Genomes Project, or Exome Aggregation Consortium).
Strong	**BS1**	Allele frequency is greater than expected for disorder.
	BS2	Observed in a healthy adult individual for a recessive (homozygous), dominant (heterozygous), or X-linked (hemizygous) disorder with full penetrance expected at an early age.
	BS3	Well-established *in vitro* or *in vivo* functional studies shows no damaging effect on protein function or splicing.
	BS4	Lack of segregation in affected members of a family.
Supporting	**BP1**	Missense variant in a gene for which primarily truncating variants are known to cause disease.
	BP2	Observed in trans with a pathogenic variant for a fully penetrant dominant gene/disorder; or observed in cis with a pathogenic variant in any inheritance pattern.
	BP3	In-frame deletions/insertions in a repetitive region without a known function.
	BP4	Multiple lines of computational evidence suggest no impact on gene or gene product (conservation, evolutionary, splicing impact, etc).
	BP5	Variant found in a case with an alternate molecular basis for disease.
	BP6	Reputable source recently reports variant as benign but the evidence is not available to the laboratory to perform an independent evaluation.
	BP7	A synonymous (silent) variant for which splicing prediction algorithms predict no impact to the splice consensus sequence nor the creation of a new splice site AND the nucleotide is not highly conserved.

ACMG: American College of Medical Genetics and Genomics.

Table 3.8. The ACMG criteria for classifying pathogenic variants (Copied and modified from Richards et al., (2015)).

Evidence of pathogenicity	Category	
Very strong	**PVS1**	Null variant (nonsense, frameshift, canonical ±1 or 2 splice sites, initiation codon, single or multiexon deletion) in a gene where LOF is a known mechanism of disease.
Strong	**PS1**	Same amino acid change as a previously established pathogenic variant regardless of nucleotide change.
	PS2	Novel (both maternity and paternity confirmed) in a patient with the disease and no family history.
	PS3	Well-established *in vitro* or *in vivo* functional studies supportive of a damaging effect on the gene or gene product.
	PS4	The prevalence of the variant in affected individuals is significantly increased compared with the prevalence in control.
Moderate	**PM1**	Located in a mutational hot spot and/or critical and well-established functional domain (e.g., active site of an enzyme) without benign variation.
	PM2	Absent from controls (or at extremely low frequency if recessive) in Exome Sequencing Project.
	PM3	For recessive disorders, detected in trans with a pathogenic variant.
	PM4	Protein length changes as a result of in-frame deletions/insertions in a nonrepeat region or stop-loss variants.
	PM5	Novel missense change at an amino acid residue where a different missense change determined to be pathogenic has been seen before.
	PM6	Assumed de novo, but without confirmation of paternity and maternity.
Supporting	**PP1**	Cosegregation with disease in multiple affected family members in a gene definitively known to cause the disease.
	PP2	Missense variant in a gene that has a low rate of benign missense variation and in which missense variants are a common mechanism of disease.
	PP3	Multiple lines of computational evidence support a deleterious effect on the gene or gene product (conservation, evolutionary, splicing impact, etc.).
	PP4	Patient's phenotype or family history is highly specific for a disease with a single genetic aetiology.
	PP5	Reputable source recently reports variant as pathogenic but the evidence is not available to the laboratory to perform an independent evaluation.

ACMG: American College of Medical Genetics and Genomics; LOF: Loss of function.

Table 3.9 The ACMG rules for combining criteria to sequence variants (Copied and modified from Richards et al., (2015)).

Pathogenic	**I.** 1 Very strong (PVS1) **and** a) ≥1 Strong (PS1–PS4) or b) ≥2 Moderate (PM1–PM6) or **II.** 1 Moderate (PM1–PM6) and 1 supporting (PP1–PP5) or a) ≥2 Supporting (PP1–PP5) b) ≥2 Strong (PS1–PS4) or **III.** 1 Strong (PS1–PS4) **and** a) ≥3 Moderate (PM1–PM6) or b) 2 Moderate (PM1–PM6) **and** ≥2 Supporting (PP1–PP5) or c) 1 Moderate (PM1–PM6) **and** ≥4 supporting (PP1–PP5)
Likely Pathogenic	**I.** 1 Very strong (PVS1) **and** 1 moderate (PM1– PM6) or **II.** 1 Strong (PS1–PS4) **and** 1–2 moderate (PM1–PM6) or **III.** 1 Strong (PS1–PS4) **and** ≥2 supporting (PP1–PP5) or **IV.** ≥3 Moderate (PM1–PM6) or **V.** 2 Moderate (PM1–PM6) **and** ≥2 supporting (PP1–PP5) or **VI.** 1 Moderate (PM1–PM6) **and** ≥4 supporting
Benign	**I.** 1 Stand-alone (BA1) or **II.** ≥2 Strong (BS1–BS4)
Likely Benign	**I.** 1 Strong (BS1–BS4) **and** 1 supporting (BP1– BP7) or **II.** ≥2 Supporting (BP1–BP7)
Uncertain Significance	**I.** Other criteria shown above are not met or **II.** the criteria for benign and pathogenic are contradictory

ACMG: American College of Medical Genetics and Genomics.

3.7.6 Phenotypic/Functional analysis of each study participant

3.7.6.1 Medical history

At the time of the recruitment of study participants we recorded the lowest FIX level recorded on their medical records, stated haemophilia severity, previous bleeding episodes, the presence and levels of inhibitors, as well as current FIX treatment documented.

3.7.6.2 FIX plasma concentration and level of severity

Whole blood collected in sodium citrate was centrifuged (Beckman Coulter Allegra X22 centrifuge) for 10 min at 2,200 x *g* in order to separate the plasma from the cellular components. The plasma was then pipetted into 2 x 500 µl aliquots into 1.5 mL tubes

and frozen at -80°C until needed for analysis. For the FIX level detection, a one-stage FIX clotting assay, as well as an enzyme-linked immunosorbent (ELISA) assay were conducted to measure the FIX plasma levels of the study participants (S1 – S21).

The one-stage FIX coagulation assay for S1 – S21 was conducted by the Free State NHLS, using the Siemens Coagulation Factor IX Deficient Plasma OTXX17 kit. The level of plasma FIX for each sample was measured using the Sysmex CS2100-I fully automated analyser (Siemens Healthcare GmbH, Germany).

Furthermore, the Human Factor IX ELISA Kit (ab188393) (Abcam, USA) was used to conduct a FIX coagulation factor ELISA assay, which was prepared according to the manufacture's protocol. The plasma samples were diluted 100-fold using the 10X Diluent M reagent and all sample assays were done in duplicate. The ELISA assay was performed at room temperature (18-25°C). Firstly, 50 µL of each FIX standard and sample was added to thoroughly coat the respective wells. A sealing tape was used to cover all the wells, which were incubated for two hours. Then, 200 µL of 1X Wash Buffer was used to manually wash each well. This washing step was repeated five times. Thereafter, 50 µL of Biotinylated Factor IX Detector Antibody was pipetted into each well, followed by incubation for one hour. A second wash step was performed in the same manner as described above. 50 µL of 1X SP Conjugate was added to each well and incubate for 30 min. Again, the microplate was then washed as described above. Next, 50 µL of Chromogen Substrate was added per well and the microplate was incubated for approximately 12 min until the optimal blue colour density developed in each well. Finally, 50 µL of Stop solution was added to each well and the blue colour in each well changed to yellow. Immediately after the colour in all the wells changed to yellow, the absorbance was read at a wavelength of 450 nm. The BIOBASE EL-10A enzyme-linked immune ELISA reader (Biobase, China) was used to quantify the FIX antigen levels for each sample, including standards, a positive control, a negative control, and the study participants. This assay was calibrated using the FIXa international standard. The controls and participant samples were all analysed at the same time, however, the sample values were only accepted if the controls passed

according to the manufacturer specifications. The LOD for this assay, as indicated by the manufacturer, is 0.24 ng/mL (Abcam, USA).

An ELISA standard curve was generated by using the concentrations (ng/mL) of the calibration standards (x-axis), which were plotted against the corresponding mean optical density (OD) values measured at 450 nm (y-axis). The concentrations of the seven standards used for the ELISA assay were 0.78 ng/mL, 1.56 ng/mL, 3.13 ng/mL, 6.25 ng/mL, 12.5 ng/mL, 25 ng/mL, and 50 ng/mL. The best-fit line for the standard curve was determined by regression analysis four-parameter logistic curve-fit. Subsequently, the unknown sample FIX concentrations (ng/mL) were determined from the ELISA standard curve, using the mean OD measurement for each sample and multiplying the corresponding FIX:C value by the dilution factor (1:100). For the ELISA results, the OD absorbance results were converted to the concentrations of test samples using the GraphPad Prism 9.4.1 software (GraphPad Software, San Diego, CA) (available at: https://www.graphpad.com/). The absorbance of each sample was converted to ng/mL, then multiplied by the dilution factor of 100. The normal 100% concentration for FIX is reported to be 5,000 ng/mL, which was used to calculate the final concentration of test samples (Ponder, 2011). Therefore, our final concentration in ng/ml was divided by 5,000 and multiplied by 100 to determine the percentage concentration. The percentage concentration (FIX:C %) is taken as equal to the IU/dL.

3.7.6.3 Presence of inhibitors against FIX

The data regarding the presence and level of FIX Inhibitors was obtained from the NHLS TrakCare Lab Webviewer system database for each participant. If FIX levels and inhibitor screening results were not available on the NHLS TrakCare system, samples were registered under the specific study location and sent for analysis to the NHLS laboratory. The NHLS reports the presence and level of FIX inhibitors using the Nijmegen-Bethesda Assay (NBA), which gives a numeric value, quantified in Nijmegen-Bethesda Units (NBU). The NBU refers to the inhibitor titre. A test result of $\leq$ 5.0 NBU demonstrates a low inhibitor titre, whereas a result of > 5.0 NBU indicates a high inhibitor titre (Collins et al., 2013; Miller et al., 2012).

3.8 Data analysis

Descriptive data analysis was conducted based on the mutational and phenotypic results obtained for each study participant. The standardised recommendations, provided by the Human Genome Variation Society (HGVS), were used for describing the *F9* sequence variants detected in our study population (Callenberg et al., 2018). Depending on the significance of the physiochemical difference between the two amino acids, the *F9* variants were classified as either radical or conservative (Smith, 2003). Distinguishing between radical and conservative *F9* variants is important for understanding the potential clinical severity of haemophilia B. Additionally, it helps in guiding treatment decisions, such as the frequency and intensity of FIX replacement therapy, as well as identifying individuals who may be at higher risk of spontaneous bleeding episodes or complications. Furthermore, knowledge of the specific mutation can aid in genetic counselling for affected individuals and their families.

The SWISS-MODEL online building software was used to perform theoretical 3D-structural analysis of the FIX variants and was assisted by the SWISS protein data bank (PDB) Viewer version 11 software (available at: https://swissmodel.expasy.org/) (Guex et al., 2009; Waterhouse et al., 2018). The 3D coordinates of three partial human FIXa crystal structures, deposited on PDB files 1NL0.pdb (GLA domain), 1EDM.pdb (EGF1 domain), and 2WPH.pdb (combination of the EGF2 and serine protease domain), were superimposed onto the porcine FIXa crystal structure (1PFX.pdb) (available at: https://www.rcsb.org/3d-view/1PFX/1).

The PolyPhen-2 online software was used to predict the potential impact of an amino acid change, due to *F9* missense variants, on the functional FIX protein (available at: http://genetics.bwh.harvard.edu/pph2/). This software additionally determines the surface accessibility of an amino acid residue. The Accessible Surface Area (ASA) of a protein may be used as a main predictor in protein folding and stability. ASA indicates if an amino acid residue within a protein is either buried or open. For the surface accessibilities of the FIXa residues, a value of zero indicates a solvent accessibility of 0 - 9%; a value of one correlates to a surface accessibility of 10-19%; a value of two corresponds to 20-29% surface accessibility, and so forth. A surface accessibility of

zero or one is indicative of a buried residue, whereas the accessibility value of exposed residues ranges from two to eight (Rallapalli et al., 2013). It has been reported that amino acid residues in buried conformation are more likely to be associated with a disease-related amino acid variant, than in an open confirmation. Furthermore, variations in the amino acids glycine, tryptophan (Trp), Tyr and cysteine (Cys) are more likely to be associated with diseases than other amino acids (Savojardo et al., 2021). Finally, based on the ACMG criteria for classifying pathogenic variants, in combination with the VarSome (https://varsome.com/) online software, we predicted the pathogenicity of confirmed *F9* variants.

For the functional analysis, all data were tabulated using Microsoft Excel, which was used to conduct the statistical analysis. A student t-test was used to determine if there is a statistically significant difference between the two assays. For the one-stage assay, using the Siemens Automated Coagulation Analyser, the lower limit of detection for the measurement of functional FIX is 0.4%. Therefore, all the FIX levels lower than the LOD were reported as <0.4%. (<0.4 IU/dL).

The ELISA assay standard curve was used to determine the diluted FIX:C (ng/mL), which was multiplied by the dilution factor (100) to obtain the undiluted FIX:C. The final FIX concentration in ng/mL was divided by 5,000 (FIX:C 100%) and multiplied by 100 to obtain the % (equivalent to IU/dL). For the statistical analysis of the FIX one-stage and ELISA assay, all FIX:C determined are presented as % (IU/dL). To determine the discrepancy between the two assays, a p-value was calculated with the student t-test, using Microsoft Excel. A p-value of <0.05 was considered to be statistically significant.

The mutational and phenotypic data obtained for each study participant were tabulated to determine the genotype-phenotype relationship. This includes the relationship between certain *F9* variants and the severity level of FIX deficiency, severity of bleeding episodes, reaction to replacement therapy, and inhibitor development. All the results recorded were compared to existing published data, to strengthen our study results as well as to identify *F9* novel variants.

CHAPTER 4: RESULTS AND DISCUSSION

INTRODUCTION

In this chapter the results of the study are presented and the data obtained is analysed, followed by an extensive discussion of the results. Furthermore, the limitations and impacts of this study are highlighted, and future recommendations are also addressed.

4.1 Study population

A total of 21 participants were enrolled in the study (Figure 4.1). This number included 18 people with haemophilia and three putative carriers. When compared to the 25 people with haemophilia B recorded in our HTC, we had a recruitment success rate of 72%. This is especially noteworthy considering that most of the recruitment coincided with the restrictions brought by the COVID-19 pandemic.

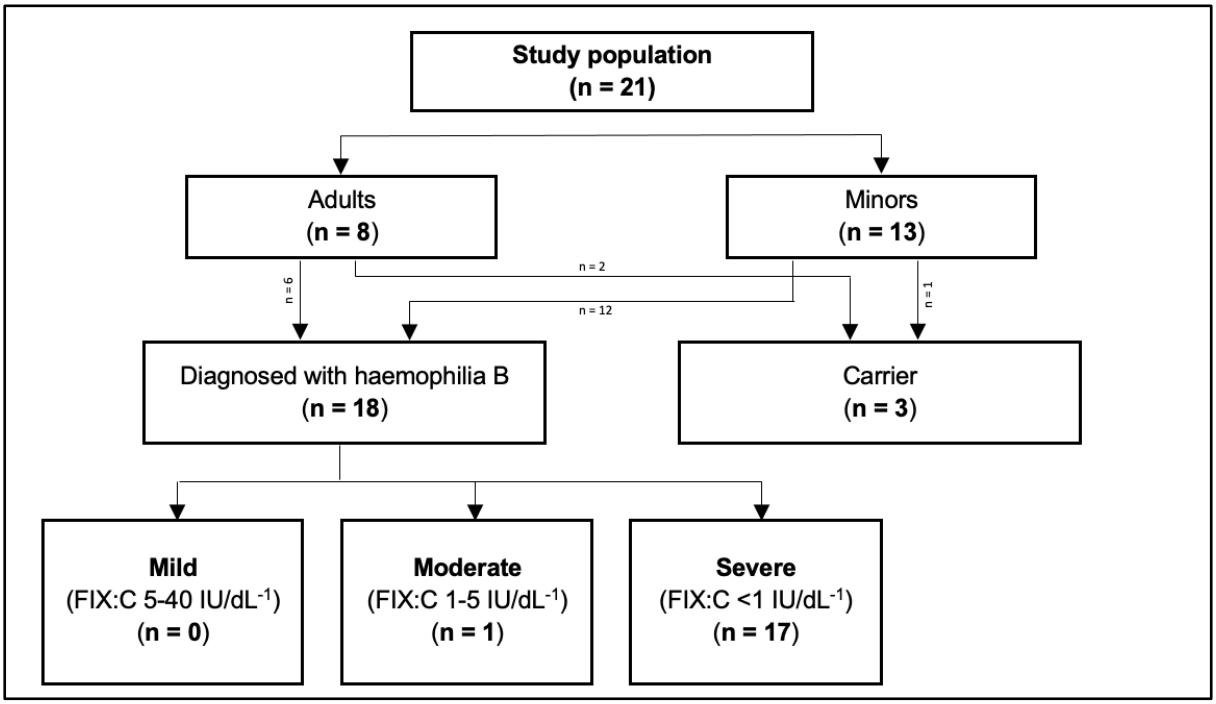

Figure 4.1. Summary of the data collected for the study population at the time of recruitment.

**dL: Decilitre; FIX:C: FIX plasma concentration; IU: International units; n: Number of study participants.*

4.2 Evaluation and optimisation of the *F9* conventional PCR detection method

4.2.1 Primer selection

For the *F9* screening method, the *F9* conventional PCR assays were optimised to successfully amplify the whole region of interest of the translated region of the *F9* gene. Previously published primers (Table 4.1) were used to optimise the PCR conditions. Ultimately, we determined the optimal PCR conditions for seven fragments spanning all eight exons of the *F9* gene.

Table 4.1. Seven *F9* primer pairs for the respective *F9* conventional PCR assays.

***F9* PCR assay** (*F9* Exon)	**Primer name**	**Primer sequence**	**Expected fragment size**
Fragment 1 (exon 1)	**F1_F** **F1_R**	5'-AGTCCAAAGACCCATTGAGG-3' 5'-GACTCTTCAATATTGCTGTCAAATC-3'	321 bp
Fragment 2 (exon 2,3)	**F2_F** **F2_R**	5'-TGCCCTAAAGAGAAATTGGC-3' 5'-TGGGTTAGAGGGTTGGACTG-3'	627 bp
Fragment 3 (exon 4)	**F3_F** **F3_R**	5'-GAGGACCGGGCATTCTAAG-3' 5'-CCAGTTTCAACTTGTTTCAGAGG-3'	236 bp
Fragment 4 (exon 5)	**F4_F** **F4_R**	5'-CCCCAATGTATATTTGACCC-3' 5'-ACTTGAATCTGCTTCCTTTTG-3'	334 bp
Fragment 5 (exon 6)	**F5_F** **F5_R**	5'-TGTAATACATGTTCCATTTGCC-3' 5'-TAGCCTCAGTCTCCCACCTG-3'	342 bp
Fragment 6 (exon 7)	**F6_F** **F6_R**	5'-TTTCTGCCAGCACCTAGAAG-3' 5'-ACCCTTCTGCCTTTAGCCC-3'	353 bp
Fragment 7 (exon 8)**	**F7_F** **F7_R**	5'-GCCAATTAGGTCAGTGGTCC-3' 5'-TGAGAGGCCCTGTTAATTTTC-3'	697 bp

***3'**: Three prime end; **5'**: Five prime end; **bp**: Base pairs; **F1_F**: Fragment 1 forward primer; **F1_R**: Fragment 1 reverse primer; **F2_F**: Fragment 2 forward primer; **F2_R**: Fragment 2 reverse primer; **F3_F**: Fragment 3 forward primer; **F3_R**: Fragment 3 reverse primer; **F4_F**: Fragment 4 forward primer; **F4_R**: Fragment 4 reverse primer; **F5_F**: Fragment 5 forward primer; **F5_R**: Fragment 5 reverse primer; **F6_F**: Fragment 6 forward primer; **F6_R**: Fragment 6 reverse primer; **F7_F**: Fragment 7 forward primer; **F7_R**: Fragment 7 reverse primer; **F9**: Factor IX gene; **PCR**: Polymerase chain reaction. Primers for exon 1-4 and exon 6-8 were published by Abla et al., (2017) and the primers for exon 5 were obtained from Radic., (2010).*

***Primer pair includes the exon 8a forward primer (HEMBex8a-M13) and the exon 8b reverse primer (HEMBex8b-M13), published by Abla et al., (2017).*

4.2.2 Optimal annealing temperatures (Appendix H)

The optimal primer annealing temperatures determined for the respective PCR assays ranged from 60°C to 62°C (Table 4.2). We defined the optimal annealing temperature as the temperature that provided a single fragment with electrophoresis, with no non-

specific fragments observed. Figure 4.2 illustrates an example of an electrophoretic gel image, depicting the temperature gradient results for the *F9* fragment 6 PCR assay.

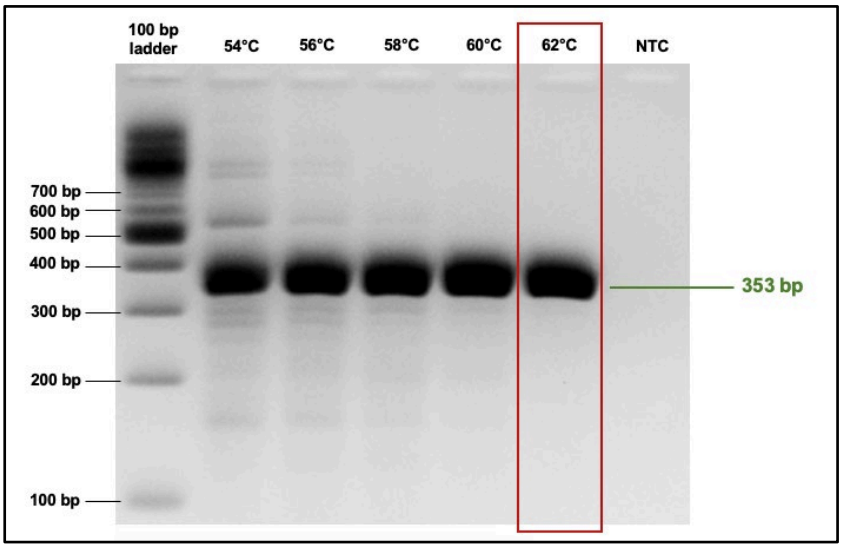

Figure 4.2. Gel image depicting the temperature gradient results of the F9 fragment 6 conventional PCR assay, with an expected fragment size of 353 bp. *A temperature gradient PCR was conducted to determine the optimal annealing temperature of the F9 fragment 6 primer pair. The temperature gradient was performed using a temperature range of 54°C, 56°C, 58°C, 60°C and 62°C. Gel electrophoretic analysis exemplified the optimal annealing temperature for the primer pair to be 62°C, as indicated by the red box.*

4.2.2.1 Troubleshooting for the Fragment 5 (exon 6) PCR assay

Following troubleshooting attempts to optimise the fragment 4 PCR assay using primers published by Abla et al., (2017) (Table 3.1), the issue of non-specific amplification persisted. Consequently, a different primer pair published by Radic, (2010) (Table 4.1) was employed to optimise the PCR assay for exon 6. During the initial temperature gradient experiment, the fragment of interest (342 bp) exhibited non-specific amplification across all tested temperatures, leading to its excision (refer to Appendix H). Subsequently, using the excised fragment as a template for a repeated temperature gradient, minimal amplification was observed at 54˚C, evident by the presence of a very faint band in the gel electrophoresis analysis. However, the assay was not repeated as amplification at 56˚C and 58˚C was lower compared to that obtained at 60˚C. Furthermore, the fragment's integrity began to deteriorate at 62˚C, prompting the determination of 62˚C as the optimal annealing temperature.

4.2.2.2 Troubleshooting for the Fragment 7 (exon 8) PCR assay

During the initial annealing temperature gradient experiment conducted for fragment 7a (Table 3.1), non-specific amplification was observed across all tested temperatures. Subsequently, several troubleshooting measures were undertaken, and gel electrophoretic analysis indicated either no amplification or non-specific binding of primers. Consequently, a modified approach was adopted, whereby the forward primer for fragment 7a and the reverse primer for fragment 7b (Table 3.1) were combined to form a novel primer pair for the amplification of the entire exon 8 (Table 4.1). Despite the presence of minimal non-specific amplification observed at the optimal annealing temperature (Appendix H), the assay reached its maximum level of optimisation. Given that Sanger sequencing generated a clean sequence from both the forward and reverse reaction, the targeted fragment was not excised for reamplification.

4.2.3 Input template DNA and the limit of detection (LOD) (Appendix I)

The LOD for each *F9* conventional PCR assay was determined using a range of six different DNA concentrations, described in section 3.4.3.2. To summarise, the LOD was determined as the lowest concentration of input DNA required for each PCR assay, without losing integrity. Each *F9* assay was conducted at optimised annealing temperatures discussed above in section 4.2.2. The electrophoretic gel image in Figure 4.3 depicts an example of the LOD results obtained for the *F9* fragment 4 conventional PCR assay. Furthermore, the LOD, summarised in Table 4.2, for each *F9* PCR assay was analysed using gel electrophoresis. The LOD ranged from 3.125 ng/μl to 12.5 ng/μl. Therefore, we can conclude that to perform a comprehensive variant detection in a person with an unknown variant, the minimum concentration of extracted DNA should not be below 12.5 ng/μl. However, if the mutation location of a familial variant is known, the LOD of the specific exon may be used as an indicator, if less than 12.5 ng/μl were extracted. This is especially relevant in participants where blood collection is difficult, and where resampling should preferably be avoided. We believe that determining the LOD for input DNA is a vital element of our method, taken that the ultimate goal is to implement this method into the diagnostic platform, thus, providing minimum sample parameters for the use of this method in different laboratories is important.

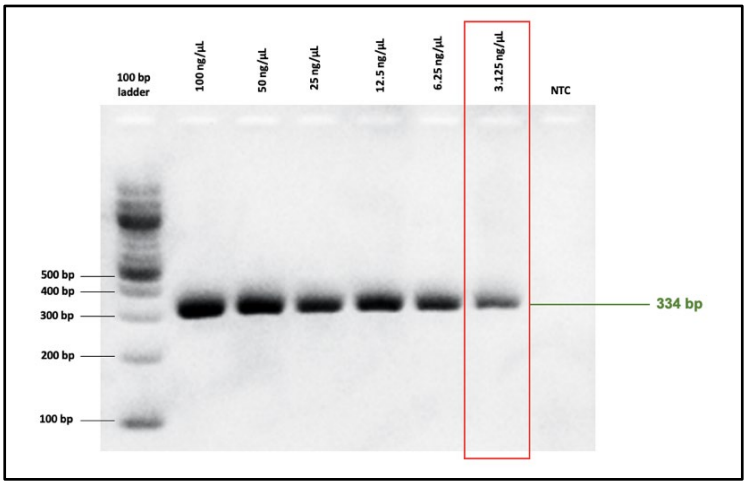

Figure 4.3. Gel image depicting the LOD results obtained for the F9 fragment 4 conventional PCR assay. *To determine the LOD for the F9 fragment 4 conventional PCR assay, a range of six different DNA concentrations was used to set up six respective PCR reactions. Furthermore, the six PCR reactions, as well as a NTC reaction, were conducted at the optimal annealing temperature determined for the PCR assay. As indicated by the red box, a DNA concentration of 3.125 ng/µL, was concluded to be the LOD for the F9 fragment 4 conventional PCR assay.*

Table 4.2. Optimised PCR conditions for each *F9* conventional PCR assay.

***F9* PCR assay** (*F9* exon)	**Optimal annealing temperature**	**Assay limit of detection (LOD)**
***F9* fragment 1** (Exon 1)	62°C	6.25 ng/∝l
***F9* fragment 2** (Exon 2;3)	62°C	6.25 ng/∝l
***F9* fragment 3** (Exon 4)	62°C	6.25 ng/∝l
***F9* fragment 4** (Exon 5)	62°C	3.125 ng/∝l
***F9* fragment 5** (Exon 6)	60°C	6.25 ng/∝l
***F9* fragment 6** (Exon 7)	62°C	3.125 ng/∝l
***F9* fragment 7** (Exon 8)	62°C	12.5 ng/∝l

F9: Factor IX gene; LOD: Limit of detection

4.2.4 Sanger sequencing (Appendix J)

Each sequencing reaction was prepared, purified and analysed as described in section 3.4.5. We deemed our Sanger sequencing reaction as successful, as we were able to create electropherograms with clear peaks and minimal non-specific background

(Figure 4.4), and we determined a 100% similarity between the normal control sample's nucleotide sequence and the reference sequence (Figure 4.5).

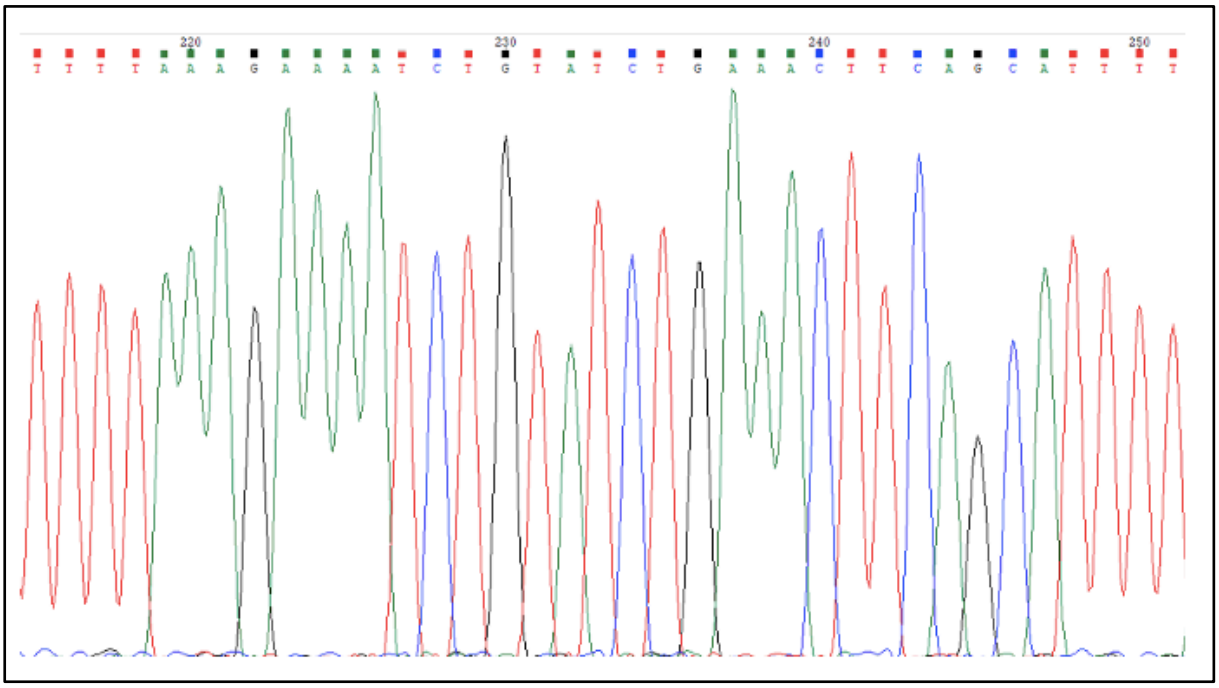

Figure 4.4. An example of the electropherogram obtained using Chromas-lite; *an example that illustrates an optimised wild-type electropherogram obtained for the forward reaction of the F9 fragment 2 PCR assay, using the control DNA.*

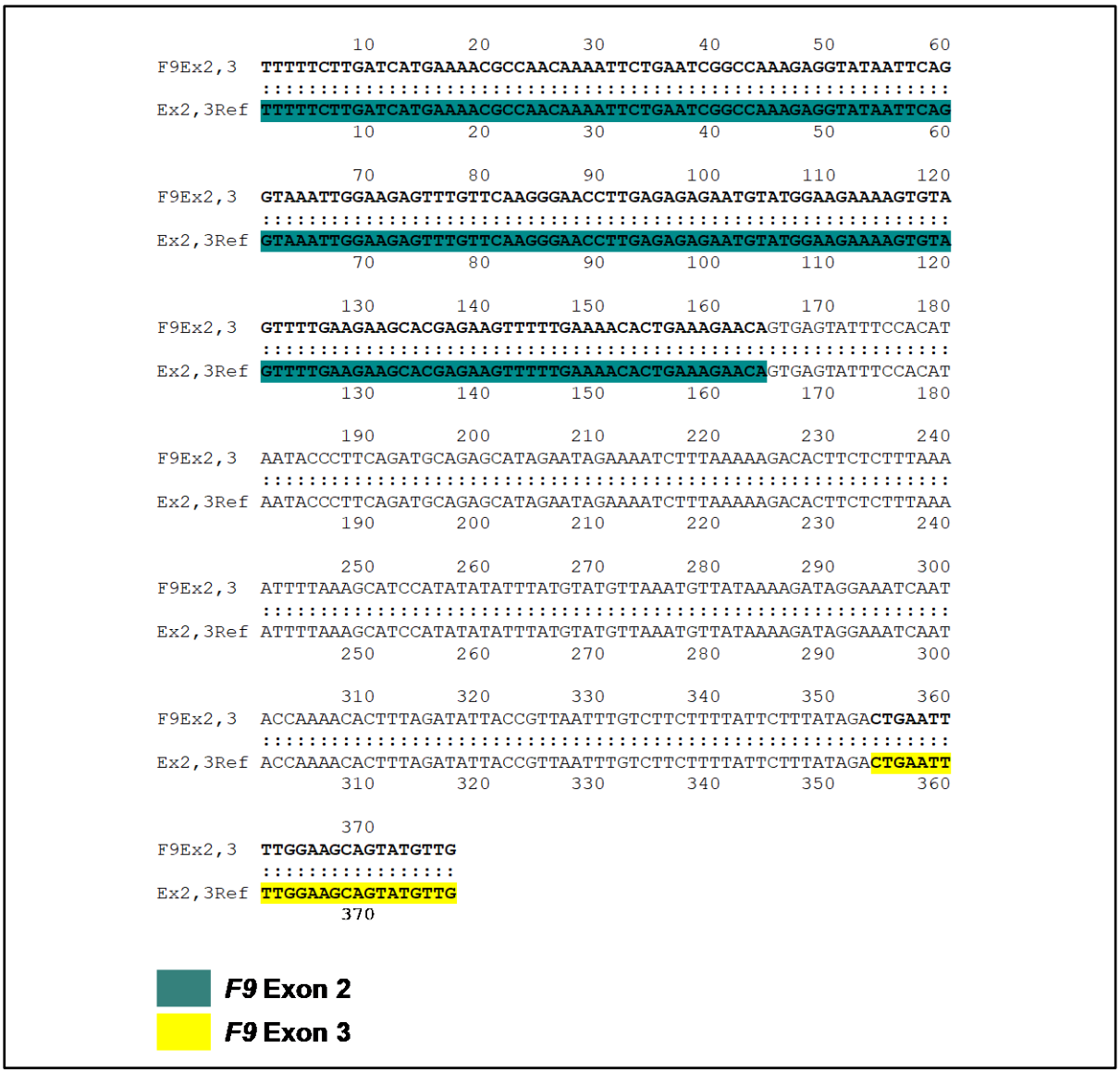

Figure 4.5. An example of the nucleotide alignment results (combined sequence of the forward and reverse reaction) obtained, using the Online Pairwise Sequence alignment software (https://www.ebi.ac.uk/Tools/psa/emboss_needle/#). *This figure depicts the results obtained for the alignment of the F9 fragment 2 (exon 2 (highlighted in teal) and exon 3 (highlighted in yellow)) sequencing reaction and its reference sequence, obtained from NCBI (NM_000133.4). The desired sequence was confirmed, as a 100% similarity was obtained.*

4.3 Screening of 21 study participants using the optimised *F9* conventional PCR assays

4.3.1 DNA isolation and the concentration of each sample

The DNA concentrations of all the samples ranged from 41.69 ng/µL to 71.25 ng/µL (Appendix K). Therefore, all our samples yielded extracted DNA with concentrations

higher than the LOD determined for the *F9* conventional PCR assays. Thus, the concentration of the extracted genomic DNA for each sample was acceptable to be used in the *F9* conventional PCR detection method.

4.3.2 *F9* conventional PCR assay fragment analysis (Appendix L)

Based on the successful amplification of the *F9* regions of interest, the optimised *F9* conventional PCR assays were demonstrated to be dependable and precise. Therefore, we believe this can be implemented in a routine diagnostic laboratory to analyse the mutational status of people affected with haemophilia B and possible carriers. Figure 4.6 is an example of an electrophoretic gel image, depicting fragments 1 to 7 of the conventional PCR assays of one of the study participants. It is clear that *F9* fragment 5 PCR assay amplifies additional non-specific fragments. However, the desired fragment of 342 bp was excised and reamplified to obtain only one fragment on the gel image (Appendix L).

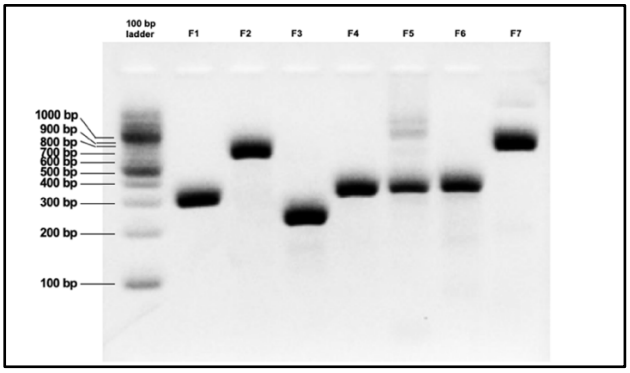

Figure 4.6. An example of gel image depicting the F9 fragment 1-7 conventional PCR assay results obtained for the study sample 11. *This figure demonstrates the successful amplification of each fragment with the respective F9 conventional PCR assays. The fragment sizes obtained with each F9 PCR assay, were as follows: F1: 321 bp; F2: 627 bp ; F3: 236 bp; F4: 334 bp; F5: 342 bp (correct fragment was excised and reamplified); F6: 353 bp; and F7: 697 bp. *bp: Base pairs; F1: Fragment 1; F2: Fragment 2; F3: Fragment 3; F4: Fragment 4; F5: Fragment 5; F6: Fragment 6; F7: Fragment 7.*

4.3.3 Sanger sequencing analysis

The Sanger sequencing alignment results (Appendix M) confirmed the *F9* variant detected for each sample (Figure 4.7). Each FIX variant will be discussed separately. In this study population of 21 individuals, ten pathogenic *F9* genetic changes were

identified. Due to the fact that two of the variants were complex variants, each with one novel and one previously described genetic change, we can conclude that in our participants there were eight pathogenic variants detected. These genetic variants included one missense variant (c.1217C>T), one nonsense variant (c.721C>T), four deletions (c.311delA; c.363_364delTG; c.1178_1180delACA; c.1376delA), and two complex variants (250A>G and 251delC; 580A>G and 726delT) (Figure 4.7). After comparing the changes to previously published data, five of the ten genetic changes (n=5/10; 50%) were found to be novel. When evaluating the variants, five of the eight variants (5/8, 62.5%) we described were found to be novel variants.

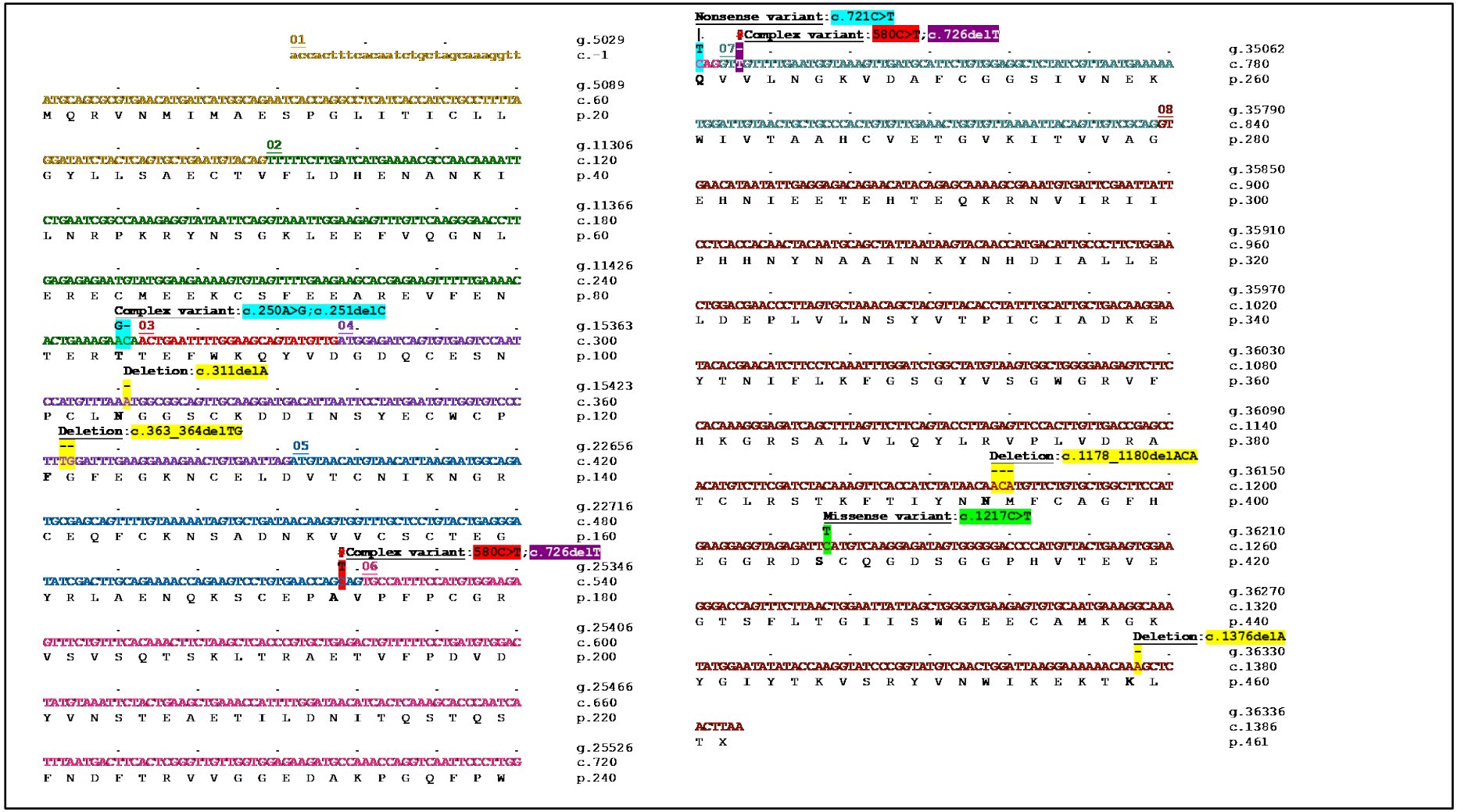

Figure 4.7. Summary of all the F9 pathogenic variants identified within the study population. **AP: Activation peptide; EGF1: Epidermal growth factor like 1 domain; EGF2: Epidermal growth factor like 2; GLA: Gamma-carboxyglutamic acid domain: SP: Serine protease.*

4.3.3.1 Missense variant

4.3.3.1.1 c.1217C>T (p.Ser^{406}Leu)

A previously described *F9* missense variant, c.1217C>T (p.Ser^{406}Leu), was identified in two of our study participants (S5 and S10) (Bicocchi et al., 2006; Chen et al., 1991; Costa et al., 2000; Ghanem et al., 1993; Saad et al., 1994). Both study participants were diagnosed with severe haemophilia B, based on the functional plasma level of FIX (FIX:C <1.0 IU/dL (S5) and FIX:C 0.6 IU/dL (S10), respectively).

This missense variant was detected in exon 8 of the *F9* gene, at position 1217 of the nucleotide sequence. Figure 4.8 depicts an example of the sequence alignment results obtained for S10, confirming the variant detected. The c.1217C>T variant results in the radical amino acid change of Ser^{406}Leu within the FIX serine protease domain (Figure 4.9). The Ser^{406} is a buried residue within the random coil region of the FIX structure, with a surface accessibility of 15.4%. Besides changing the polarity of this site from partially hydrophilic to hydrophobic, the Ser^{406}Leu variant fills this cavity intended to host the *p*-amino benzamidine inhibitor, resulting in the prevention of this molecule to access the FIX catalytic region. Figure 4.10 depicts the theoretical 3D structure created, using the SWISS-MODEL online building software to predict the effect on the FIX protein structure. Furthermore, the PolyPhen-2 online prediction tool predicted the p.Ser^{406}Leu variant to be possibly damaging with a score of 0.779 (sensitivity: 0.85; specificity: 0.93).

The ACMG criteria for classifying pathogenic variants and the categories of pathogenicity for this missense variant, are summarised in Table 4.3. Based on the ACMG rules for combining criteria for the classification of sequence variants, in combination with the online VarSome software, this *F9* missense variant (c.1217C>T), was predicted to be **pathogenic**.

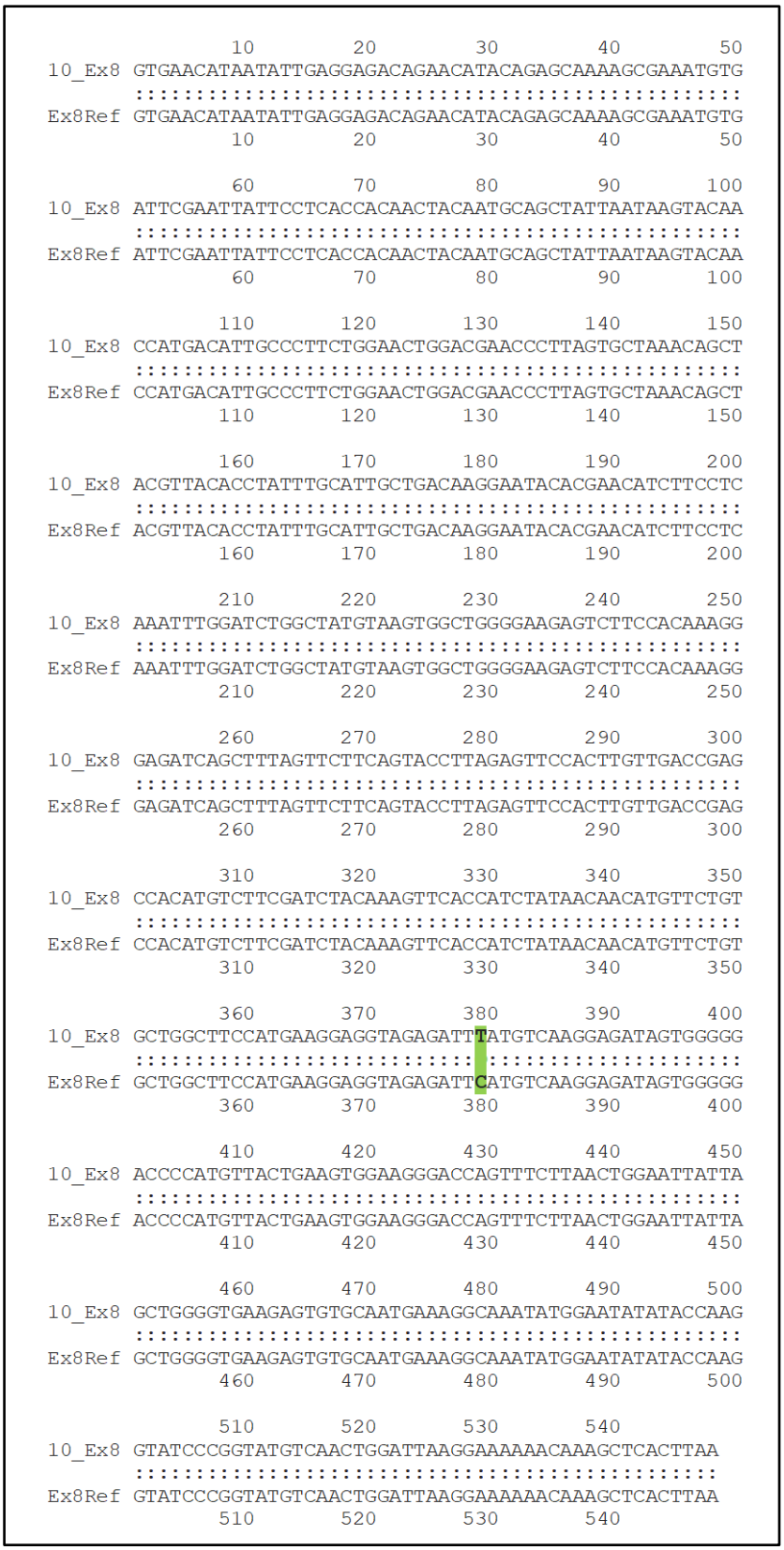

Figure 4.8. Alignment comparison of the F9 exon 8 nucleotide sequence between S10 and the corresponding reference sequence. *The sequence F9 variant, c.1217C>T, is highlighted in green. The reference sequence was obtained from NCBI (NM_000133.4).*

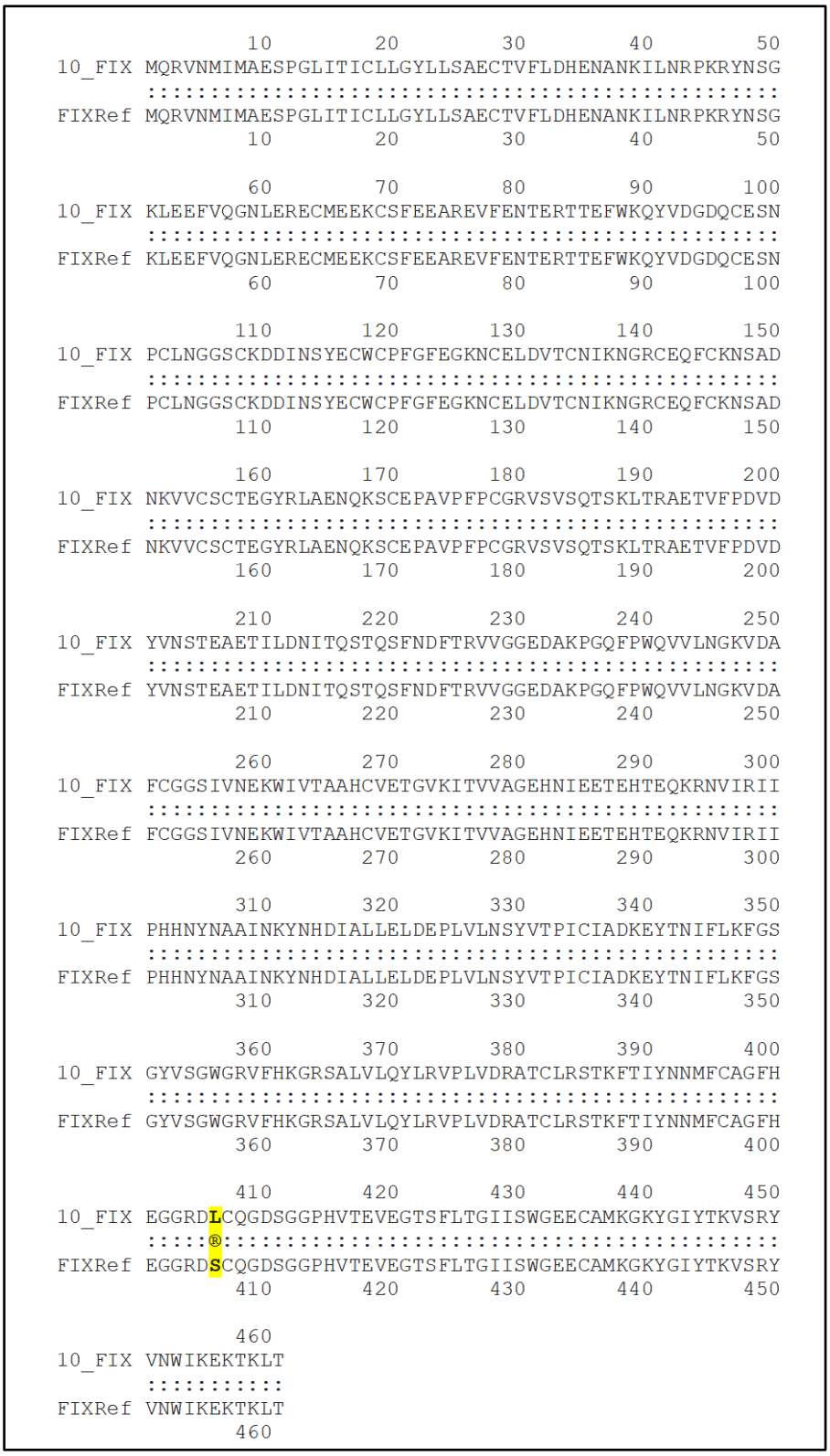

Figure 4.9. Alignment of the compromised translated FIX protein sequence for S10 and the FIX reference amino acid sequence (NP_000124.1). *The p.Ser^{406}Leu FIX amino acid variant is highlighted in yellow. *®: Radical amino acid change. The reference sequence was obtained from NCBI (NP_000124.1).*

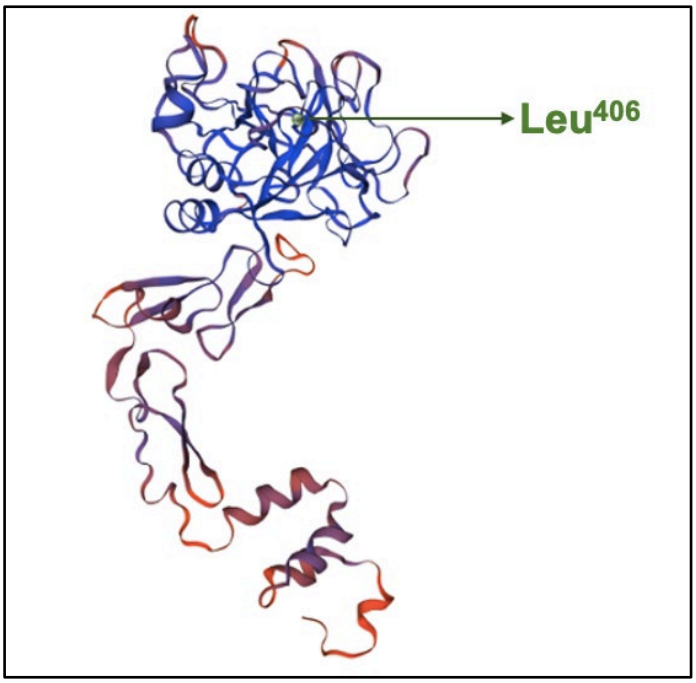

Figure 4.10. Prediction of the* altered *FIX 3D structure (p.Ser^{406}Leu).

Table 4.3. The categories of pathogenicity for *F9* variant c.1217C>T.

Categories	
PS1	Same amino acid change as a previously established pathogenic variant regardless of nucleotide change.
PS3	Well-established *in vitro* or *in vivo* functional studies supportive of a damaging effect on the gene or the gene product.
PM1	Located in a mutational hot spot and/or critical and well-established functional domain (e.g., active site of an enzyme) without benign variation. *Strong - Hot spot of length 17 amino acids has 14 missense/in-frame/non-synonymous variants (13 pathogenic, 1 uncertain and 0 benign), which qualifies as a dense hot spot.*
PM2	Absent from controls in Exome Sequencing Project, 1000 Genome Project, or Exome Aggregation Consortium.
PP3	Multiple lines of computational evidence support a deleterious effect on the gene or the gene product (conservation, evolutionary, splicing impact, etc.).
PP5	Reputable source recently reports variant as pathogenic, but the evidence is not available to the laboratory to perform an independent evaluation. *ClinVar classifies this variant as Pathogenic.*

(Categories for classification of variant pathogenicity were copied and modified from Richards et al., (2015)).

4.3.3.2 Nonsense variant

4.3.3.2.1 c.721C>T (p.Glu^{241}*)

A previously published nonsense variant, c.721C>T (p.Gln^{241}*) (Onay et al., 2003), was identified in six study participants (S2, S7, S8, S9, S12, S13). All, except S13 were diagnosed with severe haemophilia B, based on the respective FIX levels (FIX:C 0.7 IU/dL (S2); FIX:C <1.0 IU/dL (S7); <1.0 IU/dL (S8); <0.4 IU/dL (S9); 0.6 IU/dL (S12)). Furthermore, all these study participants, except S2, were related. S13 did not have a patient file at the time of recruitment, thus, she had no documented FIX plasma levels. However, as this participant is the sister of S8, a confirmed person with severe haemophilia B, it was suspected that she was an asymptomatic carrier.

The c.721C>T variant occurs in exon 6 at position 721 of the *F9* coding nucleotide sequence (Figure 4.11), resulting in the amino acid change of Gln to a premature stop (*) codon, at position 241 (Gln^{241}Stop) within the serine protease domain of the FIX heavy chain. The Gln^{241} is a buried residue, located in a beta sheet region of the FIXa structure. Consequently, the amino acid change results in the premature termination of FIX translation and the production of a FIX protein structure, comprising only 240 amino acids. Figure 4.12 shows the alignment between this truncated FIX protein and its reference sequence. According to the SWISS 3D model prediction (Figure 4.13), folding of the protein results in a further loss of 22 amino acids, resulting in a folded mature protein that is only 218 amino acids long.

The categories of pathogenicity, based on the ACMG criteria for classifying pathogenic variants for this nonsense variant, are summarised in Table 4.4. Based on the ACMG pathogenic classifying criteria, in combination with the online VarSome software, this *F9* nonsense variant (721C>T), was predicted to be **pathogenic**.

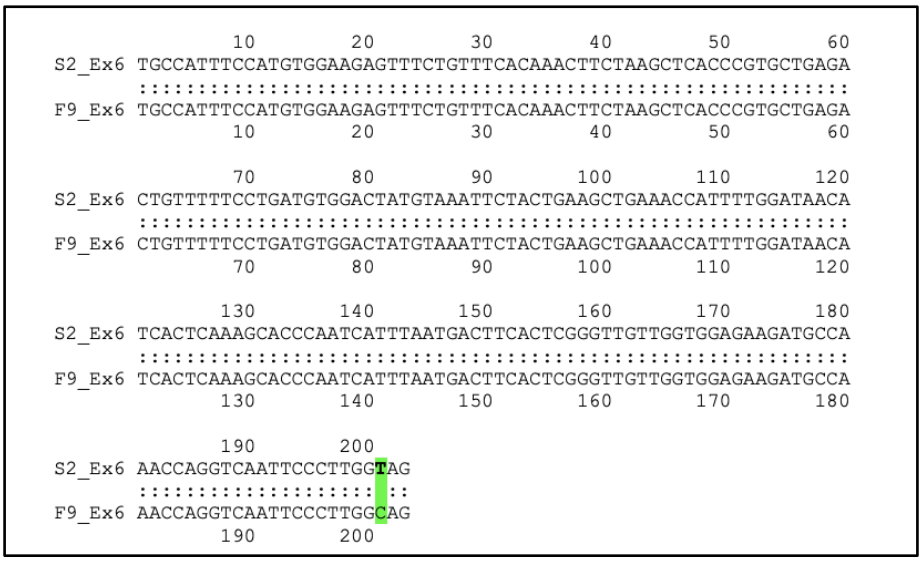

Figure 4.11. Alignment comparison of the F9 exon 6 nucleotide sequence between S2 and the corresponding reference sequence. *The sequence F9 variant, c.721C>T, is highlighted in green. The reference sequence was obtained from NCBI (NM_000133.4).*

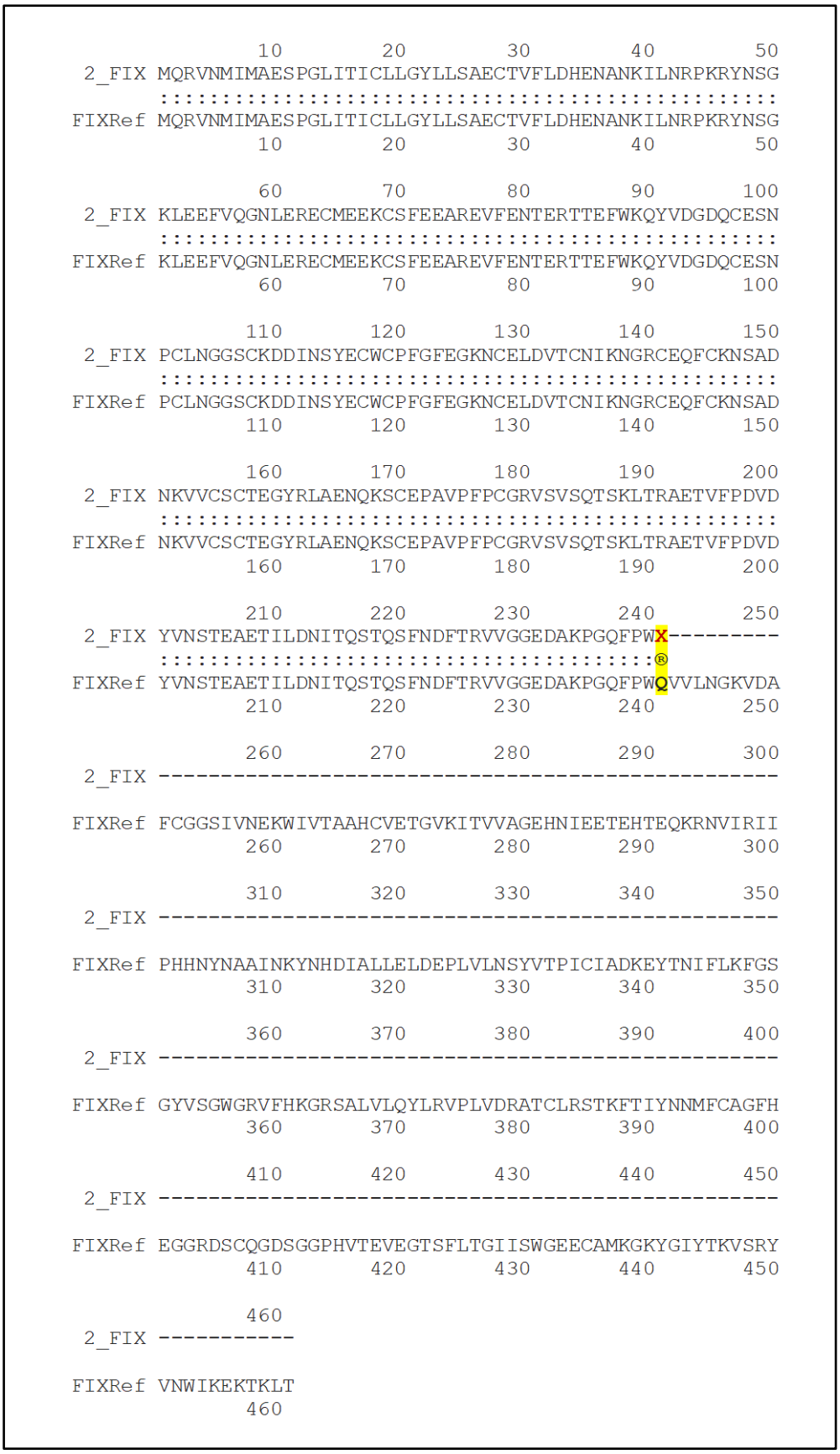
10 20 30 40 50
2_FIX MQRVNMIMAESPGLITICLLGYLLSAECTVFLDHENANKILNRPKRYNSG
FIXRef MQRVNMIMAESPGLITICLLGYLLSAECTVFLDHENANKILNRPKRYNSG
10 20 30 40 50
60 70 80 90 100
2_FIX KLEEFVQGNLERECMEEKCSFEEAREVFENTERTTEFWKQYVDGDQCESN
FIXRef KLEEFVQGNLERECMEEKCSFEEAREVFENTERTTEFWKQYVDGDQCESN
60 70 80 90 100
110 120 130 140 150
2_FIX PCLNGGSCKDDINSYECWCPFGFEGKNCELDVTCNIKNGRCEQFCKNSAD
FIXRef PCLNGGSCKDDINSYECWCPFGFEGKNCELDVTCNIKNGRCEQFCKNSAD
110 120 130 140 150
160 170 180 190 200
2_FIX NKVVCSCTEGYRLAENQKSCEPAVPFPCGRVSVSQTSKLTRAETVFPDVD
FIXRef NKVVCSCTEGYRLAENQKSCEPAVPFPCGRVSVSQTSKLTRAETVFPDVD
160 170 180 190 200
210 220 230 240 250
2_FIX YVNSTEAETILDNITQSTQSFNDFTRVVGGEDAKPGQFPWX---------
FIXRef YVNSTEAETILDNITQSTQSFNDFTRVVGGEDAKPGQFPWQVVLNGKVDA
210 220 230 240 250
260 270 280 290 300
2_FIX ----------
FIXRef FCGGSIVNEKWIVTAAHCVETGVKITVVAGEHNIEETEHTEQKRNVIRII
260 270 280 290 300
310 320 330 340 350
2_FIX ----------
FIXRef PHHNYNAAINKYNHDIALLELDEPLVLNSYVTPICIADKEYTNIFLKFGS
310 320 330 340 350
360 370 380 390 400
2_FIX ----------
FIXRef GYVSGWGRVFHKGRSALVLQYLRVPLVDRATCLRSTKFTIYNNMFCAGFH
360 370 380 390 400
410 420 430 440 450
2_FIX ----------
FIXRef EGGRDSCQGDSGGPHVTEVEGTSFLTGIISWGEECAMKGKYGIYTKVSRY
410 420 430 440 450
460
2_FIX ----------
FIXRef VNWIKEKTKLT
460

Figure 4.12. Alignment of the translated FIX protein sequence for S2 and the corresponding FIX reference amino acid sequence. *The p.Glu^{241}* FIX amino acid variant is highlighted in yellow. *®: Radical amino acid change The reference sequence was obtained from NCBI (NP_000124.1).*

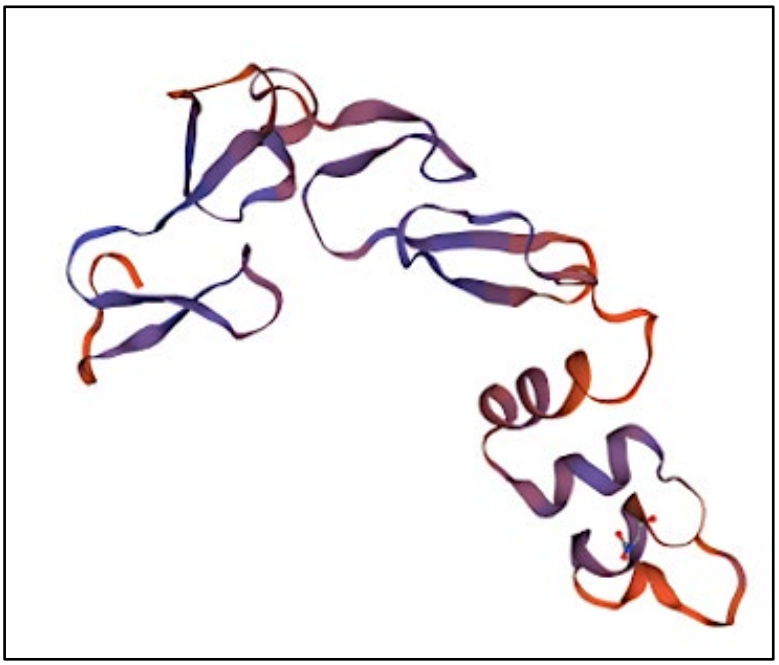

Figure 4.13. Prediction of the truncated FIX 3D structure (p.Glu^{241}*).

Table 4.4. The categories of pathogenicity for *F9* variant c.721C>T.

Categories	
PVS1	Null variant (nonsense).
PS1	Same amino acid change as a previously established pathogenic variant regardless of nucleotide change.
PS3	Well-established *in vitro* or *in vivo* functional studies supportive of a damaging effect on the gene or the gene product.
PM2	Absent from controls in Exome Sequencing Project, 1000 Genome Project, or Exome Aggregation Consortium.
PM4	Protein length changes as a result of in-frame deletions/insertions in a non-repeat region or stop-loss variants.
PP1	Cosegregation with disease in multiple affected members in a gene definitively known to cause the disease.
PP3	Multiple lines of computational evidence support a deleterious effect on the gene or the gene product.

(Categories for classification of variant pathogenicity were copied and modified from Richards et al., (2015)).

4.3.3.3 Deletions

4.3.3.3.1 c.311delA (p.Asn^{104}Metfs*31)

A novel deletion, c.311delA (p.Asn^{104}Metfs*31), was detected in four participants (S6, S14, S18, and S19). At the time of recruitment, both S6 and S14 presented with severe FIX deficiency (FIX:C <0.4 IU/dL (S6); FIX:C 0.5 IU/dL (S14)). At the time of recruitment, S18 presented with a moderate FIX level of severity (FIX:C 1.8 IU/dL). However, a severe FIX level of deficiency was obtained with the functional analysis performed in this study. Based on the FIX plasma levels, this discrepancy emphasises that the severity classification of moderate haemophilia B needs to be handled with caution, and this will be discussed in more detail later. Additionally, S19 (related to S18) was, at the time of recruitment, classified as a potential asymptomatic carrier as no FIX:C data was available at this point.

This novel variant was identified in *F9* exon 4, at position 311 of the translating nucleotide sequence (Figure 4.14). This deletion results in the radical amino acid change of Asn^{104}Met, which occurs within the EGF 1 protein domain, followed by an additional 21 radical changes (p.Gly^{105}Asn, p.Gly^{106}Asn, p.Ser^{107}Val, p.Cys^{108}Ala, p.Asp^{110}Met, p.Asp^{111}Thr, p.Asn^{113}Ile, p.Ser^{114}Pro, p.Tyr^{115}Met, p.Glu^{116}Asn, p.Cys^{117}Val, p.Trp^{118}Gly, p.Cys^{119}Val, p.Phe^{121}Leu, p.Gly^{122}Asp, p.Phe^{123}Leu, p.Gly^{125}Glu, p.Asn^{127}Thr, p.Cys^{128}Val, p.Glu^{129}Asn, and p.Asp^{131}His) five conservative changes (p.Lys^{109}Arg, p.Ile^{112}Leu, p.Glu^{121}Lys, p.Lys^{126}Arg, and p.Leu^{130}Met), and subsequently, premature termination of translation at the FIX residue 134. The Asn^{104} is an exposed residue, with a surface accessibility of 9%. Figure 4.15 demonstrates the results for the alignment between this truncated FIX protein and its reference sequence. A theoretical 3D ribbon FIXa protein structure was predicted with SWISS-MODEL online building software, shown in Figure 4.16. The complete amino acid sequence is folded into a mature FIXa protein of 133 amino acids. Summarised in Table 4.5. are the combined criteria for the prediction of variant pathogenicity, using the ACMG criteria and the VarSome online software. Finally, the c.311delA *F9* variant was predicted to be **pathogenic**.

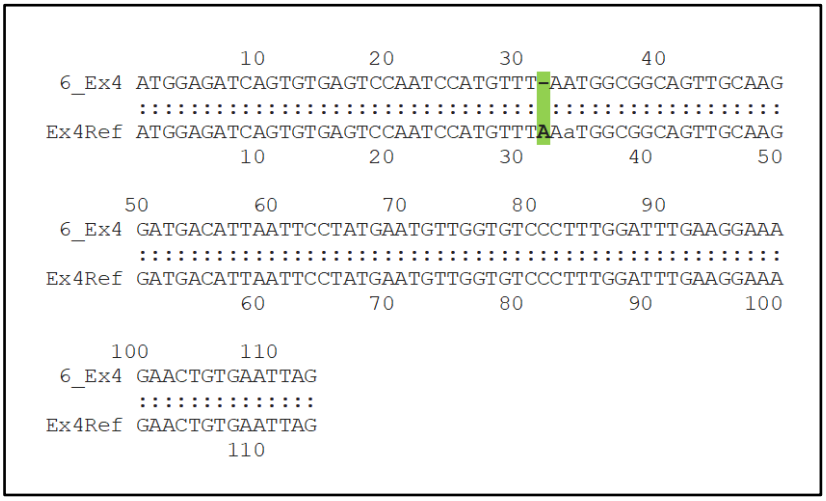

Figure 4.14. Alignment comparison of the F9 exon 4 nucleotide sequence between S6 and the corresponding reference sequence. *The sequence F9 variant, c.311delA, is highlighted in green. The reference sequence was obtained from NCBI (NM_000133.4).*

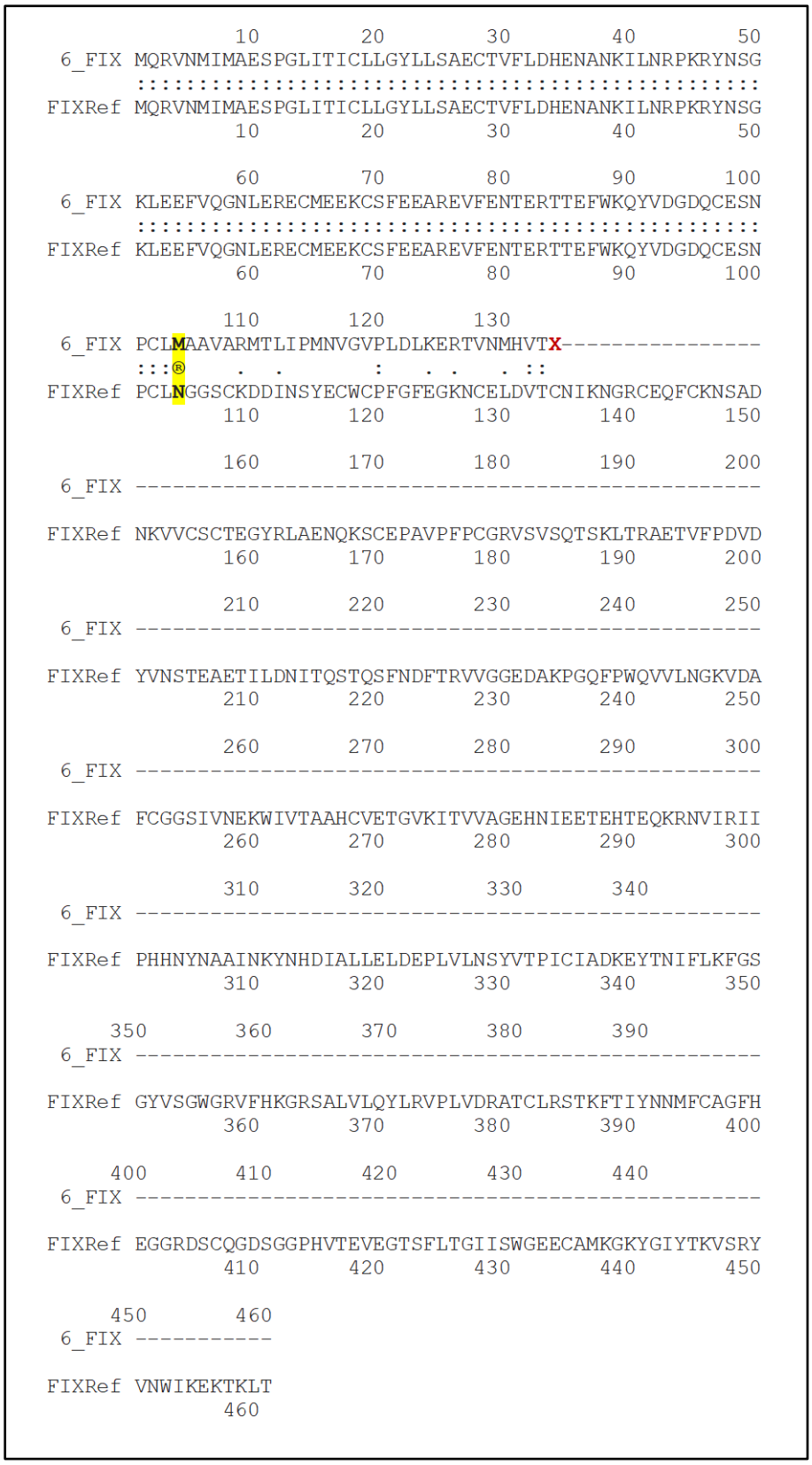

Figure 4.15. Alignment of the altered translated FIX protein sequence for S6 and the FIX reference amino acid sequence*. The radical p.Asn^{104}Metfs*31 FIX amino acid variant is highlighted in yellow. *®: Radical amino acid change; X: Stop codon. The reference sequence was obtained from NCBI (NP_000124.1).*

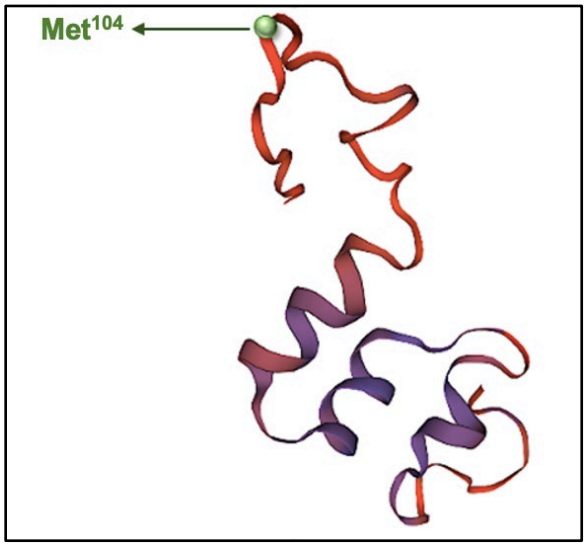

Figure 4.16. Prediction of the compromised FIX 3D structure (p.Asn^{104}Metfs*31).

Table 4.5. The categories of pathogenicity for *F9* variant c.311delA.

Categories	
PVS1	Null variant (nonsense, frameshift, canonical ± 1 or 2 spice sites, initiation codon, single or multiexon deletion) in gene where LOF is a known mechanism of disease. *Null variant (frameshift), in gene F9 for which LOF is a known mechanism of disease (gene has 45 pathogenic LOF variants and gnomAD LOF observed/expected = 0 is less than 0.763), associated with haemophilia B, due to the FIX defect and Warfarin sensitivity, X-linked.*
PS3	Well-established *in vitro* or *in vivo* functional studies supportive of a damaging effect on the gene or the gene product.
PM2	Absent from controls in Exome Sequencing Project, 1000 Genome Project, or Exome Aggregation Consortium.
PM4	Protein length changes as a result of in-frame deletions/insertions in a non-repeat region or stop-loss variants.
PM6	Assumed novel, but without confirmation of paternity or maternity.
PP3	Multiple lines of computational evidence support a deleterious effect on the gene or the gene product (conservation, evolutionary, splicing impact, etc.). *Pathogenic computational verdict based on 1 pathogenic prediction from PhyloP versus no benign predictions.*

FIX: Factor IX; LOF: Loss of function.

(Categories for classification of variant pathogenicity were copied and modified from Richards et al., (2015)).

4.3.3.3.2 c.363_364delTG (p.Phe^{121}Leufs*3)

A novel deletion, c.363_364delTG (p.Phe^{121}Leufs*3), was detected in study participant 3 (S3), who was diagnosed with a severe FIX deficiency (FIX:C 0.6 IU/dL). This novel variant was identified in *F9* exon 4, at position 363-364 of the nucleotide sequence (Figure 4.17). This results in the radical amino acid change of Phe^{121}Leu, within the EGF1 protein domain of FIX, resulting in the premature termination of FIX translation. Figure 4.18 demonstrates the results for the alignment between this truncated FIX protein and its reference sequence. Phe^{121} is a buried residue with a surface accessibility of 4%. Furthermore, Phe^{121} is a turn residue within a random coil of the FIXa structure and can compromise the folding of the truncated 122-residue FIXa molecule. (Figure 4.19).

The ACMG criteria for classifying pathogenic variants, in combination with the VarSome online prediction software, were used to predict the classification of the pathogenicity for c.363_364delTG. Based on the classification criteria, summarised in Table 4.6., the *F9* variant, c.363_364delTG, was determined to be **pathogenic**.

```
               10        20        30        40        50
 3_Ex4 ATGGAGATCAGTGTGAGTCCAATCCATGTTTAAATGGCGGCAGTTGCAAG
       ::::::::::::::::::::::::::::::::::::::::::::::::::
Ex4Ref ATGGAGATCAGTGTGAGTCCAATCCATGTTTAAATGGCGGCAGTTGCAAG
               10        20        30        40        50

               60        70        80          90
 3_Ex4 GATGACATTAATTCCTATGAATGTTGGTGTCCCTT--GATTTGAAGGAAA
       :::::::::::::::::::::::::::::::::::  :::::::::::::
Ex4Ref GATGACATTAATTCCTATGAATGTTGGTGTCCCTTTGGATTTGAAGGAAA
               60        70        80        90       100

      100       110
 3_Ex4 GAACTGTGAATTAG
       ::::::::::::::
Ex4Ref GAACTGTGAATTAG
              110
```

Figure 4.17. Alignment comparison of the F9 exon 4 nucleotide sequence between S3 and the corresponding reference sequence. *The sequence F9 variant, c.363_364delTG, is highlighted in green. The reference sequence was obtained from NCBI (NM_000133.4).*

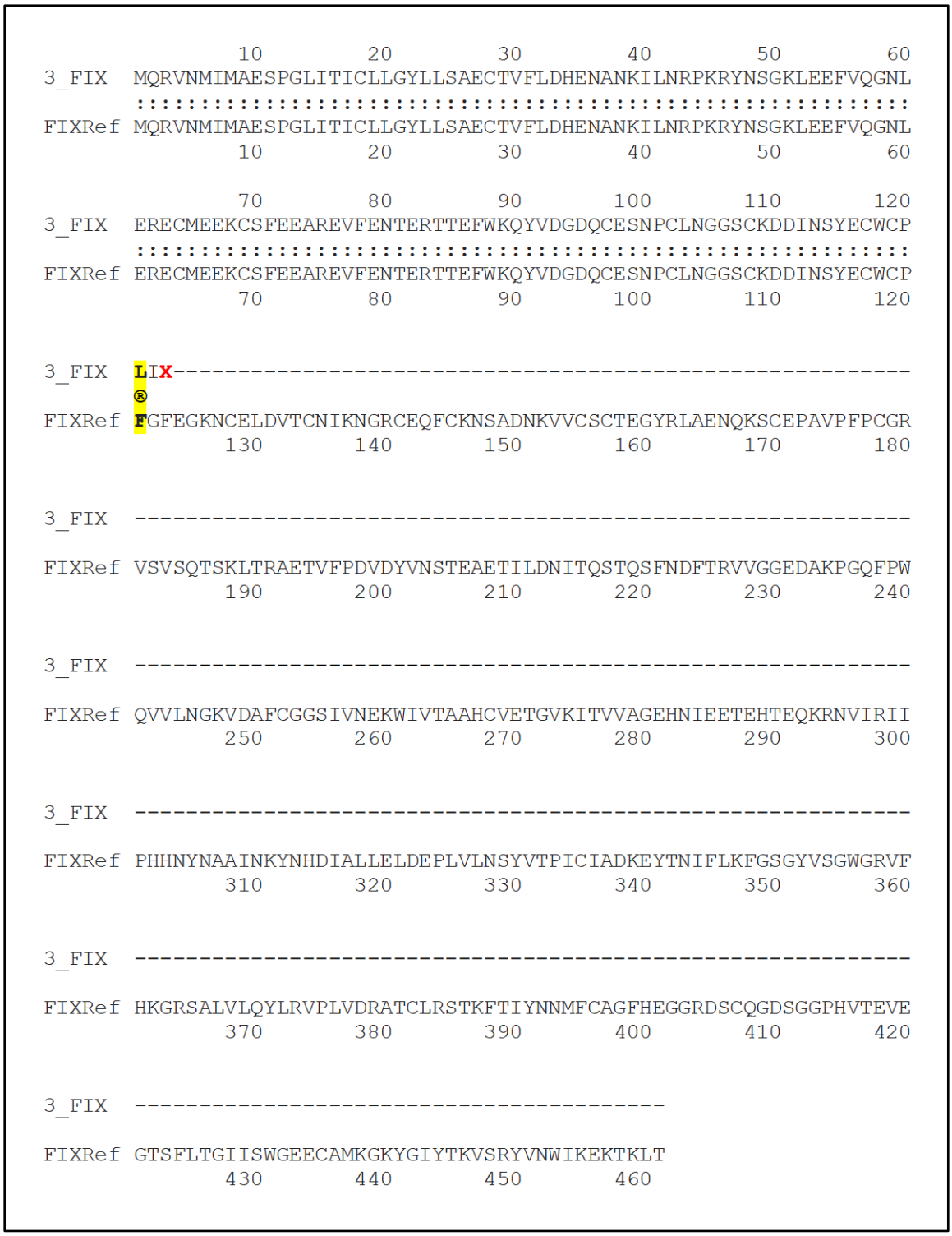

Figure 4.18. Alignment of the compromised translated FIX protein sequence for S3 and the FIX reference amino acid sequence. *The radical p.Phe[121]Leufs*3 FIX amino acid variant is highlighted in yellow. *®: Radical amino acid change; X: Stop codon. The reference sequence was obtained from NCBI (NP_000124.1).*

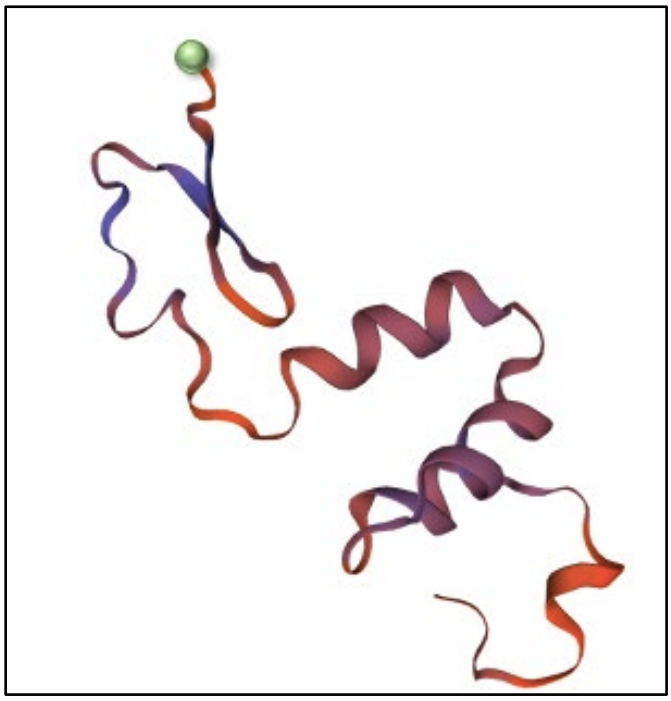

Figure 4.19. Depiction of the truncated FIX 3D structure (p.Phe121Leufs*3).

Table 4.6. The categories of pathogenicity for *F9* variant c.363_364delTG.

Categories	
PVS1	Null variant (nonsense, **frameshift**, canonical ± 1 or 2 spice sites, initiation codon, single or multiexon deletion) in gene where LOF is a known mechanism of disease.
PS3	Well-established *in vitro* or *in vivo* functional studies supportive of a damaging effect on the gene or the gene product.
PM2	Absent from controls in Exome Sequencing Project, 1000 Genome Project, or Exome Aggregation Consortium.
PM4	Protein length changes as a result of in-frame **deletions**/insertions in a non-repeat region or stop-loss variants.
PP3	Multiple lines of computational evidence support a deleterious effect on the gene or the gene product (conservation, evolutionary, splicing impact, etc.). *Pathogenic computational verdict based on 1 pathogenic prediction from PhyloP versus no benign predictions.*

***LOF: Loss of function.**

(Categories for classification of variant pathogenicity were copied and modified from Richards et al., (2015)).

4.3.3.3.3 c.1178_1180delACA (p.Asn^{393}del)

The deletion, c.1178_1180delACA, which has previously been described, was identified in three study participants (S11, S16 and S17) (Belvini et al., 2005; Miller et al., 2012; Saad et al., 1994). At the time of recruitment, two participants presented with severe FIX deficiency (FIX:C 0.9 IU/dL (S11); FIX:C <0.3 IU/dL (S16)) and S17 (FIX:C 68.4 IU/dL) was diagnosed as an asymptomatic haemophilia B carrier.

This variant is located in exon 8 of the *F9* gene, spanning from position 1178-1180 of the nucleotide sequence. Depicted in Figure 4.20, are the sequence alignment results obtained for S11, confirming the variant detected, which results in the loss of Asn^{393} within the serine protease domain (Figure 4.21). The Asn^{393} is a buried residue, located within a hydrogen bonded turn region, with a surface accessibility of zero from the mature FIXa molecule. Figure 4.22 depicts the theoretical 3D ribbon structure of this FIXa protein. Furthermore, summarised in Table 4.7, are the ACMG pathogenicity criteria, together with the online VarSome prediction software for this *F9* variant (c.1178_1180delACA), consequently classified as **pathogenic**.

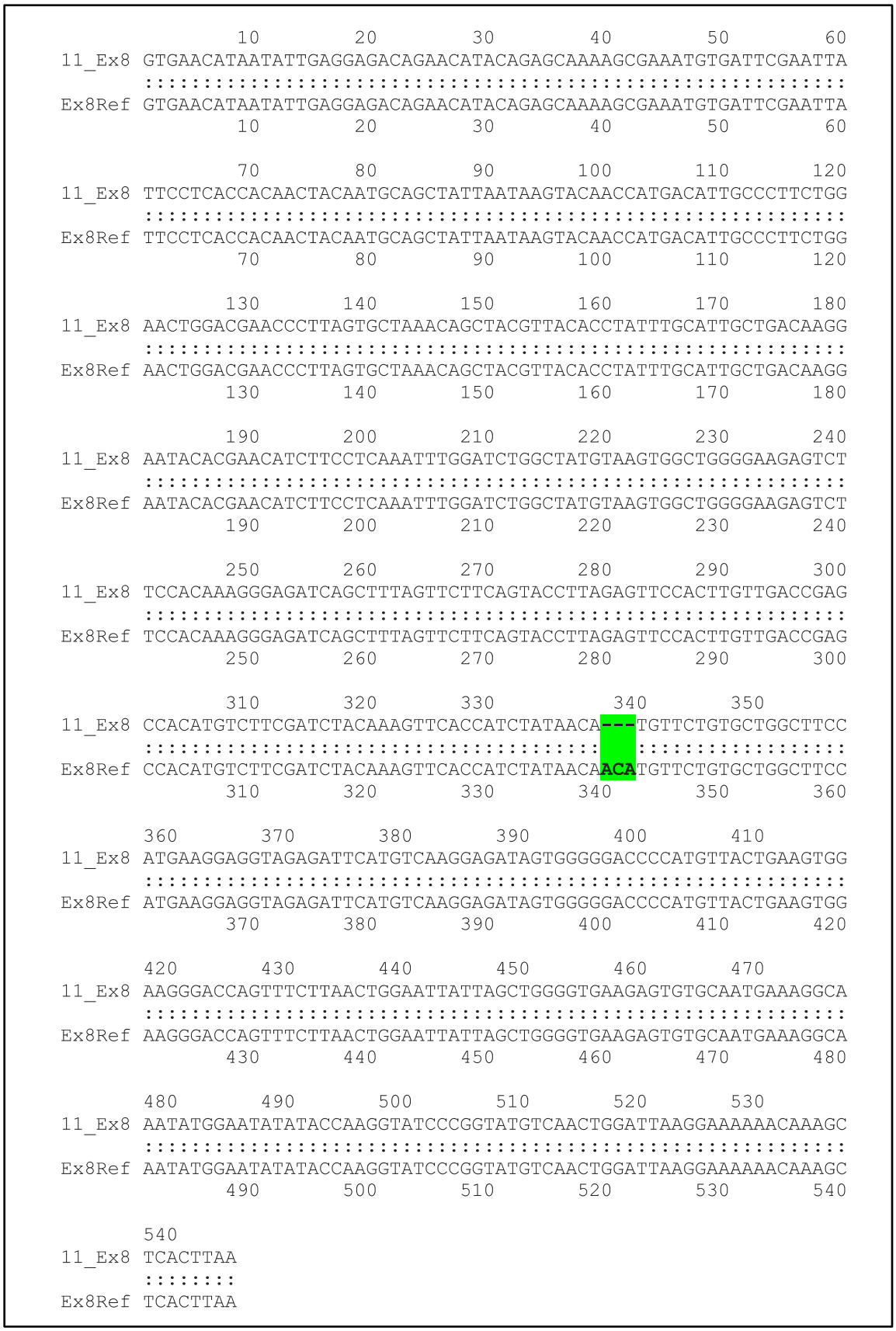

Figure 4.20. Alignment comparison of the F9 exon 8 nucleotide sequence between S11 and the corresponding reference sequence. *The sequence F9 variant, c.1178_1180delACA, is highlighted in green. The reference sequence was obtained from NCBI (NM_000133.4).*

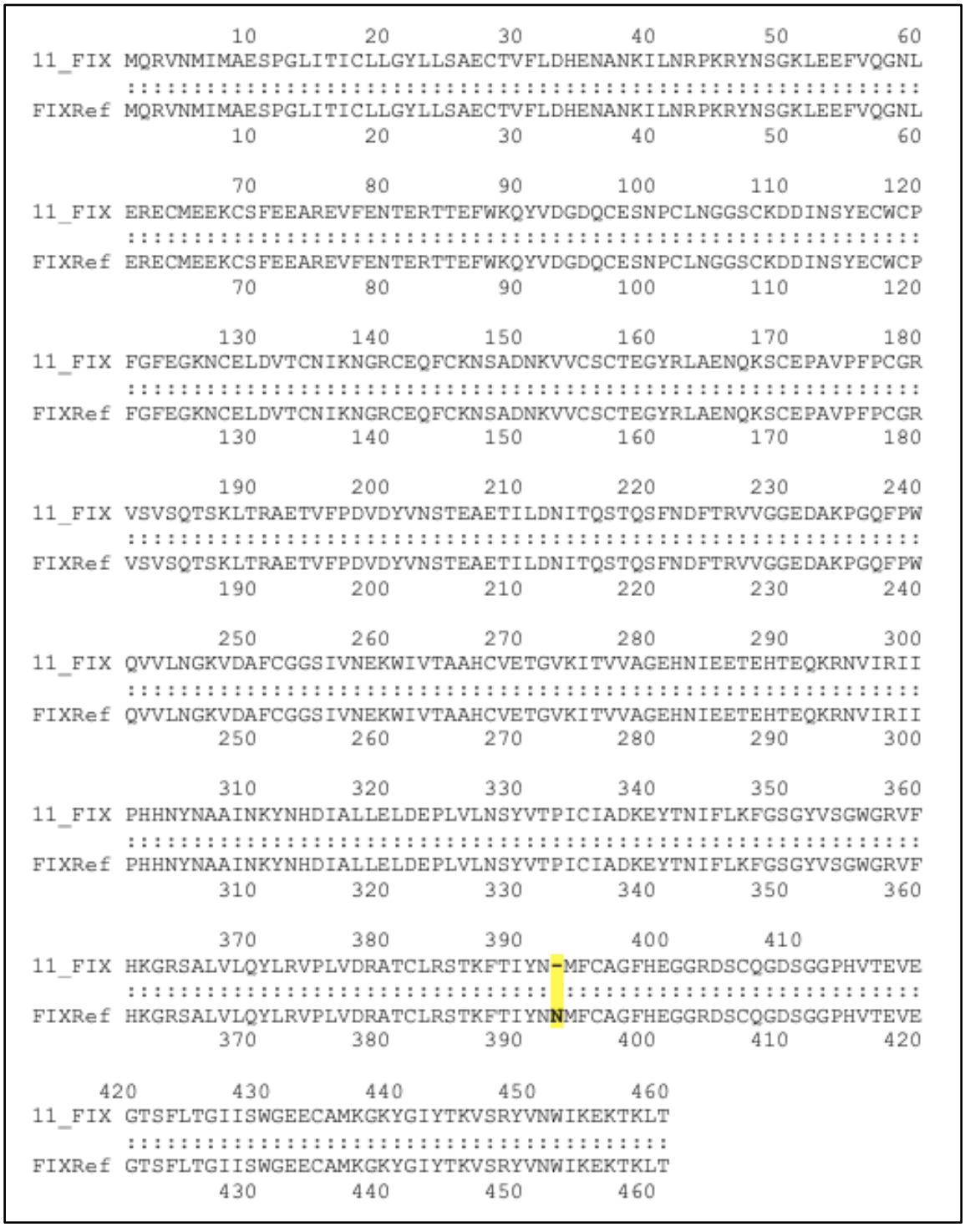

Figure 4.21. Alignment of the altered translated FIX protein sequence for S11 and the FIX reference amino acid sequence. *The p.Asn^{393}del FIX amino acid variant is highlighted in yellow. The reference sequence was obtained from NCBI (NP_000124.1).*

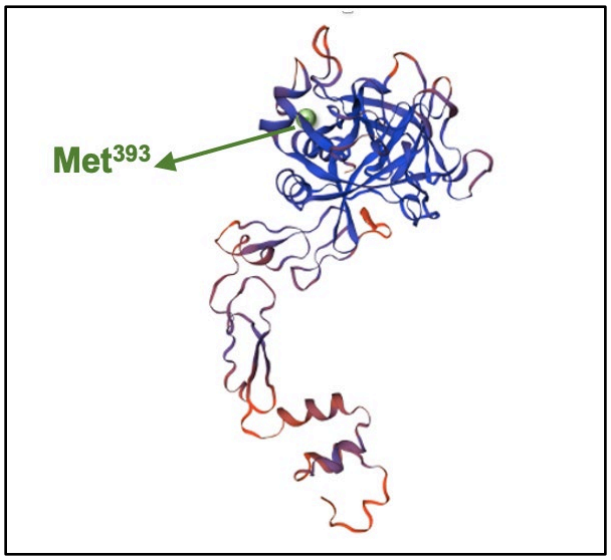

Figure 4.22. Depiction of the* altered *FIXa 3D ribbon structure (p.Asn^{393}del).

Table 4.7. The categories of pathogenicity for F9 variant c.1178_1180delACA.

Categories	
PVS1	Null variant (nonsense, **frameshift**, canonical ± 1 or 2 spice sites, initiation codon, single or multiexon deletion) in gene where LOF is a known mechanism of disease.
PS3	Well-established *in vitro* or *in vivo* functional studies supportive of a damaging effect on the gene or the gene product.
PM1	Located in a mutational hot spot and/or critical and well-established functional domain (e.g., active site of an enzyme) without benign variation. *Hot spot of length 19 amino acids has 15 missense/in-frame/non-synonymous variants (14 pathogenic, 1 uncertain and 0 benign), which qualifies as a dense hot spot.*
PM2	Absent from controls in Exome Sequencing Project, 1000 Genome Project, or Exome Aggregation Consortium.
PM4	Protein length changes as a result of in-frame deletions/insertions in a non-repeat region or stop-loss variants.
PP1	Cosegregation with disease in multiple affected members in a gene definitively known to cause the disease.
PP3	Multiple lines of computational evidence support a deleterious effect on the gene or the gene product (conservation, evolutionary, splicing impact, etc.).

LOF: Loss of function.

(Categories for classification of variant pathogenicity were copied and modified from Richards et al., (2015)).

4.3.3.3.4 c.1376delA (p.Lys^{459}Serfs*24)

A novel deletion, c.1376delA (p.Lys^{459}Serfs*24), was detected in sample 15 (S15), 20 (S20), and 21 (S21). Based on the lowest FIX level on file, at the time of recruitment, all three study participants presented with severe FIX deficiency (FIX:C 0.8 IU/dL (S15); FIX:C <0.4 IU/dL (S20); <0.3 IU/dL (S21)).

This novel *F9* variant was identified in exon 8, at position 1376 of the nucleotide sequence, resulting in a frameshift of the translating nucleotide sequence. Figure 4.23 depicts an example of the sequence alignment results obtained for S21, confirming the variant detected. This deletion results in the radical amino acid change of Lys^{459}Ser within the serine protease domain and consequently, the translation of an elongated 481-residue FIX molecule (Figure 4.24). Finally, as shown in Figure 4.25, a theoretical 3D FIXa ribbon structure was created with the SWISS-MODEL online building software. This novel variant, c.1376delA, was predicted to be **pathogenic**, based on the criteria published by the ACMG for classifying pathogenic variants. The categories of pathogenicity for this deletion, are summarised in Table 4.8.

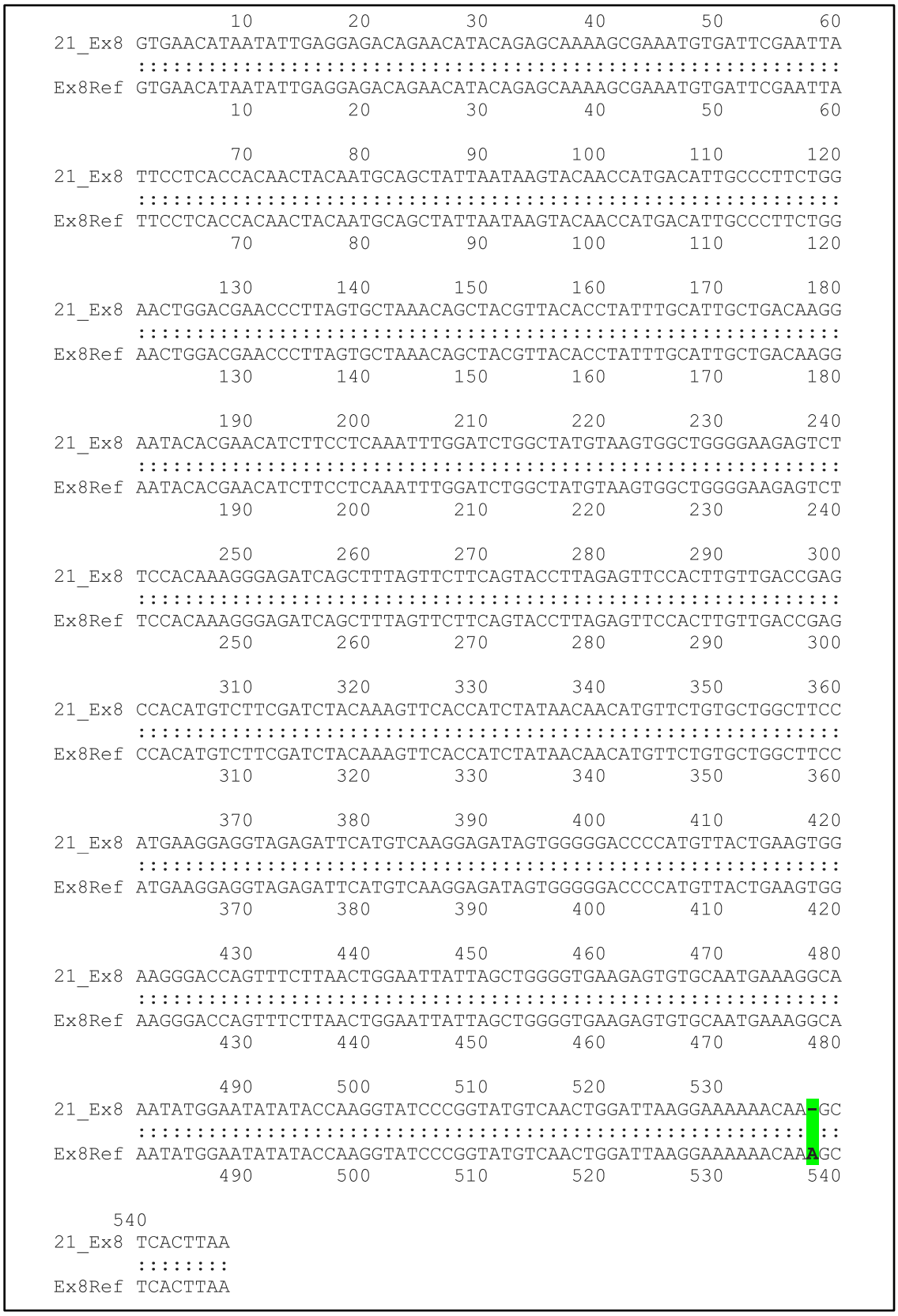

Figure 4.23. Comparison of the F9 exon 8 translating nucleotide sequence between S21 and the reference sequence. *These alignment results confirm the deletion (c.1376delA) detected in F9 exon 8, as highlighted in green. The reference sequence was obtained from NCBI (NM_000133.4).*

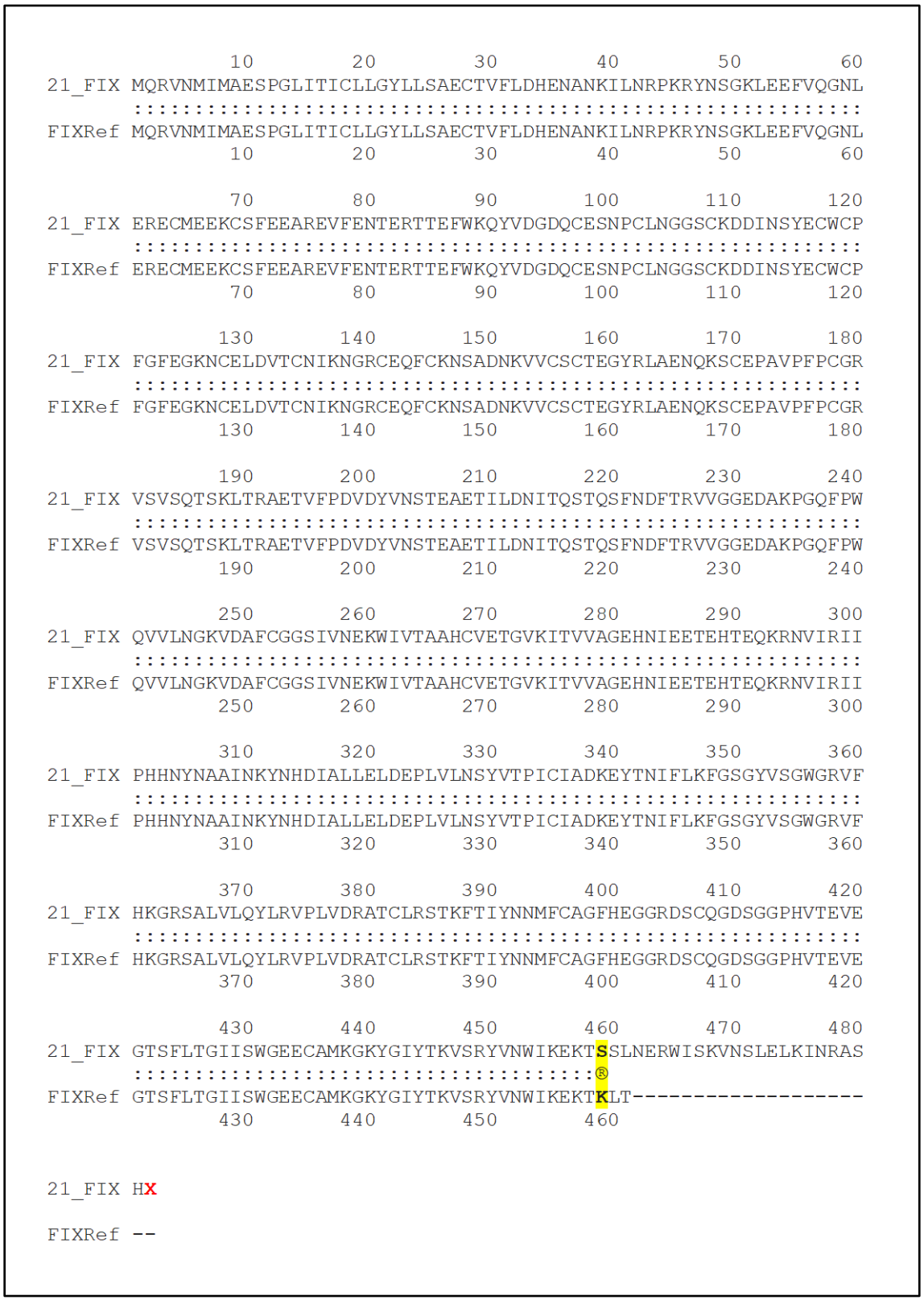

Figure 4.24. Alignment of the compromised translated FIX protein sequence for S21 and the FIX reference amino acid sequence. *The radical p.Lys^{459}Serfs*24 FIX amino acid variant is highlighted in yellow. *®: Radical amino acid change: X: Stop codon. The reference sequence was obtained from NCBI (NP_000124.1).*

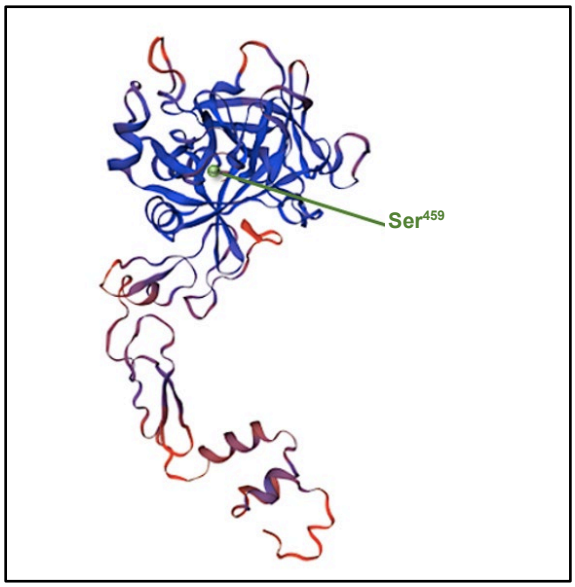

Figure 4.25. Depiction of the compromised FIX 3D ribbon structure (p.Lys^{459}Serfs*24).

Table 4.8. The categories of pathogenicity for *F9* variant c.1376delA.

Categories	
PVS1	Null variant (nonsense, frameshift, canonical ± 1 or 2 spice sites, initiation codon, single or multiexon deletion) in gene where LOF is a known mechanism of disease. *Strong because null variant (frameshift), situated only 3 amino acids from the end of the protein, in F9 gene for which LOF is a known mechanism of disease.*
PS3	Well-established *in vitro* or *in vivo* functional studies supportive of a damaging effect on the gene or the gene product.
PM2	Absent from controls in Exome Sequencing Project, 1000 Genome Project, or Exome Aggregation Consortium.
PM4	Protein length changes as a result of in-frame deletions/insertions in a non-repeat region or stop-loss variants.
PM6	Assumed novel, but without confirmation of paternity or maternity.
PP1	Cosegregation with disease in multiple affected members in a gene definitively known to cause the disease.
PP3	Multiple lines of computational evidence support a deleterious effect on the gene or the gene product (conservation, evolutionary, splicing impact, etc.). *Pathogenic computational verdict based on 1 pathogenic prediction from PhyloP versus no benign predictions.*

F9: Factor IX gene; LOF: Loss of function.

(Categories for classification of variant pathogenicity were copied and modified from Richards et al., (2015)).

4.3.3.4 Complex F9 variants

4.3.3.4.1 c.250A>G;c.251delC (p.Thr^{84}Glufs*20)

A complex *F9* variant was detected in study participant 4 (S4), which included a missense variant, c.250A>G, previously published by Wulff et al., (1999), and a novel *F9* deletion, c.251delC. At the time of recruitment, S4 presented with a severe level of FIX deficiency (FIX:C <0.3 IU/dL).

The missense variant (c.250A>G) and the novel *F9* deletion (c.251delC), are located in exon 4 of *F9*, at nucleotide position 250 and 251, respectively (Figure 4.26). Consequently, the combined effect of this complex variant results in the radical amino acid change and a frameshift (Thr^{84}Glufs*20) within the EGF1 domain of the FIX protein, as depicted in Figure 4.27. The Thr^{84} is a buried residue, within an alpha helix strand, with a surface accessibility of zero, where the value of zero corresponds to 0% – 9% solvent accessibility. As depicted in Figure 4.27, this radical variant is followed by several radical and conservative changes and ultimately, the translation of a pre-mature stop codon. The predicted 3D ribbon structure of this truncated FIXa protein is shown in Figure 4.28. The ACMG pathogenicity classification of this complex *F9* variant is summarised in Table 4.9. Based on the rules for combining criteria for pathogenic classification, this complex *F9* variant [(c.250A>G)(c.251delC)] was predicted to be **pathogenic**.

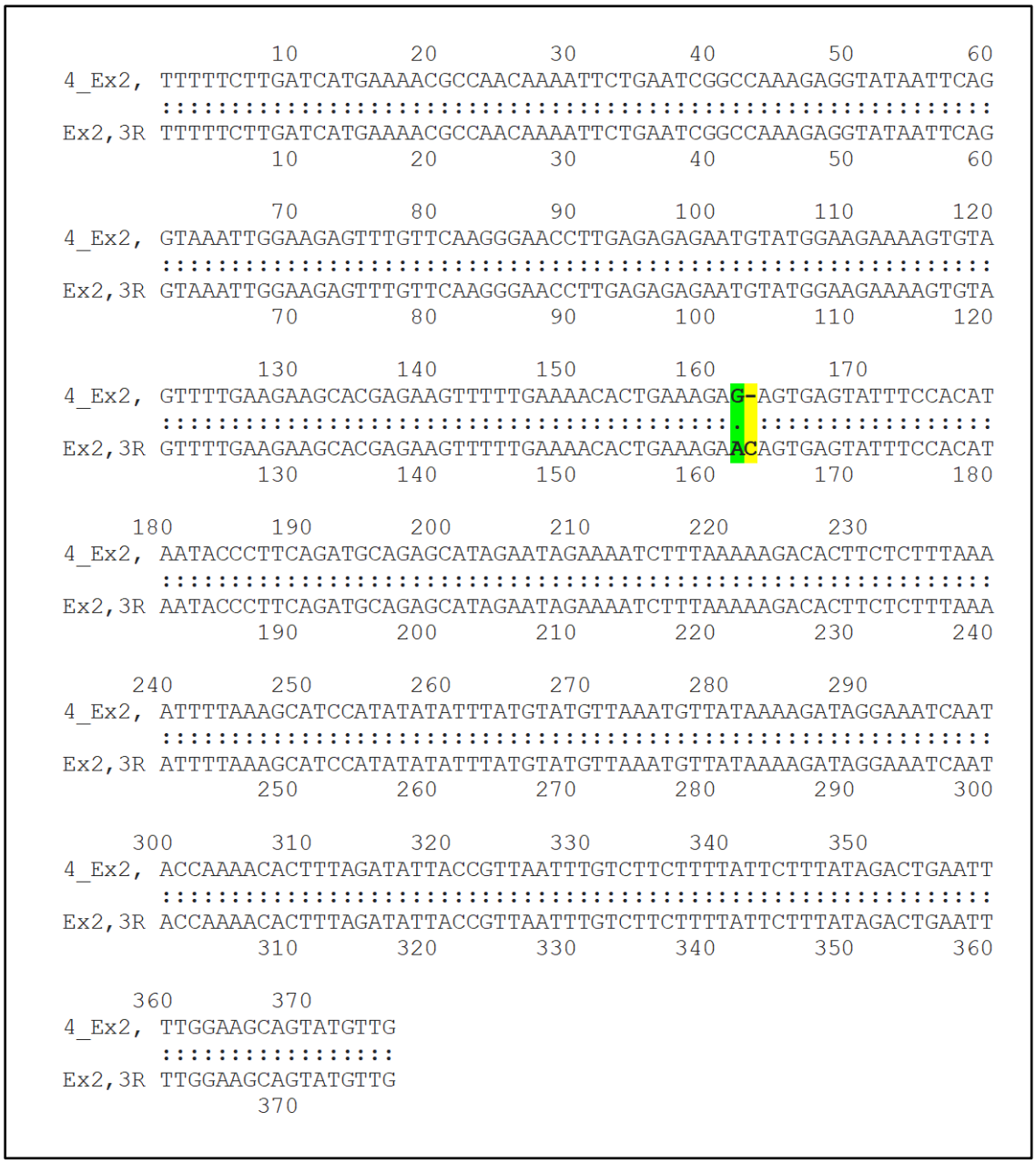

Figure 4.26. Comparison of the F9 fragment 2 (exon 2,3) translating nucleotide sequence between S4 and the reference sequence. *These alignment results confirm the complex variant detected in F9 fragment 2, as highlighted in green (c.250A>G) and yellow (c.251delC). The reference sequence was obtained from NCBI (NM_000133.4).*

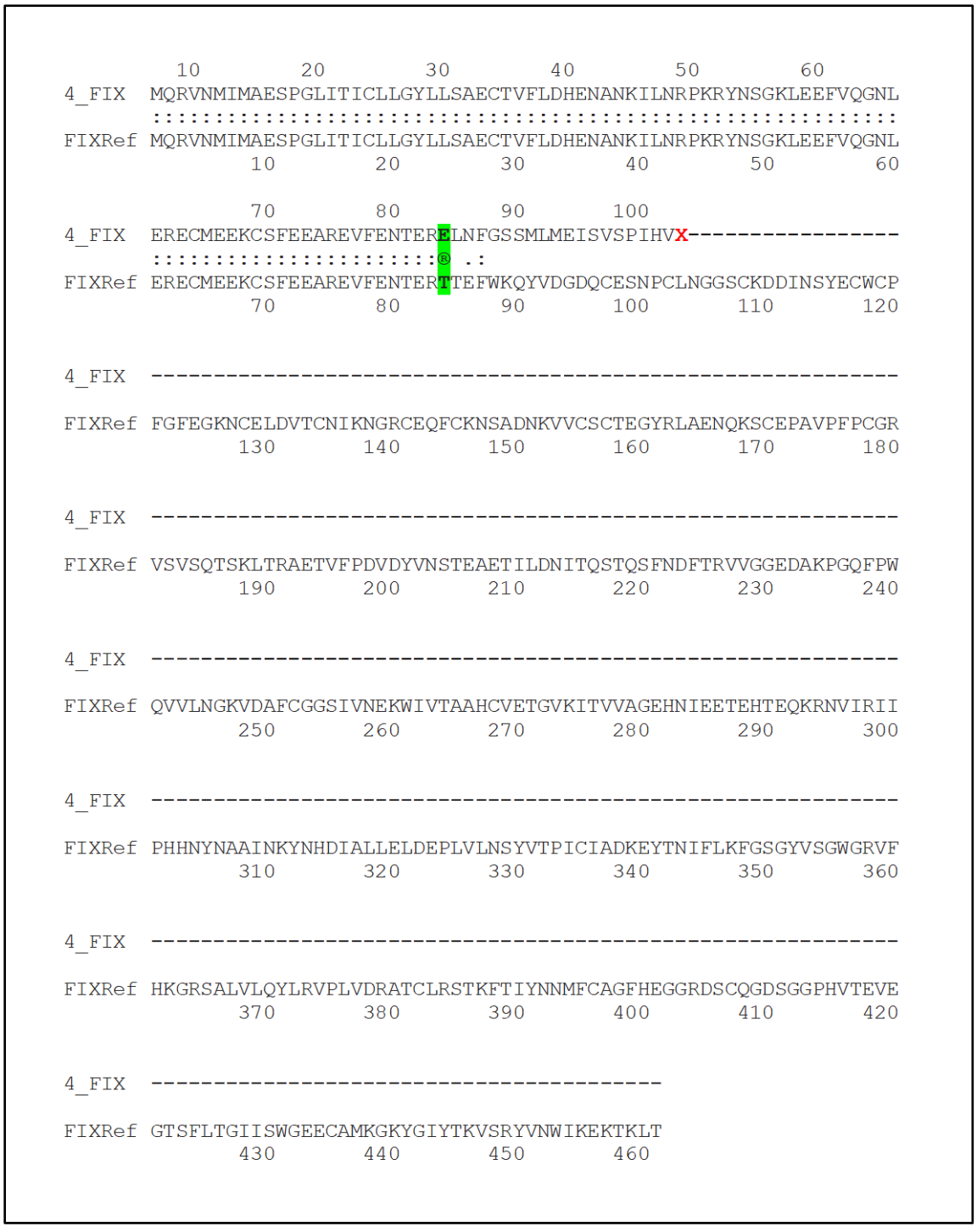

Figure 4.27 Alignment of the compromised translated FIX protein sequence for S4 and the FIX reference amino acid sequence. *The radical p.Thr^{84}Glufs*20 FIX amino acid variant is highlighted in yellow. *®: Radical amino acid change; X: Stop codon. The reference sequence was obtained from NCBI (NP_000124.1).*

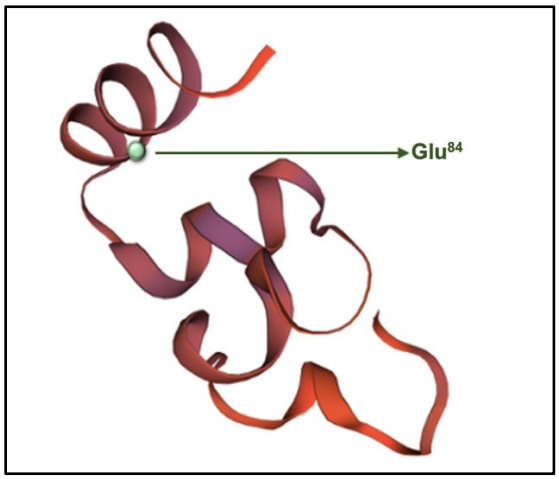

Figure 4.28. Depiction of the truncated FIX 3D structure (p. Thr^{84}Glufs*20).

Table 4.9. The combined categories of pathogenicity for the complex *F9* variant [(c.250A>G)(c.251delC)].

Categories	
c.250A>G	
PS1	Same amino acid change as a previously established pathogenic variant regardless of nucleotide change.
PS3	Well-established *in vitro* or *in vivo* functional studies supportive of a damaging effect on the gene or the gene product.
PM1	Located in a mutational hot spot and/or critical and well-established functional domain (e.g., active site of an enzyme) without benign variation.
PP2	Missense variant in a gene that has a low rate of benign missense variation and in which missense variants are a common mechanism of disease.
PP3	Multiple lines of computational evidence support a deleterious effect on the gene or the gene product (conservation, evolutionary, splicing impact, etc.).
c.251delC	
PVS1	Null variant (nonsense, **frameshift**, canonical ± 1 or 2 spice sites, initiation codon, single or multiexon deletion) in gene where LOF is a known mechanism of disease.
PS3	Well-established *in vitro* or *in vivo* functional studies supportive of a damaging effect on the gene or the gene product.
PM1	Located in a mutational hot spot and/or critical and well-established functional domain (e.g., active site of an enzyme) without benign variation.
PM4	Protein length changes as a result of in-frame deletions/insertions in a non-repeat region or stop-loss variants.
PM6	Assumed novel, but without confirmation of paternity or maternity.
PP3	Multiple lines of computational evidence support a deleterious effect on the gene or the gene product (conservation, evolutionary, splicing impact, etc.).

LOF: Loss of function.

(Categories for classification of variant pathogenicity were copied and modified from Richards et al., (2015)).

4.3.3.4.2 c.580A>G;c.726delT (p.Val^{243}Phefs*2)

A second complex *F9* variant was detected in S1, which included a missense variant, c.580A>G, previously published by McGraw et al., (1985) and a novel *F9* deletion, c.726delT. At the time of recruitment, S1 presented with a severe level of FIX deficiency (FIX:C <0.6 IU/dL).

The missense variant is located in *F9* exon 6 and the novel deletion was detected in exon 7, at nucleotide position 580 and 726, respectively (Figure 4.29a and b). The A allele in the previously published missense variant (c.580A>G), which results in a conservative amino acid change of p.Thr^{194}Ala, within the AP domain of FIXa, has a minor allele frequency (MAF) of 0.298 in European Americans. MAF is defined as the frequency of the second most prevalent allele in a specific population (Hernandez et al., 2019). Currently, there is no data available on the frequency of this variant in an African population. Furthermore, the G allele, seen in the *F9* Malmö polymorphism, c.580G>A (p.Ala^{194}Thr), has a MAF of 0.32 (Jayandharan et al., 2003). However, according to a study published by Bezemer et al., (2009), the Malmö variant has not been reported to have any association with haemophilia B (Khan and Taj, 2019).

The combined effect of this complex variant results in the amino acid change (p.Val^{243}Phefs*2) within the EGF1 domain of the FIX protein, as depicted in Figure 4.30. Val^{243} is a buried residue with a surface accessibility of zero and is located within a beta strand of the FIXa molecule. Furthermore, by using the online SWISS-MODEL online building software, a prediction of the 3D ribbon structure for the FIX protein was created using compromised FIX amino acid sequence and its reference sequence (Figure 4.31). Table 4.10 summarises the ACMG categories for pathogenic sequence variants. The combined criteria for pathogenic classification predicted this complex *F9* variant [(c.580A>G)(c.726delT)] to be **pathogenic**. According to previous publications, the c.580A>G variant is associated with a mild haemophilia B and classified as “benign” (Goodeve, 2015) and “likely benign” (Kulkarni et al., 2021). Therefore, we can hypostasise that the severe phenotype observed in this study might be due to the novel deletion,

resulting in a radical amino acid change (Tyr^{243}Phe) rather than the conservative amino acid variant (Thr^{194}Ala).

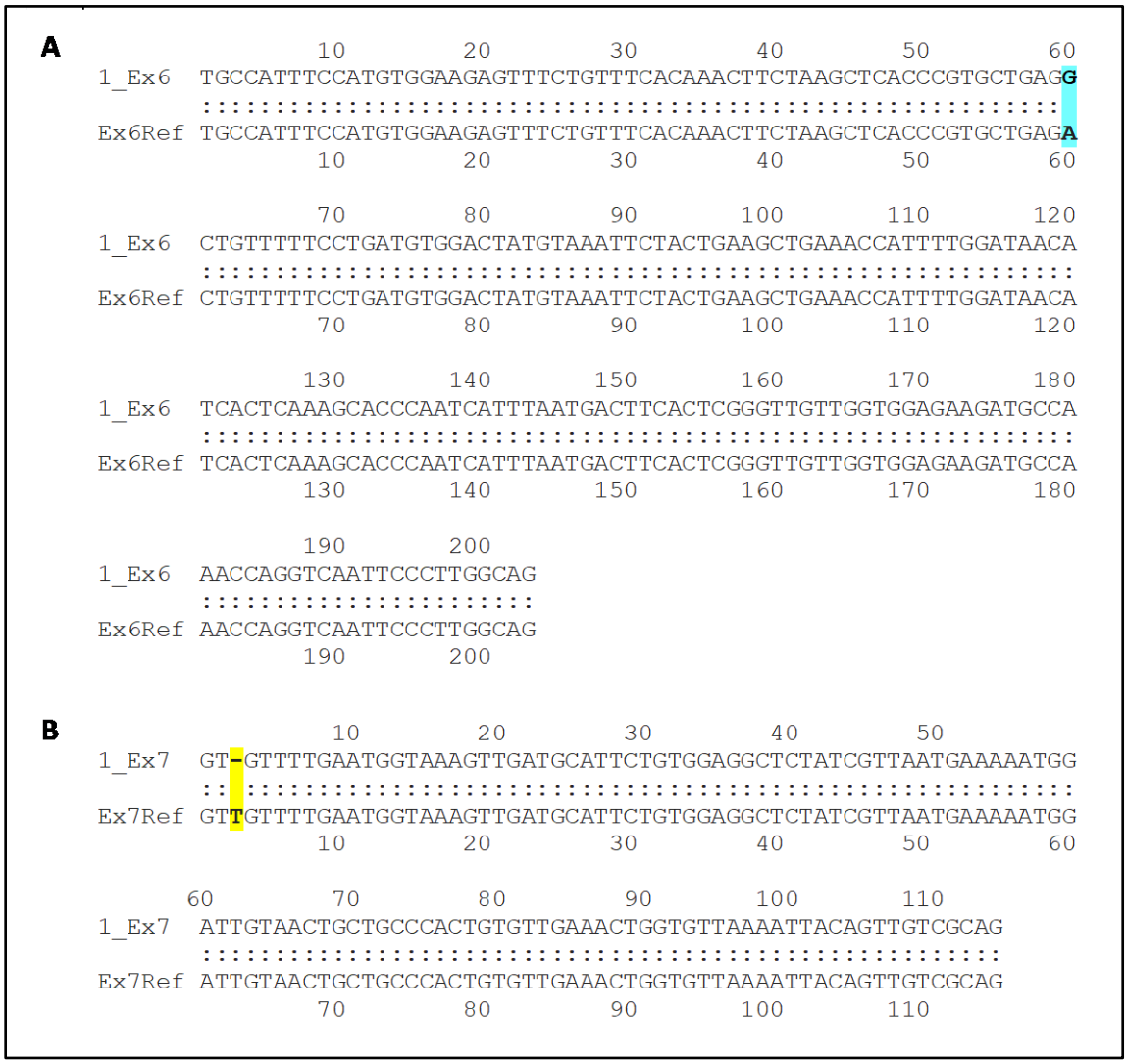

Figure 4.29. Comparison of the F9 (A) exon 6 and (B) exon 7 translating nucleotide sequence between S1 and the reference sequence (NM_000133.4)*. These alignment results confirm the complex variant detected in F9 exon 6 (c.580A>G) and 7 (726delA), as highlighted in turquoise and yellow, respectively. The reference sequence was obtained from NCBI (NM_000133.4).*

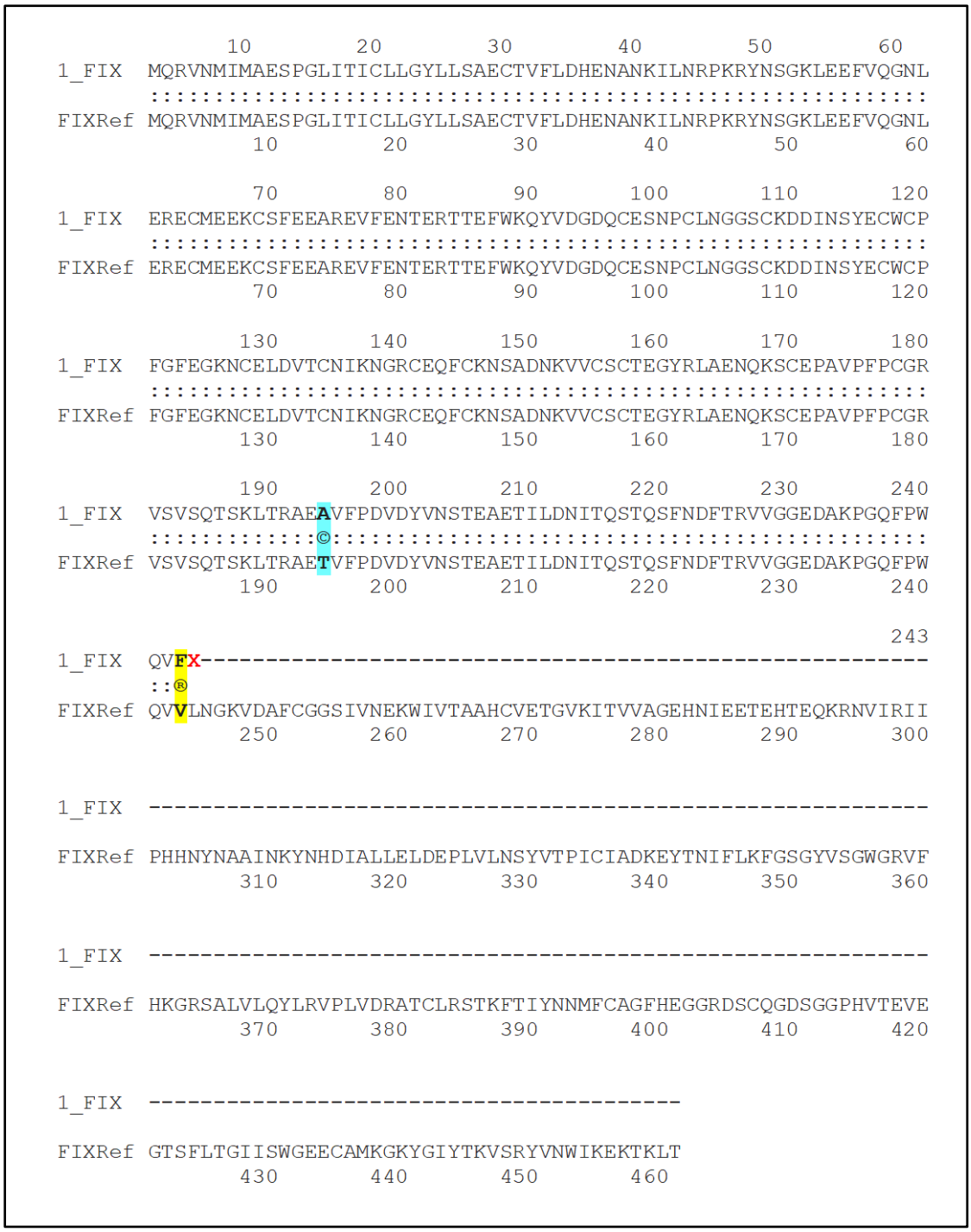

Figure 4.30. Depiction of the compromised translated FIX protein sequence for S1 and the FIX reference amino acid sequence. *conservative amino acid variant (p.Thr^{194}Ala), caused by the missense F9 variant is highlighted in turquoise. The radical p.Val^{243}Phefs*2 amino acid variant (highlighted in yellow), is followed by a stop codon, indicated in red. *©: Conservative amino acid change; ®: Radical amino acid change; X: Stop codon. The reference sequence was obtained from NCBI (NP_000124.1).*

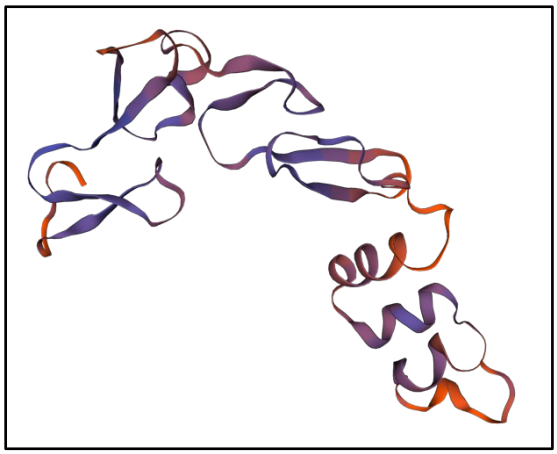

Figure 4.31. Depiction of the truncated FIX 3D structure (p.Val^{243}Phefs*2).

Table 4.10. The combined categories of pathogenicity for the complex *F9* variant [(c.580A>G);(c.726delT)].

Categories	
c.580A>G	
PS1	Same amino acid change as a previously established pathogenic variant regardless of nucleotide change.
PS3	Well-established *in vitro* or *in vivo* functional studies supportive of a damaging effect on the gene or the gene product.
PM1	Located in a mutational hot spot and/or critical and well-established functional domain (e.g., active site of an enzyme) without benign variation.
PP2	Missense variant in a gene that has a low rate of benign missense variation and in which missense variants are a common mechanism of disease.
PP3	Multiple lines of computational evidence support a deleterious effect on the gene or the gene product (conservation, evolutionary, splicing impact, etc.).
BA1	Allele frequency is >5% in Exome Sequencing Project, 1000 Genomes Project or Exome Aggregation Consortium. *GnomAD exomes allele frequency = 0.217 is greater than the 0.05 threshold.*
BP6	Reputable source recently reports variant as benign, but the evidence is not available to the laboratory to perform an independent evaluation.
c.251delC	
PVS1	Null variant (nonsense, **frameshift**, canonical ± 1 or 2 spice sites, initiation codon, single or multiexon deletion) in gene where LOF is a known mechanism of disease.
PS3	Well-established *in vitro* or *in vivo* functional studies supportive of a damaging effect on the gene or the gene product.
PM2	Absent from controls in Exome Sequencing Project, 1000 Genome Project, or Exome Aggregation Consortium.
PM4	Protein length changes as a result of in-frame deletions/insertions in a non-repeat region or stop-loss variants.
PM6	Assumed novel, but without confirmation of paternity or maternity.

LOF: Loss of function.

(Categories for classification of variant pathogenicity were copied and modified from Richards et al., (2015)).

4.4 Functional analysis

The clinical data (lowest FIX level on file, previous bleeding episodes and current treatment) for each study participant are summarised in Table 4.11. Unfortunately, an accurate annual bleeding rate could not be established.

Table 4.11. Summary of the functional data of S1 - S21 at the time of recruitment.

Sample	Adult/ Minor	Previous bleeding event(s)	Current FIX Replacement treatment	FIX plasma level	FIX Inhibitors	FIX Inhibitor titre	Severity
S1	Adult	Spontaneous bleed in right elbow (target joint) and shoulder. Bleeds in both knees, with severe bilateral knee deformities.	Prophylaxis 1500 IU weekly	0.6 IU/dL	Negative	N/A	Severe
S2	Adult	Bleeding events in left elbow (target joint), as well as the left knee and ankle.	Episodic 1500 IU	0.7 IU/dL	Negative	N/A	Severe
S3	Adult	Intracranial bleed as a child, resulting in slow intellectual development. Severe bleed in right elbow.	Episodic 2000 IU	0.6 IU/dL	Negative	N/A	Severe
S4	Adult	Bleeding events in right elbow and wrist, resulting in difficulty to use hand. Bleeds in both knees and presenting with chronic swelling of both knees. Multiple nasal bleeds.	Episodic 1500 IU	<0.3 IU/dL	Positive	21.50 NBU	Severe
S5	Minor	Bleed in right forearm and received on-demand. Thereafter, patient started prophylaxis treatment and had no bleeds since.	Prophylaxis 1500 IU weekly	<1.0 IU/dL	Negative	N/A	Severe
S6	Minor	Experienced bleeding events in right upper arm, left thigh (more than once), right knee and right ankle (more than once and received 5000 IU FIX on demand after last bleed). Patient also experienced heavy bleeding after a tooth came loose.	Episodic 2000 IU	<0.4 IU/dL	Negative	N/A	Severe
S7	Minor	No major bleeding events. One bleed in right knee, resulting in a small bruise. Small haematoma on scalp.	Prophylaxis 500 IU weekly	<1.0 IU/dL	Negative	N/A	Severe
S8	Minor	Major bleed in right knee (target joint), which is very swollen and patient presents with chronic synovitis. Bleed in the right elbow.	Prophylaxis 1000 IU weekly	<1.0 IU/dL	Negative	N/A	Severe
S9	Minor	Bleed in left knee (target joint) and patient presents with chronic swollen knee.	Prophylaxis 1000 IU 2x/week	<0.4 IU/dL	Negative	N/A	Severe
S10	Minor	Multiple bleeding events in right knee (target joint). One bleed in left elbow and left ankle.	Prophylaxis 2000 IU 2x/week	<0.6 IU/dL	Negative	N/A	Severe
S11	Minor	Multiple nose bleeds. No other information available in patient file.	Episodic 1000 IU	0.9 IU/dL	*Unknown*	N/A	Severe
S12	Minor	Two bleeds in right shoulder (target joint) and shoulder is swollen and patient experiences chronic pain in joint. One bleeding event in left shoulder (swollen and painful). One bleed in right knee (swollen). Patient also presents with osteoarthropathy and previous intra-articular rifampicin.	Episodic 1200 IU (target joint) 1000 IU (other bleeding events)	0.6 IU/dL	Negative	N/A	Severe
S13	Minor	No previous bleeding events. Patient is a carrier.	N/A	*Unknown*	*Unknown*	N/A	*Unknown*
S14	Adult	Bleeding event in left knee. Patient complains about a chronic feeling of numbness in left leg.	Prophylaxis 1500 2x/week +400 IU for Episodic use	0.5 IU/dL	Negative	N/A	Severe
S15	Minor	*Unknown*	Prophylaxis 1000 IU 2x/week	0.8 IU/dL	Negative	N/A	Severe
S16	Minor	No previous bleeding events (newborn).	*Unknown*	<0.3 IU/dL	*Unknown*	*Unknown*	Severe
S17	Adult	No previous bleeding events. Patient is a carrier of haemophilia B.	N/A	68.4 IU/dL	*Unknown*	N/A	Carrier
S18	Minor	Bleeding event on head when baby was injured during a caesarean section.	*Unknown*	1.8 IU/dL	*Unknown*	N/A	Moderate
S19	Adult	No previous bleeding events. Patient is a carrier of haemophilia B.	N/A	*Unknown*	*Unknown*	N/A	*Unknown*
S20	Minor	Bleed in the left buttock due to injury during soccer game. Multiple bleeding events in the right shoulder (target joint). Overall, bleeding is well controlled.	Prophylaxis 1000 IU weekly	<0.4 IU/dL	Negative	N/A	Severe
S21	Adult	Multiple bleeding events in left elbow (target joint) and patient presents with haematoma. Bleed in left shoulder, left elbow, left hip, left groin (1500 IU FIX received afterwards), and left thumb (chronic swelling). Patient also experienced a mild intracranial bleed as a child, as well as a iliopsoas bleed.	Episodic 500 IU	<0.3 IU/dL	Negative	N/A	Severe

4.4.1 One-stage FIX assay

The one-stage assay FIX levels determined for each sample at the time of recruitment (this level may differ from the lowest level on file, due to treatment), are summarised in Table 4.12. For the people with haemophilia B, the one-stage FIX levels ranged from <0.4% to 18.8%. The lower limit of detection of the one-stage FIX assay determined on the Siemens Automated Coagulation Analyser Model CS-2100i is 0.4%. Thus, all levels lower than this cut-off value were reported as <0.4%.

For possible carriers, the one-stage FIX results ranged from 49.3 IU/dL to 93.3 IU/dL. Based the modified classification communicated by the ISTH, participant S19, with a FIX level of 49.3% and no history of previous bleeding events, could be classified as an asymptomatic carrier of haemophilia B. However, given that this result is close to the upper limit of the woman with mild haemophilia range (5 – 40 IU/dL), and taking biological and intra-assay variation into consideration, it is recommended to repeat the test on a fresh sample to confirm the results. We did not repeat the test, as the objective of this part of the study was not to make a diagnosis, but to compare the one-stage and ELISA methods.

It was determined that the one participant who was classified with moderate haemophilia (S18, FIX:C 1.8 IU/dL) had a one-stage FIX level of 0.4 IU/dL. Therefore, the participant was re-classified with severe haemophilia B. When further evaluating the specific case, the original result was derived from a sample taken shortly after birth, as the mother was a known carrier of haemophilia B. The sample for this study was taken a few days later, before any FIX replacement therapy was initiated. Taken that the variant detected in both the mother and baby was detected in other study participants with severe haemophilia B, we can hypothesise that the slightly elevated FIX level detected shortly after birth might be due to residual FIX derived from the mother, and that our lower result is more indicative of the endogenous FIX of the baby. Nonetheless, it is recommended to re-evaluate FIX levels three to six months after birth to confirm the haemophilia diagnosis (Indiana Hemophilia and Thrombosis Center, 2022). All subsequent results of this specific case were much higher than both

the FIX level at birth and the FIX level obtained in this study, as the baby was placed on prophylactic FIX replacement treatment.

4.4.2 FIX ELISA assay

The ELISA assay standard curve is depicted in Figure 4.32. The four-parameter standard curve, using regression analysis, ranged from 0.78 ng/mL to 50 ng/mL. The FIX ELISA assay results of the possible carriers ranged from 28.4 IU/dL to 74.3 IU/dL. Based on the ISTH classification, and the ELISA results, participant S13 can be classified as a girl with mild haemophilia B. The ELISA assay does not measure the levels of functional FIX, therefore, intra-assay variance should again be considered for appropriate classification.

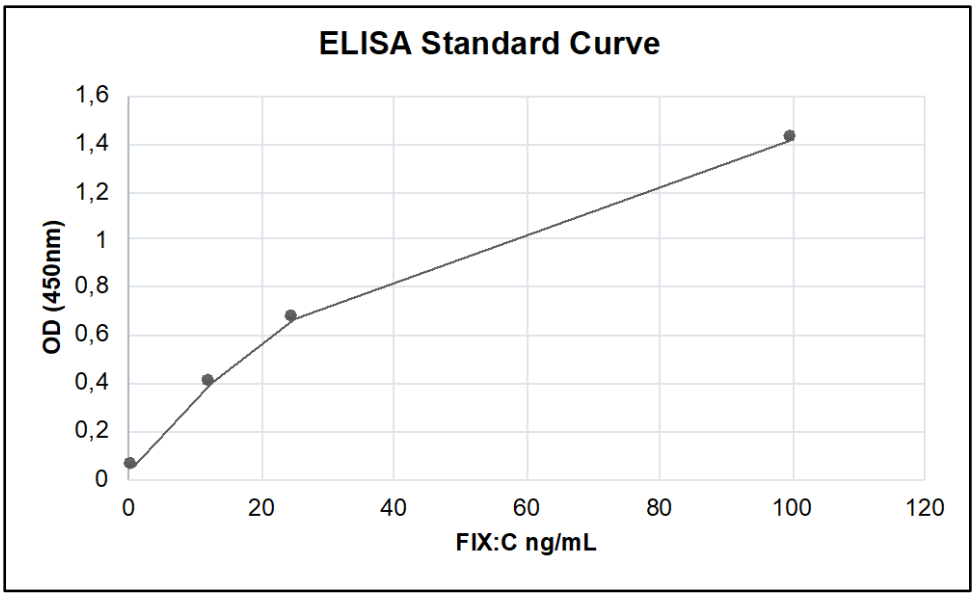

Figure 4.32. Standard curve for the FIX ELISA assay. **ELISA: Enzyme-linked immunosorbent assay; FIX:C: Plasma concentration of FIX coagulant activity; OD: Optical density.*

Table 4.12. FIX level analysis using two different detection methods.

	FIX Plasma level (IU/dL)		
Study Participant	Lowest on file	One stage	ELISA
S1	0.6	<0.4	0.7
S2	0.7	2.7	0.1
S3	0.6	2.0	2.0
S4	<0.3	<0.4	0.3
S5	<1	3.6	2.9
S6	<0.4	2.7	1.3
S7	<1	5.1	4.0
S8	<1	<0.4	0.9
S9	<0.4	18.8	11.1
S10	0.6	1.4	0.9
S11	0.9	<0.4	0.8
S12	0.6	13.4	12.2
S13	-	93.3	28.4
S14	0.5	0.7	0.1
S15	0.8	<0.4	46.3
S16	<0.3	-	-
S17	68.4	54.5	74.2
S18	1.8	<0.4	1.2
S19	-	49.3	57.0
S20	<0.4	<0.4	0.2
S21	<0.3	<0.4	0.4

4.4.3 Discrepancies between the FIX one-stage and ELISA assay

The FIX one-stage assay measures the functional levels of FIX based on its ability to form a blood clot, whereas the ELISA assay is not a functional assessment tool as it only measures the actual quantity of FIX (Miller, 2018). The statistical difference between the two FIX:C assays was not significant (p = 0.473). However, 45% of the participants (S1, S2, S6, S8, S11, S13, S14, S15 and S18) demonstrated classic inter-assay discrepancy, defined by having a FIX one-stage/ELISA ratio >2.0 (Kihlberg et al., 2017) (Table 4.13). Another noteworthy finding is that for participant S15, the ELISA method determined the FIX level at near normal levels of 46.3 IU/dL. This level does not correlate clinically to the bleeding phenotype and history of this participant, with the one-stage level of <0.4 IU/dL being a more accurate reflection of the phenotype. Therefore, taken together with the inter-assay discrepancy, we believe that the ELISA method is not a useful tool in haemophilia B diagnostics, and that the one-stage method remains the gold-standard for FIX level detection.

It must be noted that the results determined in this study are expected to differ from the lowest FIX levels reported in the medical records of the participants, as the participants may be at different time points regarding post-FIX replacement therapy. Therefore, the purpose of this part of our study was not to determine the severity level of the participants, but to determine if the two detection methods are comparable. Furthermore, the comparison was also to identify any genetic variant that may only have an effect on the functionality of the FIX, and not the physical quantity of the protein.

Table 4.13. FIX:C one-stage/ELISA assay discrepancies.

		FIX:C (IU/dL)		
Sample	**FIX Replacement treatment**	**One-stage**	**ELISA**	**One-stage/ELISA ratio >2.0**
S1	Prophylaxis: 1000 IU/week	<0.4	**0.7**	1 : **>2**
S2†	Episodic: 1500 IU	**2.7**	0.1	**27** : 1
S6*	Episodic: 2000 IU	**2.7**	1.3	**2.1** : 1
S8†	Prophylaxis: 1000 IU/2x/week	**<0.4**	**0.9**	1 : **>2.25**
S11	Unknown	<0.4	**0.8**	1 : **>2**
S13†	**Unknown**	**93.3**	28.4	**~3.29** : 1
S14*	Prophylaxis: 1500 IU/2x/week	**0.7**	0.1	**7** : 1
S15	Prophylaxis: 1000 IU/2x/week	**<0.4**	**46.3**	>1 : **>115.75**
S18*	Unknown	<0.4	**1.2**	>1 : **3**

†***Nonsense variant (c.721C>T) identified; *Deletion (c.311delA) identified***

4.5 Mutational and functional relationship

For an appropriate and comprehensive analysis of the mutational and functional relationship, a comprehensive approach was followed which included the patients' medical history, genetic variant status, and functional analysis.

The previously described missense variant, c.1217C>T (p.Ser^{406}Leu), identified in the serine protease domain of FIXa, was found in S5 and S10, who both presented with severe FIX deficiency at the time of recruitment. This correlates with the previous published data regarding this missense variant (Bicocchi et al., 2006; Chen et al., 1991; Costa et al., 2000; Ghanem et al., 1993; Saad et al., 1994). However, both individuals revealed a higher FIX:C deficiency level for the one-stage and ELISA assay (Table 4.12), which is evident of the prophylactic replacement received by both participants twice a week.

The previously described nonsense variant, c.721C>T (pGlu^{241}*) was identified in six study participants (S2, S7, S8, S9, S12, S13). All the related individuals, except S13 (carrier), are receiving prophylactic replacement therapy of 1,000 IU twice a week, whereas S2 is being administered episodic FIX replacement therapy of 1,500 IU. Based on the data presented in Table 4.13, S8 and S13 revealed discrepancies between the one-stage and ELISA FIX assays. S8 was the only participant with this nonsense variant, who revealed a higher FIX:C for the ELISA than the one-stage assay. We hypothesised that a potential reason for the higher FIX levels observed with the ELISA assay could be the presence of interfering substances in S8. These interfering substances may affect the reliability of the one-stage assay, resulting in a decreased FIX level detected. However, it is also possible that the ELISA assay might be less susceptible to these substances, resulting in an artificially higher level of FIX being detected. Further investigations in required to identify these interfering factors present in the patient sample to evaluate the impact on the ELISA assay. These investigations may involve conducting additional test, for example, diluting the sample or performing alternative quantification assays to confirm the discrepancy observed between the ELISA and one-stage assay, as well as determine the underlying cause. When evaluating the combined analysis for the variant-phenotypic relationship of

these six participants (Table 4.14), our data correlates with the published data (Onay et al., 2003). Looking at the FIX:C and inhibitor development post-treatment, these findings also suggest that there is no significant difference between the FIX:C levels and inhibitor formation regardless of whether a patient received episodic or prophylactic replacement therapy. Nonetheless, since various other environmental and epigenetic factors may contribute to the formation of inhibitors, these results should be interpreted with caution. However, given the rarity of inhibitor development in individuals with haemophilia B, the limited size of the study population presents challenges in conducting feasible investigations into immunogenicity.

A novel deletion, c.311delA (p.Asn^{104}Metfs*31), within the EGF1 domain was detected in four study participants. Ultimately, three of the participants (S6, S14 and S18) had severe FIX deficiency (FIX:C <1 IU/dL) and S19, the mother of S18, was diagnosed as a potential haemophilia B carrier, since no FIX:C data was available at the time of commencing the study. S18 presented with a moderate FIX:C level of 1.8 IU/dL at the time of recruitment, as well as for the ELISA assay (FIX:C 1.2 IU/dL) (Table 4.12). Both related individuals (S18 and S19), as highlighted in Table 4.14, revealed a higher level of functional FIX:C levels measured by quantitative ELISA assay compared to the one-stage assay. The one-stage assay revealed a severe FIX deficiency for S18. However, S6, who was receiving episodic replacement therapy of 2,000 IU at the time of a bleeding episode, presented a higher level of FIX for the one-stage assay than with the ELISA. With reference to Table 4.13., discrepancies were identified between the one-stage and ELISA FIX assay, with a FIX one-stage/ELISA ratio of >2, for both S6 and S18. Furthermore, to conclude what the effect of replacement therapy is on these individuals is limited to the variant-phenotypic relationship documented for S6 (Table 4.14), since no treatment data for S18 and S19 were available at the time of recruitment.

A previously described deletion, c.1178_1180delACA (p.Asn^{393}del) (Belvini et al., 2005; Miller et al., 2012; Saad et al., 1994), located in the serine protease domain of FIXa, was identified in three study participants. At the time of recruitment, correlating the previous published data, two individuals (S11 and S16) were diagnosed with

severe haemophilia B. Additionally, the functional FIX one-stage assay for S11, with an unknown treatment history, also revealed a severe FIX deficiency. Due to the lack of treatment data for S11 and S16, a complete understanding regarding this variant's clinical presentation for each individual, was inconclusive. However, the data we do have correlates with previously published data (Belvini et al., 2005; Miller et al., 2012; Saad et al., 1994), where severe haemophilia B is described. Based on the lowest FIX level on file, the one-stage and ELISA FIX assay results, S17 is classified as a carrier of haemophilia B.

Another novel *F9* deletion c.1376del A (p.Lys^{459}Serfs*24), also detected in the serine protease domain, was found in three study participants (S15, S20 and S21). All three participants presented with severe haemophilia B at the time of recruitment. However, only S20 and S21, who are related, as shown in Table 4.14, presented with severe FIX deficiency using the one-stage and ELISA FIX assay. However, the ELISA assay for S15 revealed a normal FIX:C plasma level of 46.3 IU/dL but a severe level of deficiency (FIX:C <0.4) using the functional one-stage assay. The discrepancy in results may indicate that the amino acid change may destabilise the protein in such a manner, that in some cases (such as S15) the epitope for the anti-FIX antibody used in the ELISA assay may become exposed, resulting in an elevated estimation of the non-functional protein levels. This hypothesis can be tested in future by performing more in-depth protein structure crystallography. S15 and S20 are both treated with FIX prophylaxis (1,000 IU per week), whereas S21 receives episodic replacement (1,500 IU) treatment at the time of bleeding episodes.

A complex variant [(c.250A>G; c.251delC) (p.Thr^{84}Glufs*2)], located in the EGF1 domain of FIXa, was identified in S4, the only study participant who presented with FIX inhibitors against FIX replacement treatment (episodic), summarised in Table 4.14. The level of haemophilia B severity presented by S4 correlates with a previously published case for the missense variant (Wulff et al., 1999). However, the data published by Wulff et al. (1999), did not present with FIX inhibitors against the replacement therapy. Therefore, we can predict that the additional, novel deletion (c.251delC) may cause further instability to the FIXa molecule, resulting in a more

identifiable protein, and consequently the risk of eliciting an immune response with resultant inhibitor development. No discrepancy was identified between the one-stage and ELISA FIX assays for the measurement of FIX levels in this participant.

A second complex variant [(c.580A>G; c.726delT) (p.Val^{243}Phefs*2)], also containing a known missense variant, c.580A>G (resulting in a conservative amino acid change), and a novel deletion (c.726delT), was detected in S1 (Figure 4.30). Despite the FIX prophylaxis of 1,000 IU per week, FIX:C levels at the time of recruitment as well as the results obtained with the FIX one-stage and ELISA assay, revealed a severe level of FIX:C deficiency. The results for this missense variant and the corresponding level of severity, correlate with the data published by McGraw et al. (1998).

The genotype-phenotype results suggested that no variant consistently resulted in more or less bleeding episodes. However, as an accurate historic annual bleeding rate could not be established for all participants, this finding would need to be confirmed in another study. Unfortunately, the historic annual bleed rate was not available or complete for all participants, thus, these results need to be interpreted with caution, as a definite conclusion cannot be made. Nonetheless, it is evident that all of these variants are associated with a severe bleeding history, when present in males. Furthermore, it was encouraging to find that none of the carriers presented with a history of excessive bleeding, indicating that the presence of at least one wild-type *F9* allele is sufficient to maintain the haemostatic balance in women. The variants also had a diverse range of treatment options used, with no variant having a clear preference for a specific treatment modality. Bi-weekly prophylaxis remained the treatment of choice (9/18), closely followed by episodic treatment (7/18). It is also apparent that the variants could not predict a possible discrepancy between the one-stage functional FIX levels and the physical protein levels as determined by the ELISA method.

The low incidence of inhibitors in our cohort also indicates that inhibitor formation in people with haemophilia B may not be as significant a challenge as seen in people with haemophilia A, possibly due to the much smaller FIX protein compared to the

FVIII protein. However, taken that the only participant who had inhibitors had a complex variant, we hypothesise that the presence of more complex genetic variants in the *F9* gene may serve as a positive predictor for FIX inhibitor formation. However, future studies are required to confirm this, as there are numerous risk factors, not related to *F9*, that can cause inhibitor development. Thus, we recommend that an even more cautious treatment approach should be followed in patients where a complex variant has been detected. This further illustrates the vital role genetic testing may play in the management of people with haemophilia.

Table 4.14. Summary of genotypic and phenotypic data.

F9 variant					FIX Plasma level (IU/dL)				
c.	p.	Novel	FIX domain	Sample	Lowest on file	One-stage	ELISA	Inhibitors	Related individuals
c.311delA	p.Asn^{104}Metfs*31	Yes	EGF1	S6	<0.4	2.7	1.3	-	
				S14	0.5	0.7	0.1	-	
				S18	1.8	<0.4	1.2	-	S18 and S19
				S19	-	49.3	57.0	-	
c.262_364delAT	p.Phe^{121}Leufs*3	Yes	EGF1	S3	0.6	2.0	2.0	-	
c.721C>T	p.Glu^{241}*	No	Serine Protease	S2	0.7	2.7	0.1	-	
				S7	<1	5.1	4.0	-	S7, S8, S9, S12 and S13
				S8	<1	<0.4	0.9	-	
				S9	<0.4	18.8	11.1	-	
				S12	0.6	13.4	12.2	-	
				S13	-	93.3	28.4	-	
c.1178_1180delACA	p.Asn^{393}del	No	Serine Protease	S11	0.9	<0.4	0.8	-	
				S16	<0.3	-	-	-	S16 and S17
				S17	68.4	54.5	74.2	-	
c.1217C>T	p.Ser^{406}Leu	No	Serine Protease	S5	<1	3.6	2.9	-	
				S10	0.6	1.4	0.9	-	
c.1376delA	p.Lys^{459}Serfs*24	Yes	-	S15	0.8	<0.4	46.3	-	
				S20	<0.4	<0.4	0.2	-	S20 and S21
				S21	<0.3	<0.4	0.4	-	
c.250A>G c.251delC	p.Thr84Glufs*20	No Yes	GLA	S4	<0.3	<0.4	0.3	Positive 21.5 BU	
c.580A>G c.726delT	p.Val^{243}Phefs*2	No Yes	Linker Serine Protease	S1	0.6	<0.4	0.7	-	

CHAPTER 5: CONCLUSION

Haemophilia B (Christmas disease), an X-linked recessive coagulation disorder, is characterised by the deficiency of coagulation FIX, caused by a broad spectrum of *F9* gene variants. FIX, the largest vitamin K-dependent protein synthesised by the liver, is translated from the *F9* gene which is located on chromosome Xq27.1. Haemophilia B is caused by a heterogeneous spectrum of pathogenic variants distributed throughout the coding and non-coding regions of *F9.* According to the EAHAD-CPDB, more than 1,200 unique pathogenic *F9* variants have been identified in 4,713 respective haemophilia B cases. Despite the extensive heterogeneity of haemophilia B, at the time of writing, no routine genetic testing to identify causative *F9* variants within a South Africa population was available. Needless to say, very limited genotype-phenotype data have been published in our population. The justification to perform this study arose from a South African publication by Stones and McGill (2000), highlighting the importance of genetic testing for a more comprehensive clinical diagnosis, as well as prediction of inhibitor development, and improved patient management (Stones and McGill, 2000).

Therefore, it is vital to determine whether there is a link between certain *F9* variants and inhibitor development and consequently anaphylaxis. The detection of known and novel *F9* variants is an important aspect in assisting with the improvement of haemophilia B diagnosis, prediction of inhibitor development against FIX, as well as improved treatment models and patient management. Additionally, the ability to identify pathogenic variants can potentially enhance the undertaking of prenatal diagnosis and diagnosis of carriers and women/girls with haemophilia B (Salviato et al., 2019).

In this study, we optimised a *F9* variant detection method, and subsequently, successfully screened 21 study participants. We thereby identified eight pathologic *F9* variants, distributed throughout the FIX protein domains, with no mutational hotspot recognised, which is consistent with findings described in literature (Radic et al., 2013;

McVay et al., 2020). We detected previously published variants including the missense variants c.1217 (p.Ser^{406}Leu) and c.580A>G (p.Thr^{194}Ala), the nonsense variant c.721C>T (p.Glu^{241}*), and the deletion c.1178_1180delACA (p.Asn^{393}del). Additionally, we detected five novel variants including the deletions c.311delA (p.Asn^{104}Metfs*31), c.363_364delAT (p.Phe^{121}Leufs*3), c.1376delA (p.Lys^{459}Serfs*24), 726delT (p.Val^{243}Phefs*2), and c.251delC (Thr^{84}Glufs*20).

Two complex *F9* variants were identified in our study population. The complex variant included one existing missense variant (c.580A>G) and an additional novel deletion (726delT), which resulted in a radical amino acid change, p.Val^{243}Phefs*2. We also confirmed the detection of a complex variant, containing one known missense variant (c.250A>G) as well as a novel deletion (c.251delC) and consequently, a radical amino acid change, Thr^{84}Glufs*20.

Based on the ACMG criteria for the classification of sequence variants, in combination with the online VarSome software, all of these confirmed radical *F9* variants were predicted to be pathogenic. Interestingly, the previously published missense variant detected in S4 (c.250A>G), who was the only individual who presented with the presence of FIX inhibitors, was not previously associated with inhibitor development. Therefore, the additional novel deletion (c.251delC) is a very exciting finding, as it might be a potential predictor of inhibitor development against FIX replacement therapy. This finding highlights the importance of genetic testing for appropriate patient management of individuals diagnosed with haemophilia B.

Some major discrepancies were found between the one-stage and the ELISA FIX assays. The ELISA assay FIX levels, which were highly elevated in comparison to the one-stage assay in some cases, and even came close to the borderline of normal FIX levels, did not correlate with the bleeding phenotype in these individuals. Therefore, taking all discrepancies between the one-stage FIX and the ELISA assay into account, it was concluded that, in our setting, the ELISA assay is not suitable for the measurement of FIX:C in the process of diagnosing haemophilia B. As a result, we confirm that the one-stage FIX assay should remain the gold standard and the method

of choice in laboratories performing routine haemophilia diagnostics. However, it must be noted that a chromogenic FIX assay may be a valuable diagnostic addition when one-stage results and phenotypic presentation do not correlate.

To conclude, the fact that we identified five novel variants in our study further confirms how vital genetic screening is in our population. It emphasises how the African continent can contribute to a better understanding of haemophilia B. However, this can only be achieved when more directed studies are performed, and when genetic testing becomes more accessible to the population in developing countries.

5.1 Limitations and Impacts of the study

5.1.1 Limitations

The first limiting factor of this study was a small study population group. A larger study population would have aided in a comprehensive and accurate representation of haemophilia B in the larger South African context. Secondly, another limitation was, in some cases, the insufficient recordkeeping of clinical data, such as bleeding events and treatment history. This could have compromised the accuracy of the determination of a genotype/phenotype relationship for a study participant. In addition, most of the study participants were recruited during the restrictive time of the COVID-19 pandemic, resulting in certain patients not attending follow-up appointments. This could have contributed to a small study population, as well as precluding thorough documentation of clinical data. A further limitation of this study could be the lack of consistency in the recorded FIX level at the time of diagnosis.

5.1.2 Impacts and future research

In South Africa, there is currently no routine genetic testing done for haemophilia B diagnosis. The results of this study highlight the possible positive contribution of genetic testing to haemophilia B patient management. Furthermore, mutational and phenotypic data obtained, including the detection of *F9* novel variants, will contribute to the disorder-specific database and contribute to both the local and global understanding of haemophilia B.

We recommend that future studies be done to evaluate the specific impact of the novel variants on the FIX crystal structure. In addition, we recommend ongoing development and refinement of reliable genetic assays to establish efficient and cost-effective variant detection methods which can be applied on the routine diagnostic platform in future.

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APPENDICES

Appendix A: The Health Science Research Ethics Committee (HSREC) Approval Letter

UNIVERSITY OF THE FREE STATE
UNIVERSITEIT VAN DIE VRYSTAAT
YUNIVESITHI YA FREISTATA

UFS·UV
HEALTH SCIENCES
GESONDHEIDSWETENSKAPPE

Health Sciences Research Ethics Committee

06-Nov-2019

Dear **Ms Chené Bester**

Ethics Clearance: **Mutational analysis of a South African haemophilia B population.**
Principal Investigator: **Ms Chené Bester**
Department: **Haematology and Cell Biology Department (Bloemfontein Campus)**

APPLICATION APPROVED

Please ensure that you read the whole document

With reference to your application for ethical clearance with the Faculty of Health Sciences, I am pleased to inform you on behalf of the Health Sciences Research Ethics Committee that you have been granted ethical clearance for your project.

Your ethical clearance number, to be used in all correspondence is:**UFS-HSD2019/1569/2611**

The ethical clearance number is valid for research conducted for one year from issuance. Should you require more time to complete this research, please apply for an extension.

We request that any changes that may take place during the course of your research project be submitted to the HSREC for approval to ensure we are kept up to date with your progress and any ethical implications that may arise. This includes any serious adverse events and/or termination of the study.

A progress report should be submitted within one year of approval, and annually for long term studies. A final report should be submitted at the completion of the study.

The HSREC functions in compliance with, but not limited to, the following documents and guidelines: The SA National Health Act. No. 61 of 2003; Ethics in Health Research: Principles, Structures and Processes (2015); SA GCP(2006); Declaration of Helsinki; The Belmont Report; The US Office of Human Research Protections 45 CFR 461 (for non-exempt research with human participants conducted or supported by the US Department of Health and Human Services- (HHS), 21 CFR 50, 21 CFR 56; CIOMS; ICH-GCP-E6 Sections 1-4; The International Conference on Harmonization and Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH Tripartite), Guidelines of the SA Medicines Control Council as well as Laws and Regulations with regard to the Control of Medicines, Constitution of the HSREC of the Faculty of Health Sciences.

For any questions or concerns, please feel free to contact HSREC Administration: 051-4017794/5 or email EthicsFHS@ufs.ac.za.

Thank you for submitting this proposal for ethical clearance and we wish you every success with your research.

Yours Sincerely

Dr. SM Le Grange
Chair : Health Sciences Research Ethics Committee

Health Sciences Research Ethics Committee
Office of the Dean: Health Sciences
T: +27 (0)51 401 7795/7794 | E: ethicsfhs@ufs.ac.za
IRB 00006240; REC 230408-011; IORG0005187; FWA00012784
Block D, Dean's Division, Room D104 | P.O. Box/Posbus 339 (Internal Post Box G40) | Bloemfontein 9300 | South Africa
www.ufs.ac.za

Appendix B: Approval letters from the Free State Department of Health and the Northern Cape Department of Health

health
Department of Health
FREE STATE PROVINCE

28 October 2019

Dr C Bester
Dept. of Haematology and Cell Biology
UFS

Dear Dr C Bester

Subject: Mutational analysis of a South African haemophilia B population.

- Please ensure that you read the whole document, Permission is hereby granted for the above – mentioned research on the following conditions:
- Participation in the study must be voluntary.
- A written consent by each participant must be obtained.
- Serious Adverse events to be reported to the Free State department of health and/ or termination of the study
- Ascertain that your data collection exercise neither interferes with the day to day running of **Universitas Hospital** nor the performance of duties by the respondents or health care workers.
- Confidentiality of information will be ensured and please do not obtain information regarding the identity of the participants.
- **Research results and a complete report should be made available to the Free State Department of Health on completion of the study (a hard copy plus a soft copy).**
- Progress report must be presented not later than one year after approval of the project to the Ethics Committee of the University of the Free State and to Free State Department of Health.
- Any amendments, extension or other modifications to the protocol or investigators must be submitted to the Ethics Committee of the University of the Free State and to Free State Department of Health.
- **Conditions stated in your Ethical Approval letter should be adhered to and a final copy of the Ethics Clearance Certificate should be submitted to** sebeelats@fshealth.gov.za/ makenamr@fshealth.gov.za **before you commence with the study**
- No financial liability will be placed on the Free State Department of Health
- **Please discuss your study with Institution Manager on commencement for logistical arrangements see 2nd page for contact details.**
- Department of Health to be fully indemnified from any harm that participants and staff experiences in the study
- Researchers will be required to enter in to a formal agreement with the Free State department of health regulating and formalizing the research relationship (document will follow)
- **As part of feedback you will be required to present your study findings/results at the Free State Provincial health research day**

Trust you find the above in order.

Kind Regards

Dr D Motau
HEAD: HEALTH
Date: 28/10/2019

FREE STATE PROVINCIAL TREASURY
RECEIVED
29-10-2019
10h40
OFFICE OF THE HOD

Head : Health
PO Box 227, Bloemfontein, 9300
4th Floor, Executive Suite, Bophelo House, cnr Maitland and Harvey Road, Bloemfontein
Tel: (051) 408 1648 Fax: (051) 406 1556 e-mail khusenj@fshealth.gov.za/dfshealth.gov.za/chikobvup@fshealth.gov.za

www.fs.gov.za

DEPARTMENT OF HEALTH
LEFAPHA LA BOPHELO BO BOTLE
DEPARTEMENT VAN GESONDHEID
ISEBE LENKONZO ZENTLALONTLE

Robert Mangaliso
Sobukwe Hospital

Head Clinical Management: Medical

Du Toitspan Road
Private Bag X5021
Kimberley

Tel: 053 802 2147
Fax: 053 832 9435 /
086 617 4089

Reference / Tshupelo / Verwysings / Isalathiso :

Date / Leshupelo / Datum / Umhla : 21st October 2019 Dr H Saeed

TO: Ms C Bester

RE: Permission to do research

Permission is hereby granted to conduct a medical research project at Robert Mangaliso Sobukwe Hospital, title proposed: *"Mutational analysis of a South African haemophilia B population"*

Please submit proof of ethics clearance, before commencing with the research.
Kindly submit research protocol to the Northern Cape Provincial Health Research and Ethics Committee for approval.

Contact Details:
Dr E Worku
Email address: eworku@ncpg.gov.za

Mr Mashute
Email address: bmashute@ncpg.gov.za
Tel: (053) 8302134

Dr H Saeed
MBBS,H.Dip.Int.Med.(CMSA),M.Fam.Med.(UFS),
Specialist Family Physician, Affiliate Lecturer – UFS
Acting Head Clinical Management: Medical

21/10/19
Date:

Appendix C: Example of an informed consent form signed by adult study participants

INFORMED CONSENT FORM

Title of the research project: *Mutational analysis of a South African haemophilia B population.*

Principal investigator: **Chené Bester**
Address: **Department of Haematology and Cell Biology**
Faculty of Health Sciences
University of the Free State
Bloemfontein, 9301
Contact number: **071 628 3552**

To Project Participant:

A. PURPOSE AND BACKGROUND

You are invited to take part in a research project conducted in the Human Molecular Biology Unit at the University of the Free State, Bloemfontein, South Africa. We (Me Chené Bester, together with Dr Janse van Rensburg and Mr Kloppers) are planning to conduct a research project wherein we will attempt to detect mutations involved in haemophilia B and to establish how these mutations influence the functions of the blood clotting system.
Haemophilia B is a hereditary rare bleeding disorder that a person is born with and where a person's blood can't clot due to a deficiency or defect of an important protein that plays a role in blood clotting. This protein is known as coagulation factor IX (FIX). Mutations in the FIX gene may prevent certain functions or production of this protein, causing a person with haemophilia B to bleed for longer. Genes are what you inherit from your parents. It is important that these mutations are diagnosed as they may play a role in the treatment of the disorders.

B. PROCEDURES

We need (15 ml) of blood and your time involvement will approximately be five (5) minutes as the blood is drawn. There will be no further engagements, remunerations (payment for service) or costs involved.

C. RISKS

The risks should not exceed those that are normally expected in donating blood samples for scientific research. You may experience a slight degree of pain and discomfort during the procedure and adverse effects like a slight bruise or swelling at the site where the blood was drawn.
We do not expect any of these adverse medical effects to occur. However, if you do experience any medical problems, because of your participation, the necessary medical treatment will be provided.

D. CONFIDENTIALITY

Reports resulting from this study might in future be used for scientific publication purposes but will not identify you as a participant. All information gathered in this study will remain confidential and be given out only with your permission or as required by law. If you give us permission by signing this consent form, we will protect your confidentiality.

E. VOLUNTARY PARTICIPATION

Your decision whether to participate in this study is voluntary. If you choose not to participate, there will be no penalty or loss of benefits that you are entitled to. If you choose to participate in this study, you can withdraw your consent and discontinue participation at any time without prejudice.

F. QUESTIONS

If you have any questions about this research at any time, please call Ms Chené Bester (**071 628 3552**). You may contact the Secretariat of the Health Sciences Research Ethics Committee of the Faculty of Health Sciences, University of the Free State at telephone number **0514052812** if you have questions regarding your right as a participant in this research project.

G. CONSENT

By signing this consent form, you indicate that you have read the form and agree that you voluntarily participate in the study. If you choose not to participate in this study there will be no penalty or loss of benefits to which you are entitled to. If you agree to take part, you are free to withdraw your consent from it at any time. Likewise, no penalty or loss of benefits to which you are otherwise entitled to will occur.
The research study, including the above mentioned information has been verbally described to me. I agree to voluntarily participate in the study, as set out above.

Signed at (*place*) ..... on (*date*): .....

....

Signature of participant **Signature of witness**

.....

Signature of researcher

Appendix D: Example of the information document for genetic research signed by adult participants

INFORMATION DOCUMENT FOR GENETIC RESEARCH: ADULT PARTICIPANTS

Title of the research project: *Mutational analysis of a South African haemophilia B population.*

Principal investigator: **Chené Bester**
Address: **Department of Haematology and Cell Biology**
Faculty of Health Sciences
University of the Free State
Bloemfontein, 9301
Contact number: **071 628 3552**

To Project Participant:

A. PURPOSE AND BACKGROUND

We are planning to conduct a research project wherein we will attempt to detect mutations involved in haemophilia B and to establish how these mutations influence the functions of the blood clotting system. Haemophilia B is a hereditary rare bleeding disorder that a person is born with and where a person's blood can't clot due to a deficiency or defect of an important protein that plays a role in blood clotting. This protein is known as coagulation factor IX (FIX). Mutations in the FIX gene may prevent certain functions or production of this protein, causing a person with haemophilia B to bleed for longer. Genes are what you inherit from your parents. It is important that these mutations are diagnosed as they may play a role in the treatment of the disorders.

B. PROCEDURES

We request your permission to draw blood (15 mL) and to use your blood and DNA (blood) for future laboratory analysis. We will determine which mutations may be responsible for your haemophilia B, and how this mutation is affecting the function of your blood clotting system. The findings of this study will not have direct bearing on your management of this bleeding disorder however, it will allow the diagnosis of mutations that causes haemophilia B to be detected. These mutations might play a role in how haemophilia B is treated in the future. You are free to refuse consent and you do not have to give reasons for doing so.

C. CONFIDENTIALITY

The following arrangements have been made to ensure privacy and confidentiality of your genetic information:

- Your blood sample will be marked with a code and not your name. Researchers will, therefore, be able to identify the sample, but not technicians working with the sample. \

D. RESULTS OF RESEARCH STUDY

If this research generates information about you which may be of relevance to the health of other family members, your consent will be sought before offering to disclose such information to the family members concerned, except if such disclosure is compulsory as determined by the law.

E. STORAGE OF BLOOD AND DNA

We would like to retain your blood and DNA (blood) for possible future research.
The duration of storage will be maximum fifteen (15) years.
Your blood and DNA sample will be marked with a code to protect your identity.

If you are unhappy to have your blood and DNA stored for future research, your genetic material and information will be disposed of at the end of this study, once the sample storage and record-keeping requirements of good research practice have been met.

Do you have any sensitivity on how your blood should be disposed of? If so, what are they?

These will be recorded and considered at the time of disposal.
We can dispose your genetic material even after the research has started, since the samples are stored in an identifiable form.

F. VOLUNTARY PARTICIPATION

You are not obligated to participate in this research and if you choose to participate in this study, you can withdraw your consent and discontinue participation at any time without prejudice. Your decision whether to participate in this study is voluntary and your routine medical treatment will not be compromised if you choose not to participate.

G. RISKS

The risks should not be more that those which are normally expected during the donation of blood samples for scientific research.
You may experience a small degree of pain and/or discomfort during the blood drawing procedure. Possible undesirable affects, such as a slight bruise or swelling at the sight where your blood was drawn, may occur. We do not expect any of these adverse medical effects to occur. However, if you do experience any medical problems, because of your participation, the necessary medical treatment will be provided.

H. PUBLICATION OF DATA

Reports resulting from this study might in future be used for scientific publication purposes but will not identify you as a participant. All information gathered in this study will remain confidential and be given out only with your permission or as required by law. If you give us permission by signing this consent form, we will protect your confidentiality.

I. CONTACT INFORMATION

If you have any questions about this research at any time, please call Chené Bester on **0716283552**. You may contact the Secretariat of the Health Sciences Research Ethics Committee of the Faculty of Health Sciences, University of the Free State at telephone number **0514052812** if you have questions regarding your right as a participant in this research project.

J. CONSENT

By signing this consent form, you indicate that you have read the form and agree that you voluntarily participate in the study. If you choose not to participate in this study there will be no penalty or loss of benefits to which you are entitled to. If you agree to take part, you are free to withdraw your consent from it at any time. Likewise, no penalty or loss of benefits to which you are otherwise entitled to will occur.
The research study, including the above-mentioned information has been verbally described to me. I agree to voluntarily participate in the study, as set out above

PLEASE SELECT THE OPTIONS BELOW:

I give consent to participate in the study	Yes / No
I give consent for collection of genetic material	Yes / No
I give consent that my genetic material may be stored	Yes / No
I give consent that stored material may be used in future haemophilia related studies	Yes / No

Signed at (*place*) ..... on (*date*): .....

....

Signature of participant **Signature of resea**

.....

Signature of researcher

Appendix E: Example of an informed consent for signed by the parents/legal guardians of minor participants

TITLE OF THE RESEARCH PROJECT: Mutational analysis of a South African haemophilia B population.

PRINCIPAL INVESTIGATOR: Chené Bester

ADDRESS: Department of Haematology and Cell Biology
Faculty of Health Sciences
University of the Free State
Bloemfontein, 9301

CONTACT NUMBER: 071 628 3552

To Project Participant:

A. PURPOSE AND BACKGROUND

Your child is invited to take part in a research project conducted in the Human Molecular Biology Unit at the University of the Free State, Bloemfontein, South Africa. We (Me Chené Bester, together with Dr Janse van Rensburg and Mr Kloppers) are planning to conduct a research project wherein we will attempt to detect mutations involved in haemophilia B and to establish how these mutations influence the functions of the blood clotting system.
Haemophilia B is a hereditary rare bleeding disorder that a person is born with and where a person's blood can't clot due to a deficiency or defect of an important protein that plays a role in blood clotting. This protein is known as coagulation factor IX (FIX). Mutations in the FIX gene may prevent certain functions or production of this protein, causing a person with haemophilia B to bleed for longer. Genes are what you inherit from your parents. It is important that these mutations are diagnosed as they may play a role in the treatment of the disorders.

B. PROCEDURES

We need (15 ml) of blood and your child's time involvement will approximately be five (5) minutes as the blood is drawn. There will be no further engagements, remunerations or costs involved.

C. RISKS

The risks should not exceed those that are normally expected in donating blood samples for scientific research. Your child may experience a slight degree of pain and discomfort during the procedure and adverse effects like a slight bruise or swelling at the site where the blood was drawn. We do not expect any of these adverse medical effects to occur. However, if your child do experience any medical problems as a result of his or her participation, the necessary medical treatment will be provided.

D. CONFIDENTIALITY

Reports resulting from this study might in future be used for scientific publication purposes but will not identify your child as a participant. All information gathered in this study will remain confidential and be given out only with your permission or as required by law. If you give us permission by signing this consent form, we will protect your child's confidentiality.

E. VOLUNTARY PARTICIPATION

Your decision whether or not to allow your child to participate in this study is voluntary. If you choose not let you child participate, there will be no penalty or loss of benefits that your child is entitled to. If you choose to allow your child to participate in this study, you can withdraw your consent and discontinue your child's participation at any time without prejudice.

F. QUESTIONS

If you or your child have any questions about this research at any time, please call Ms Chené Bester (**071 628 3552**). You may contact the Secretariat of the Health Sciences Research Ethics Committee of the Faculty of Health Sciences, University of the Free State at telephone number **0514052812** if you have questions regarding your child's right as a participant in this research project.

G. CONSENT

By signing this consent form, you indicate that you have read the form and agree that your child can voluntarily participate in the study. If you choose not to allow your child to participate in this study, there will be no penalty or loss of benefits to which you or your child are entitled to. If you agree to let your child take part, you are free to withdraw your child from it at any time. Likewise, no penalty or loss of benefits to which you or your child are otherwise entitled to will occur.
The research study, including the above-mentioned information has been verbally described to me. I agree to let my child voluntarily participate in the study, as set out above.

Signed at (*place*) ..... on (*date*): .....

....

Signature of parent/legal guardian ***Signature of witness***

.....

Signature of investigator

Appendix F: Example of an assent form signed by minor participants

Researcher's name(s):	**Miss Chené Bester**
	Dr WJ Janse van Rensburg
	Mr JF Kloppers
Address:	Department of Haematology and Cell Biology
	Francois Retief Building, Second Floor, Room 409A
	University of the Free State
	DF Malherbe avenue
	Bloemfontein, South Arica
Contact number:	**051 405 3098 / 071 628 3552**

WHAT IS RESEARCH?

Research is something we do to find new knowledge about the way things (and people) work. We use research projects or studies to help us find out more about disease or illness. Research also helps us to find better ways of helping or treating children who are sick.

WHAT IS THIS RESEACH STUDY ALL ABOUT?

In our research study, we would like to find out new things about the disease, known as haemophilia B. We want to develop a new test so that we will be able to find specific mutations (*change/different working of your gene*) that is the reason that you have haemophilia B. If we can do this, we can help more people, like you, to get better treatment and care.

WHY HAVE I BEEN INVITED TO TAKE PART IN THIS RESEARCH STUDY?

You have been invited to participate in this research study because you have haemophilia B.

WHO IS DOING THE RESEACH?

The research will be done by Chené Bester, who is a student at the medical school at the University of the Free State. This research project is being supervised Dr Janse van Rensburg and Mr Kloppers, who is also working for the University of the Free State.

WHAT WILL HAPPEN TO ME IN THIS STUDY?

A doctor will draw a little blood from you. This is all that will happen to you in this project.

CAN ANYTHING BAD HAPPEN TO ME?

There is a risk that you might feel a bit of pain where the blood is drawn, and it might cause a bruise. If you feel any pain or discomfort during or after blood is drawn you should tell the doctor or your parents about it immediately.

CAN ANYTHING GOOD HAPPEN TO ME?

There is no direct benefit for you in this study. Your participation will however help to get a better understanding about the illness (haemophilia B) that cause bleeding and in future help to develop a possible cure.

WILL ANYONE KNOW THAT I AM IN THIS STUDY?

Only you, your parents and the researchers that you are participating in this study. Nobody else will know that you are in this study unless you tell them.

WHO CAN I TALK TO ABOUT THE STUDY?

If you have any questions about this research study, you can contact Chené Bester directly on 071 628 3552. Emails can be sent to chenebester40@gmail.com.
Alternatively, you can also contact Dr Janse van Rensburg on 051 405 3116.

WHAT IF I DO NOT WHAT TO DO THIS?

You can refuse to take part in this study, even if your parents agree that you can participate. You can also withdraw from the study at any time you want, and you will not get in trouble for doing so.

Do you understand the research study and are you willing to take part?

Has the researcher answered all your questions?

Do you understand that you can pull out of the study at any time?

Signed at (*place*): ..... On (*date*): .....

....

Signature/mark of child ***Signature of witness***

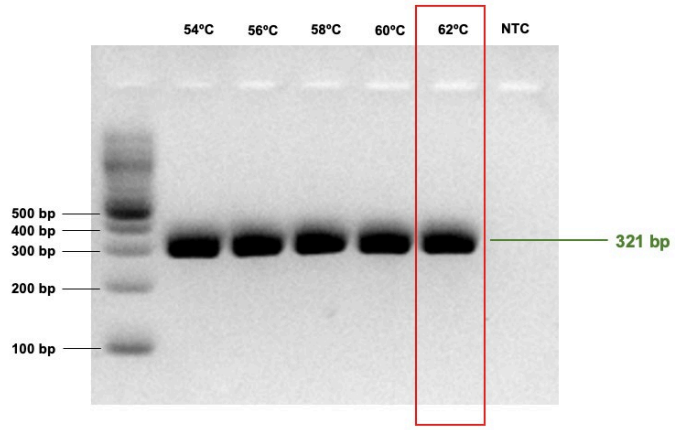
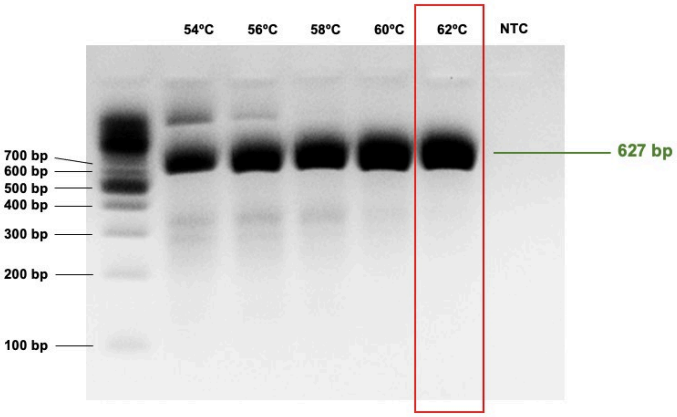
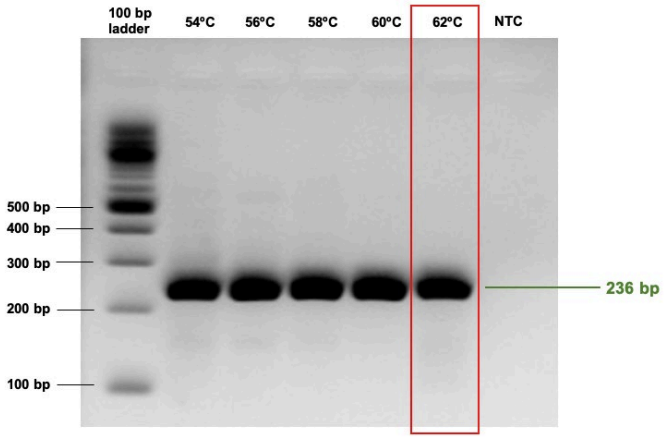
.....

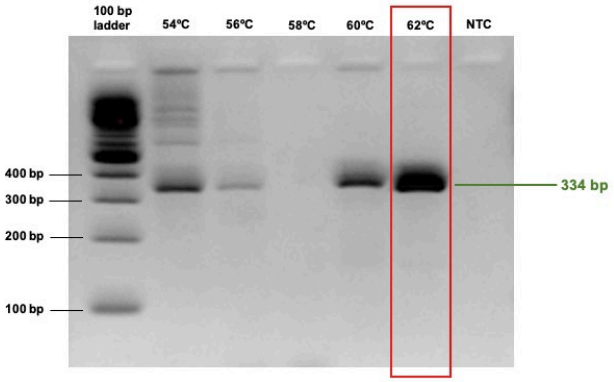
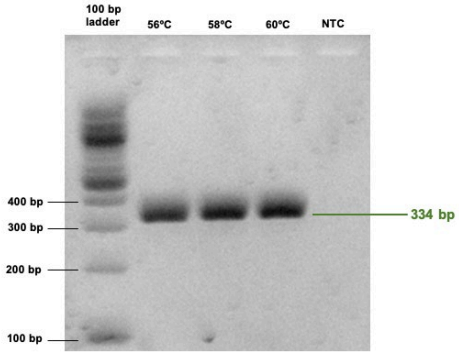
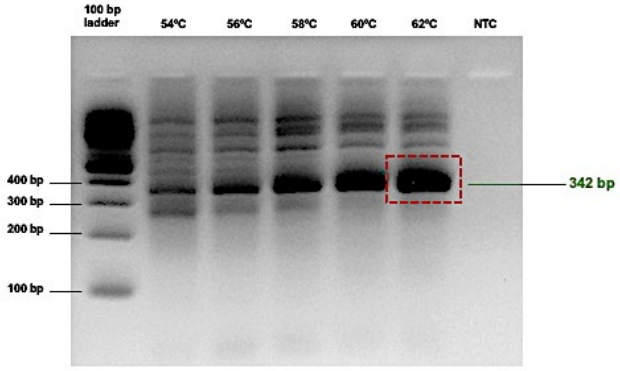
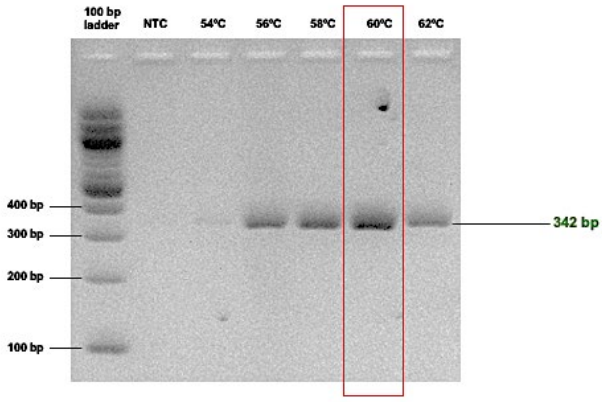
Signature of researcher

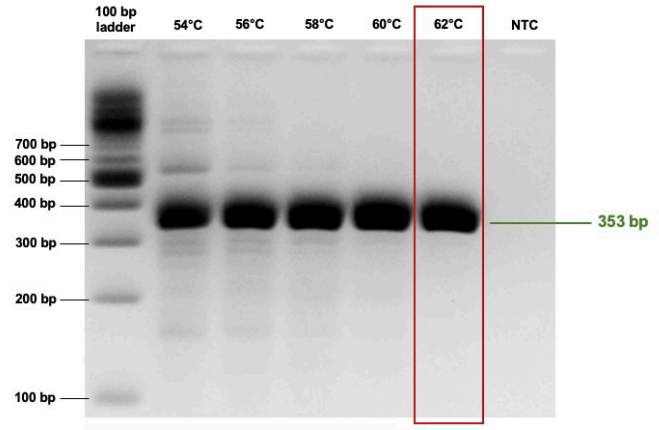
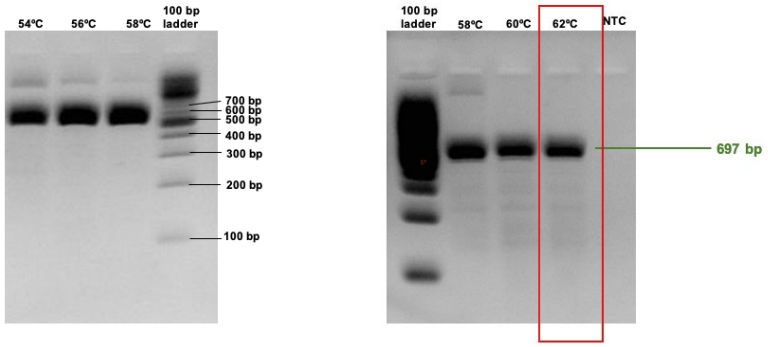
Appendix G: Reference sequence for *F9* fragment 1 - 7, demonstrating the forward and reverse primer sequences (red), as well as the *F9* exon sequence within the respective fragment

F9 fragment	Reference Sequence	*F9* exon nt positions		Fragment size
		Start	End	
F1 (exon 1)	**agtccaaagacccattgagg**gagatggacattatttcccagaagtaaatacagctcagcttgtacttt ggtacaactaatcgaccttaccactttcacaatctgctagcaaaggttatgcagcgcgtgaacatgat catggcagaatcaccaggcctcatcaccatctgccttttaggatatctactcagtgctgaatgtacag gtttgtttccttttttaaaatacattgagtatgcttgccttttagatatagaaatatctgatgctgtc ttcttcactaaattttgattacat**gatttgacagcaatattgaagagtc**	5001	5117	321 bp
F2 (exon 2;3)	**tgccctaaagagaaattggc**tttcagattatttggattaaaaacaaagacttttcttaagagatgtaaa attttcatgatgttttctttttgctaaaactaaagaattattcttttacatttcagttttcttgat catgaaaacgccaacaaaattctgaatcggccaaagaggtataattcaggtaaattggaagagtttgt tcaagggaaccttgagagagaatgtatggaagaaaagtgtagttttgaagaagcacgagaagttttg aaaacactgaaagaacagtgagtatttccacataataccttcagatgcagagcatagaatagaaaat ctttaaaaagacacttctctttaaaatttttaaagcatccatatatatttatgtatgttaaatgttata aaagataggaaatcaataccaaaacactttagatattaccgttaatttgtcttcttttattctttata gactgaattttggaagcagtatgttggtaagcaattcatttttatcctctagctaatatatgaaacata tgagaattatgtgggtttttctctgcataaatagataatatattaaactttgtcaaaaggactcaga aagat**cagtccaaccctctaaccca**	11275	11438	627 bp
F3 (exon 4)	**gaggaccgggcattctaag**cagtttacgtgccaattcaatttcttaacctatctcaaagatggagatc agtgtgagtccaatccatgtttaaatggcggcagttgcaaggatgacattaattcctatgaatgttgg tgtccctttggatttgaaggaaagaactgtgaattaggtaagtaactatttttgaatactcatggtt caaagtttc**cctctgaaacaagttgaaactgg**	11627	11651	236 bp
F4 (exon 5)	**cccccaatgtatatttgaccc**atacatgagtcagtagttccatgtacttttttagaaatgcatgttaaa tgatgctgttactgtctatttttgcttcttttagatgtaacatgtaacattaagaatggcagatgcgag cagttttgtaaaaatagtgctgataacaaggtggtttgctcctgtactgagggatatcgacttgcaga aaaccagaagtcctgtgaaccagcaggtcataatctgaataagatttttttaaagaaaatctgtatctg aaacttcagcattttaacaaacctacataatttttaattccta**cttgaatctgcttcctttttg**	22628	22756	334 bp
F5 (exon 6)	**tgtaatacatgttccatttgcc**aatgagaaatatcaggttactaatttttcttctatttttctagtgc catttccatgtggaagagtttctgtttcacaaacttctaagctcacccgtgctgagactgtttttcct gatgtggactatgtaaattctactgaagctgaaaccattttggataacatcactcaaagcacccaatc atttaatgacttcactcgggttgttggtggagaagatgccaaaccaggtcaattcccttggcagtac tttatactgatggtgtgtcaaaactggagctcagctggcaagacacaggc**caggtgggagactgaggc ta**	25327	25529	342 bp
F6 (exon 7)	**tttctgccagcacctagaag**ccaatattttgcctattcctgtaaccagcacacatatttatttttttc tagatcaaatgtattatgcagtaagagtcttaattttgttttcacaggttgttttgaatggtaaagtt gatgcattctgtggaggctctatcgttaatgaaaaatggattgtaactgctgcccactgtgttgaaac tggtgttaaaattacagttgtcgcaggtaaatacacagaaagaataataatctgcagcaccactagct ctttaatatgattggtacaccatattttactaaggtctaataaaattgttgttgaataaatt**gggcta aaggcagaagggt**	35006	35120	353 bp
F7 (exon 8)	**gccaattaggtcagtggtcc**caagtagtcacttagaaaatctgtgtatgtgaaatactgtttgtgact taaaatgaaatttatttttaatagtgaacataatattgaggagacagaacatacagagcaaaagcga aatgtgattcgaattattcctcaccacaactacaatgcagctattaataagtacaaccatgacattgc ccttctggaactggacgaacccttagtgctaaacagctacgttacacctatttgcattgctgacaagg aatacacgaacatcttcctcaaatttggatctggctatgtaagtggctggggaagagtcttccacaaa gggagatcagctttagttcttcagtaccttagagttccacttgttgaccgagccacatgtcttcgatc tacaaagttcaccatctataacaacatgttctgtgctggcttccatgaaggaggtagagattcatgtc aaggagatagtgggggacccccatgttactgaagtggaagggaccagtttcttaactggaattattagc tggggtgaagagtgtgcaatgaaaggcaaatatggaatatataccaaggtatcccggtatgtcaactg gattaaggaaaaaacaaagctcacttaatgaaagatggatttccaaggttaattcattggaatt**gaaa attaacagggcctctca**	35789	37723	679

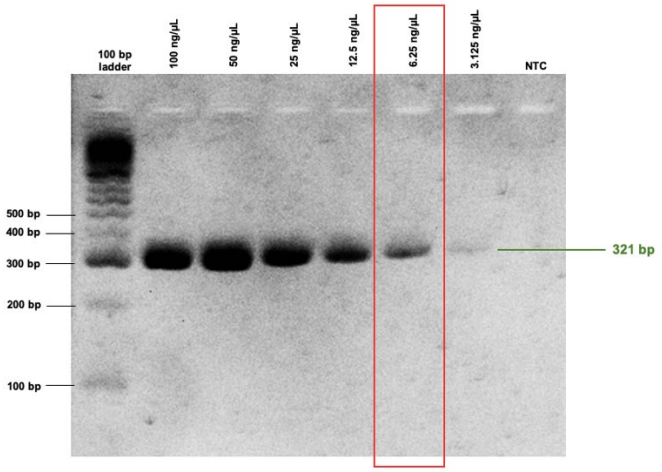
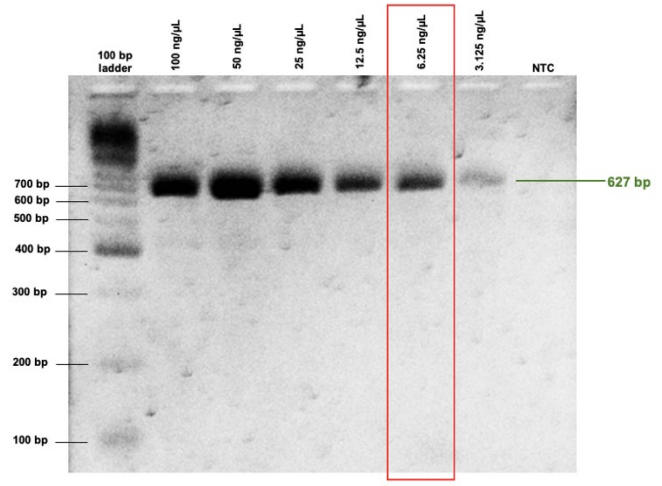
Appendix H: Gel images depicting the optimal annealing temperature determined for each primer pair of the respective *F9* conventional PCR assays

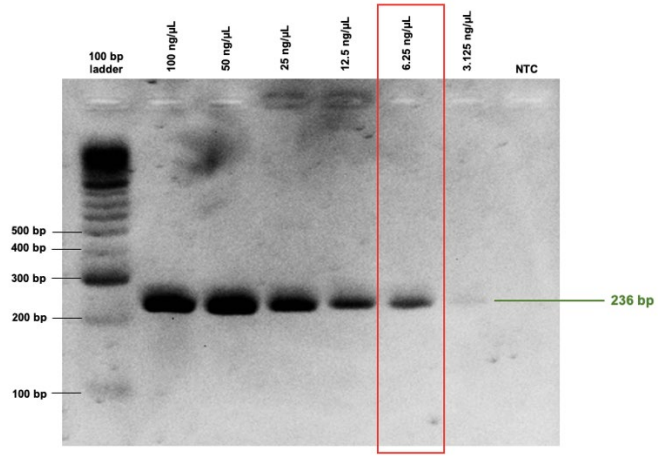
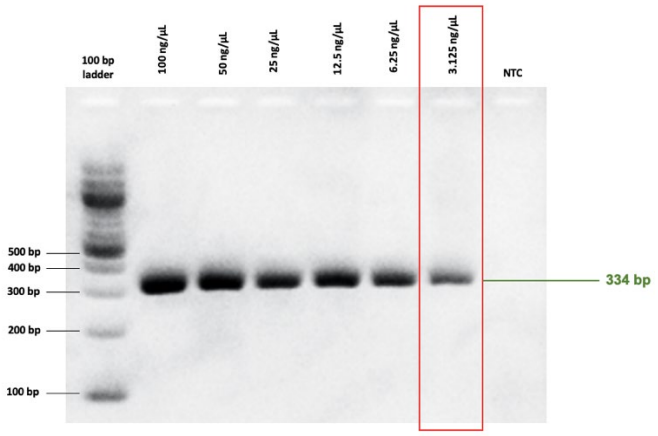
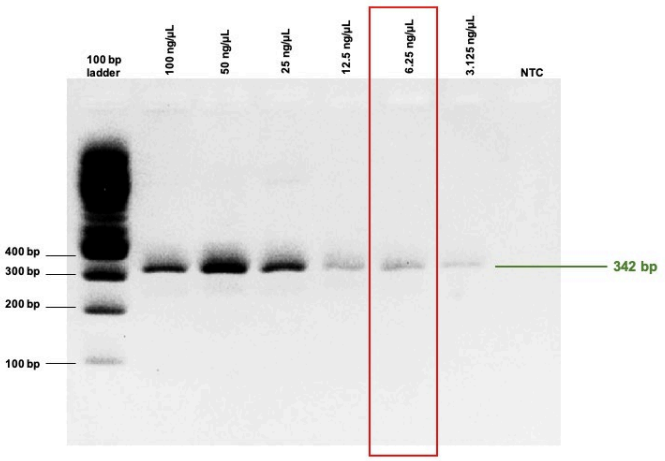
F9 Fragment	Fragment size	Gel image	Optimal annealing temperature
1	321 bp		62°C
2	627 bp		62°C
3	236 bp		62°C

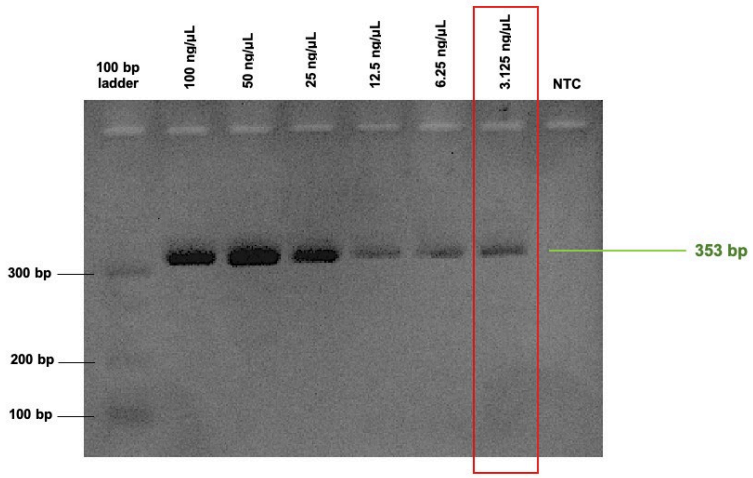
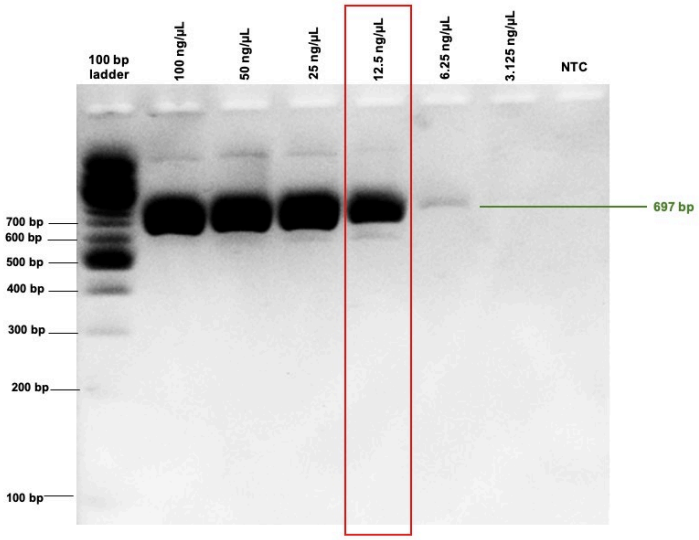
4	334 bp	 *Repeated reactions at 56°C, 58°C, and 60°C*  *The fragment obtained at 62°C in the first temperature gradient was still the darkest and therefore the optimal annealing temperature*	**62°C**
5	342 bp	 *Correct fragment (342 bp) amplified at 62°C was excised and used as template DNA to repeat the temperature gradient* 	**60°C**

6	353 bp		**62ºC**
7	697 bp		**62ºC**

Appendix I: Gel images depicting the level of detection determined for each primer pair of the respective *F9* conventional PCR assays

F9 Fragment	Fragment size	Gel image	LOD
1	321 bp		6.25 ng/µL
2	627 bp		6.25 ng/µL

3	236 bp		6.25 ng/µL
4	334 bp		3.125 ng/µL
5	342 bp		6.25 ng/µL

6	353 bp		3.125 ng/µL
7	697 bp		12.5 ng/µL

Appendix J: Sanger sequencing analysis of the *F9* conventional PCR products obtained at optimal cycling conditions

F9 fragment 1 (Exon 1)

```
               10        20        30        40        50        60
S1Ex1   ATGCAGCGCGTGAACATGATCATGGCAGAATCACCAGGCCTCATCACCATCTGCCTTTTA
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex1Ref  ATGCAGCGCGTGAACATGATCATGGCAGAATCACCAGGCCTCATCACCATCTGCCTTTTA
               10        20        30        40        50        60

               70        80
S1Ex1   GGATATCTACTCAGTGCTGAATGTACAG
        :::::::::::::::::::::::::::
Ex1Ref  GGATATCTACTCAGTGCTGAATGTACAG
               70        80
```

F9 fragment 2 (Exon 2,3)

```
                10        20        30        40        50        60
F9Ex2,3  TTTTTCTTGATCATGAAAACGCCAACAAAATTCTGAATCGGCCAAAGAGGTATAATTCAG
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref TTTTTCTTGATCATGAAAACGCCAACAAAATTCTGAATCGGCCAAAGAGGTATAATTCAG
                10        20        30        40        50        60

                70        80        90       100       110       120
F9Ex2,3  GTAAATTGGAAGAGTTTGTTCAAGGGAACCTTGAGAGAGAATGTATGGAAGAAAAGTGTA
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref GTAAATTGGAAGAGTTTGTTCAAGGGAACCTTGAGAGAGAATGTATGGAAGAAAAGTGTA
                70        80        90       100       110       120

               130       140       150       160       170       180
F9Ex2,3  GTTTTGAAGAAGCACGAGAAGTTTTTGAAAACACTGAAAGAACAGTGAGTATTTCCACAT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref GTTTTGAAGAAGCACGAGAAGTTTTTGAAAACACTGAAAGAACAGTGAGTATTTCCACAT
               130       140       150       160       170       180

               190       200       210       220       230       240
F9Ex2,3  AATACCCTTCAGATGCAGAGCATAGAATAGAAAATCTTTAAAAAGACACTTCTCTTTAAA
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref AATACCCTTCAGATGCAGAGCATAGAATAGAAAATCTTTAAAAAGACACTTCTCTTTAAA
               190       200       210       220       230       240

               250       260       270       280       290       300
F9Ex2,3  ATTTTAAAGCATCCATATATATTTATGTATGTTAAATGTTATAAAAGATAGGAAATCAAT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref ATTTTAAAGCATCCATATATATTTATGTATGTTAAATGTTATAAAAGATAGGAAATCAAT
               250       260       270       280       290       300

               310       320       330       340       350       360
F9Ex2,3  ACCAAAACACTTTAGATATTACCGTTAATTTGTCTTCTTTTATTCTTTATAGACTGAATT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref ACCAAAACACTTTAGATATTACCGTTAATTTGTCTTCTTTTATTCTTTATAGACTGAATT
               310       320       330       340       350       360

               370
F9Ex2,3  TTGGAAGCAGTATGTTG
         :::::::::::::::::
Ex2,3Ref TTGGAAGCAGTATGTTG
               370
```

F9 Exon 2
F9 Exon 3

F9 fragment 3 (Exon 4)

```
               10        20        30        40        50        60
F9Ex4   ATGGAGATCAGTGTGAGTCCAATCCATGTTTAAATGGCGGCAGTTGCAAGGATGACATTA
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex4Ref  ATGGAGATCAGTGTGAGTCCAATCCATGTTTAAATGGCGGCAGTTGCAAGGATGACATTA
               10        20        30        40        50        60

               70        80        90       100       110
F9Ex4   ATTCCTATGAATGTTGGTGTCCCTTTGGATTTGAAGGAAAGAACTGTGAATTAG
        :::::::::::::::::::::::::::::::::::::::::::::::::::
Ex4Ref  ATTCCTATGAATGTTGGTGTCCCTTTGGATTTGAAGGAAAGAACTGTGAATTAG
               70        80        90       100       110
```

F9 fragment 4 (Exon 5)

```
               10        20        30        40        50        60
F9Ex5   ATGTAACATGTAACATTAAGAATGGCAGATGCGAGCAGTTTTGTAAAAATAGTGCTGATA
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex5Ref  ATGTAACATGTAACATTAAGAATGGCAGATGCGAGCAGTTTTGTAAAAATAGTGCTGATA
               10        20        30        40        50        60

               70        80        90       100       110       120
F9Ex5   ACAAGGTGGTTTGCTCCTGTACTGAGGGATATCGACTTGCAGAAAACCAGAAGTCCTGTG
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex5Ref  ACAAGGTGGTTTGCTCCTGTACTGAGGGATATCGACTTGCAGAAAACCAGAAGTCCTGTG
               70        80        90       100       110       120


F9Ex5   AACCAGCAG
        :::::::::
Ex5Ref  AACCAGCAG
```

F9 fragment 5 (Exon 6)

```
               10        20        30        40        50        60
F9Ex6   TGCCATTTCCATGTGGAAGAGTTTCTGTTTCACAAACTTCTAAGCTCACCCGTGCTGAGA
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex6Ref  TGCCATTTCCATGTGGAAGAGTTTCTGTTTCACAAACTTCTAAGCTCACCCGTGCTGAGA
               10        20        30        40        50        60

               70        80        90       100       110       120
F9Ex6   CTGTTTTTCCTGATGTGGACTATGTAAATTCTACTGAAGCTGAAACCATTTTGGATAACA
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex6Ref  CTGTTTTTCCTGATGTGGACTATGTAAATTCTACTGAAGCTGAAACCATTTTGGATAACA
               70        80        90       100       110       120

              130       140       150       160       170       180
F9Ex6   TCACTCAAAGCACCCAATCATTTAATGACTTCACTCGGGTTGTTGGTGGAGAAGATGCCA
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex6Ref  TCACTCAAAGCACCCAATCATTTAATGACTTCACTCGGGTTGTTGGTGGAGAAGATGCCA
              130       140       150       160       170       180

              190       200
F9Ex6   AACCAGGTCAATTCCCTTGGCAG
        :::::::::::::::::::::::
Ex6Ref  AACCAGGTCAATTCCCTTGGCAG
              190       200
```

F9 fragment 6 (Exon 7)

```
              10        20        30        40        50        60
F9Ex7  GTTGTTTTGAATGGTAAAGTTGATGCATTCTGTGGAGGCTCTATCGTTAATGAAAAATGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex7Ref GTTGTTTTGAATGGTAAAGTTGATGCATTCTGTGGAGGCTCTATCGTTAATGAAAAATGG
              10        20        30        40        50        60

              70        80        90       100       110
F9Ex7  ATTGTAACTGCTGCCCACTGTGTTGAAACTGGTGTTAAAATTACAGTTGTCGCAG
       :::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex7Ref ATTGTAACTGCTGCCCACTGTGTTGAAACTGGTGTTAAAATTACAGTTGTCGCAG
              70        80        90       100       110
```

F9 fragment 7 (Exon 8)

```
              10        20        30        40        50        60
F9Ex8  GTGAACATAATATTGAGGAGACAGAACATACAGAGCAAAAGCGAAATGTGATTCGAATTA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref GTGAACATAATATTGAGGAGACAGAACATACAGAGCAAAAGCGAAATGTGATTCGAATTA
              10        20        30        40        50        60

              70        80        90       100       110       120
F9Ex8  TTCCTCACCACAACTACAATGCAGCTATTAATAAGTACAACCATGACATTGCCCTTCTGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref TTCCTCACCACAACTACAATGCAGCTATTAATAAGTACAACCATGACATTGCCCTTCTGG
              70        80        90       100       110       120

             130       140       150       160       170       180
F9Ex8  AACTGGACGAACCCTTAGTGCTAAACAGCTACGTTACACCTATTTGCATTGCTGACAAGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref AACTGGACGAACCCTTAGTGCTAAACAGCTACGTTACACCTATTTGCATTGCTGACAAGG
             130       140       150       160       170       180

             190       200       210       220       230       240
F9Ex8  AATACACGAACATCTTCCTCAAATTTGGATCTGGCTATGTAAGTGGCTGGGGAAGAGTCT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref AATACACGAACATCTTCCTCAAATTTGGATCTGGCTATGTAAGTGGCTGGGGAAGAGTCT
             190       200       210       220       230       240

             250       260       270       280       290       300
F9Ex8  TCCACAAAGGGAGATCAGCTTTAGTTCTTCAGTACCTTAGAGTTCCACTTGTTGACCGAG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref TCCACAAAGGGAGATCAGCTTTAGTTCTTCAGTACCTTAGAGTTCCACTTGTTGACCGAG
             250       260       270       280       290       300

             310       320       330       340       350       360
F9Ex8  CCACATGTCTTCGATCTACAAAGTTCACCATCTATAACAACATGTTCTGTGCTGGCTTCC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref CCACATGTCTTCGATCTACAAAGTTCACCATCTATAACAACATGTTCTGTGCTGGCTTCC
             310       320       330       340       350       360

             370       380       390       400       410       420
F9Ex8  ATGAAGGAGGTAGAGATTCATGTCAAGGAGATAGTGGGGGACCCCATGTTACTGAAGTGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref ATGAAGGAGGTAGAGATTCATGTCAAGGAGATAGTGGGGGACCCCATGTTACTGAAGTGG
             370       380       390       400       410       420

             430       440       450       460       470       480
F9Ex8  AAGGGACCAGTTTCTTAACTGGAATTATTAGCTGGGGTGAAGAGTGTGCAATGAAAGGCA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref AAGGGACCAGTTTCTTAACTGGAATTATTAGCTGGGGTGAAGAGTGTGCAATGAAAGGCA
             430       440       450       460       470       480

             490       500       510       520       530       540
F9Ex8  AATATGGAATATATACCAAGGTATCCCGGTATGTCAACTGGATTAAGGAAAAAACAAAGC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref AATATGGAATATATACCAAGGTATCCCGGTATGTCAACTGGATTAAGGAAAAAACAAAGC
             490       500       510       520       530       540

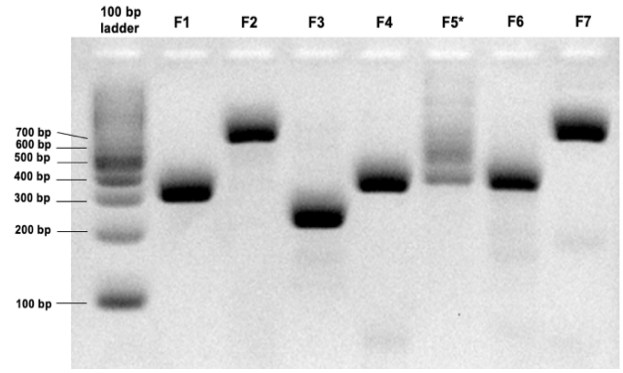

F9Ex8  TCACTTAA
       ::::::::
Ex8Ref TCACTTAA
```

Appendix K: DNA concentration measurements for S1 – S21

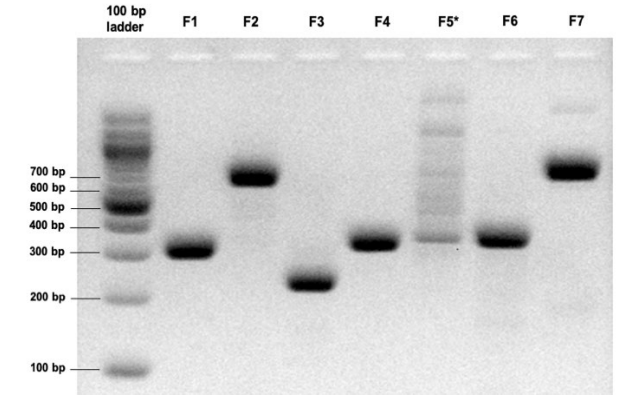
Study participant	DNA Concentration measurements (ng/µL)		
	1st Measurement	2nd Measurement	Mean value
Participant 1 (S1)	56.82	57.20	**57.01**
Participant 2 (S2)	**49.11**	50.07	**49.59**
Participant 3 (S3)	43.14	40.77	**41.96**
Participant 4 (S4)	**69.73**	69.46	**69.59**
Participant 5 (S5)	70.12	68.98	**69.55**
Participant 6 (S6)	**44.72**	41.64	**43.18**
Participant 7 (S7)	45.08	45.31	**45.19**
Participant 8 (S8)	**59.99**	57.83	**58.91**
Participant 9 (S9)	62.24	66.04	**64.14**
Participant 10 (S10)	**61.73**	61.27	**61.50**
Participant 11 (S11)	70.40	72.09	**71.25**
Participant 12 (S12)	**55.02**	57.38	**56.20**
Participant 13 (S13)	66.81	67.02	**66.92**
Participant 14 (S14)	**49.72**	52.75	**51.24**
Participant 15 (S15)	53.44	53.01	**53.23**
Participant 16 (S16)	**51.90**	54.57	**53.24**
Participant 17 (S17)	61.72	63.11	**62.42**
Participant 18 (S18)	**58.32**	57.07	**57.70**
Participant 19 (S19)	69.89	70.54	**70.22**
Participant 20 (S20)	56.97	56.71	**56.84**
Participant 21 (S21)	47.70	47.13	**47.42**

Appendix L: Gel electrophoretic analysis of *F9* fragment 1 – 7 conventional PCR assays conducted for the 21 study participants

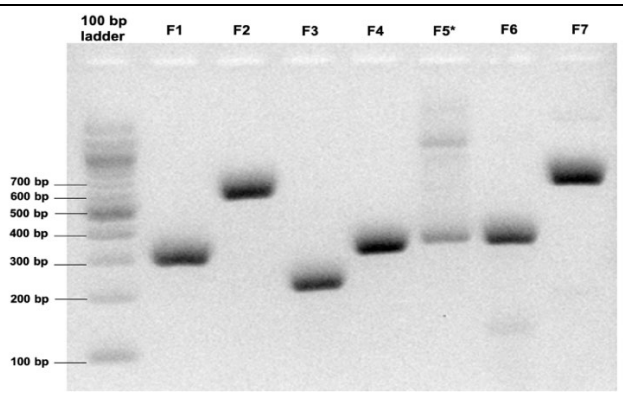
S1



S2



S3

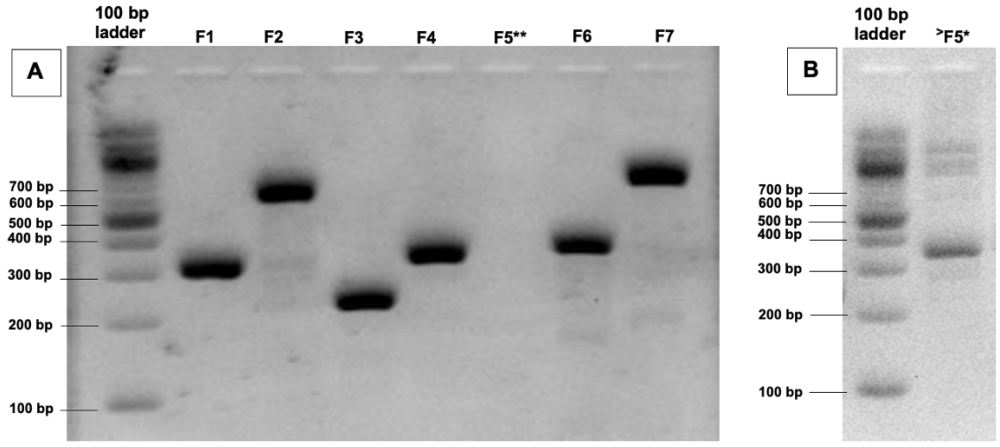


Fragment sizes:* *F1: *321 bp;* ***F2:*** *627 bp ;* ***F3:*** *236 bp;* ***F4:*** *334 bp;* ***F5*:*** *342 bp (before correct fragment was excised and reamplified);* ***F6:*** *353 bp; and* ***F7:*** *697 bp.*

Correct* ***F5* *fragment (342 bp) was excised and reamplified for S1, S2 and S3.*

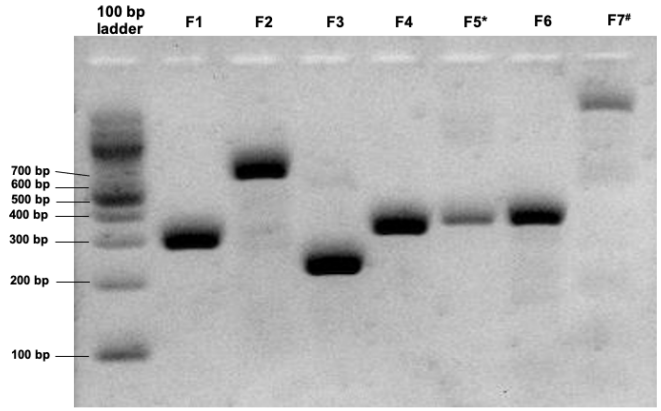
NOTE: Gel images of all reamplification results are presented later in the Appendix L section.

S4

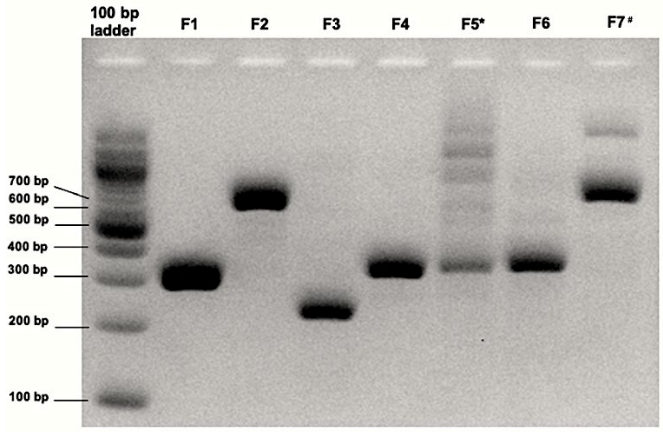


(A) ***Failed F5 PCR reaction was repeated;* ***(B)*** *>Successful reamplification of F5*

S5



S6

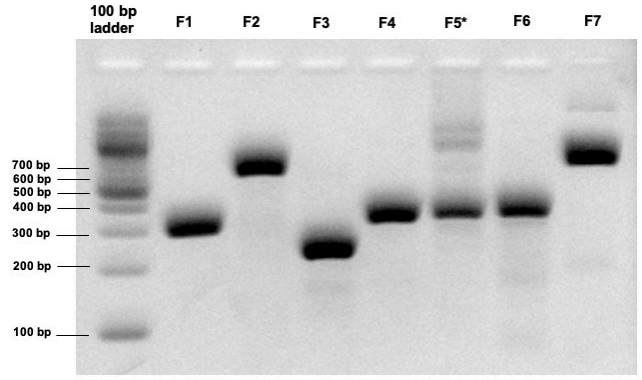


Fragment sizes: F1: *321 bp;* ***F2:*** *627 bp ;* ***F3:*** *236 bp;* ***F4:*** *334 bp;* ***F5*:*** *342 bp (before correct fragment was excised and reamplified);* ***F6:*** *353 bp; and* ***F7:*** *697 bp.*

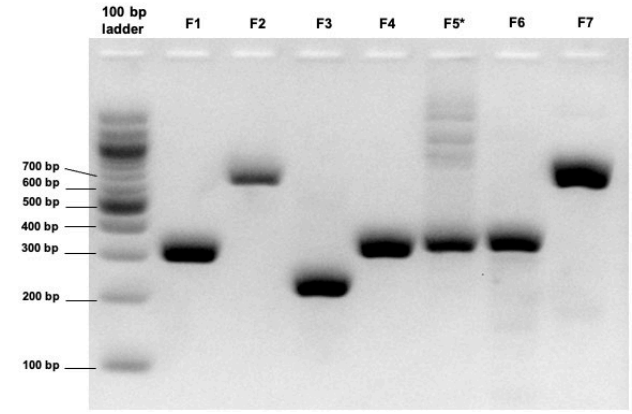
Correct* ***F5* *fragment (342 bp) was excised and reamplified for S4, S5 and S6.*

#F7 *PCR reactions were repeated for S5 and S6.*

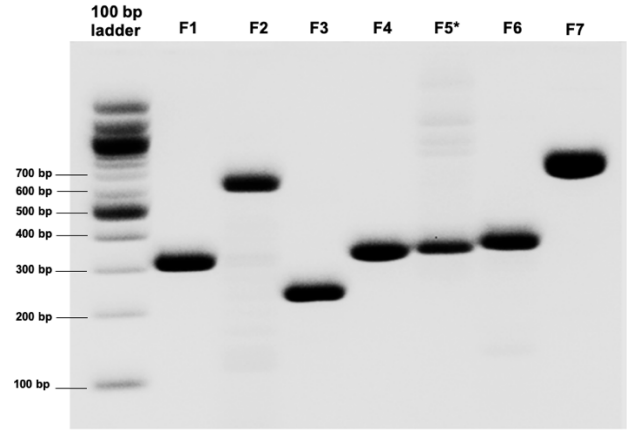
S7



S8



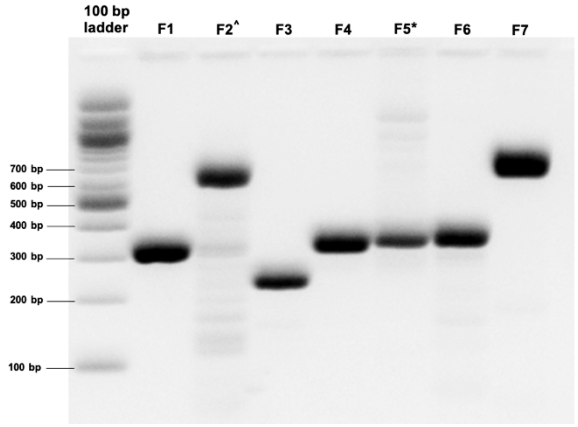
S9



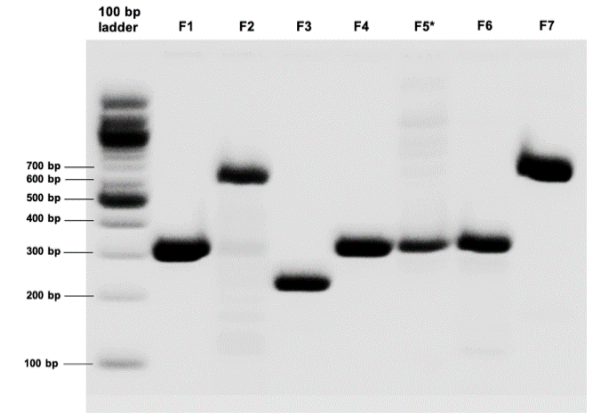
***Fragment sizes:* F1:** *321 bp;* **F2:** *627 bp ;* **F3:** *236 bp;* **F4:** *334 bp;* **F5*:** *342 bp (before correct fragment was excised and reamplified);* **F6:** *353 bp; and* **F7:** *697 bp.*

Correct* **F5 *fragment (342 bp) was excised and reamplified for S7, S8 and S9..*

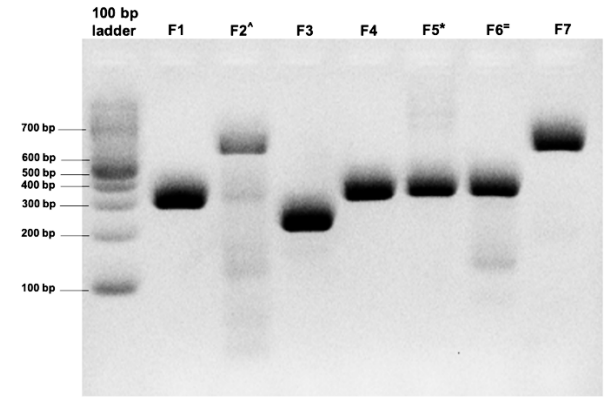
S10



S11



S12



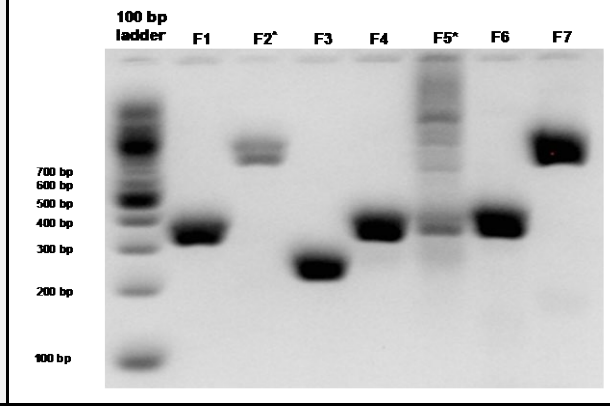
Fragment sizes: F1: *321 bp;* ***F2:*** *627 bp;* ***F3:*** *236 bp;* ***F4:*** *334 bp;* ***F5*:*** *342 bp (before correct fragment was excised and reamplified);* ***F6:*** *353 bp; and* ***F7:*** *697 bp.*

Correct* ***F5* *fragment (342 bp) was excised and reamplified for S10, S11 and S12.*

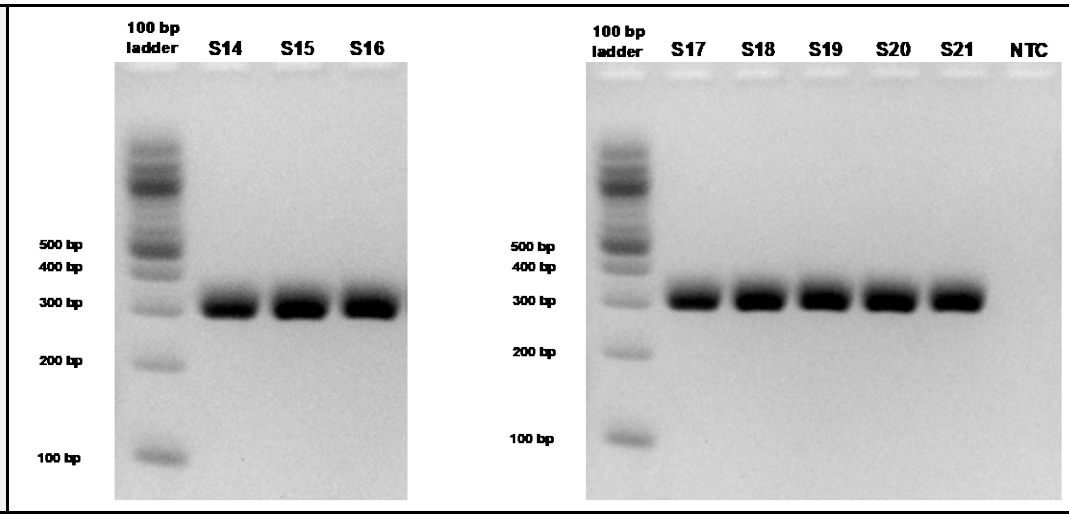
^F2 *PCR reaction was repeated for S10 and S12.*

=F6 *PCR reaction was repeated for S12.*

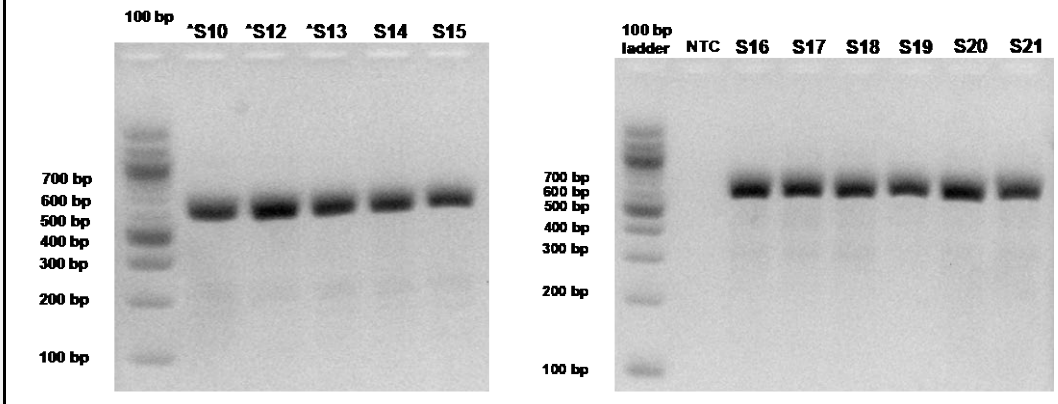
S13



F1 (S14 – S21)



F2 (627 bp) (^S10; ^S12; ^S13; S14 – S21)

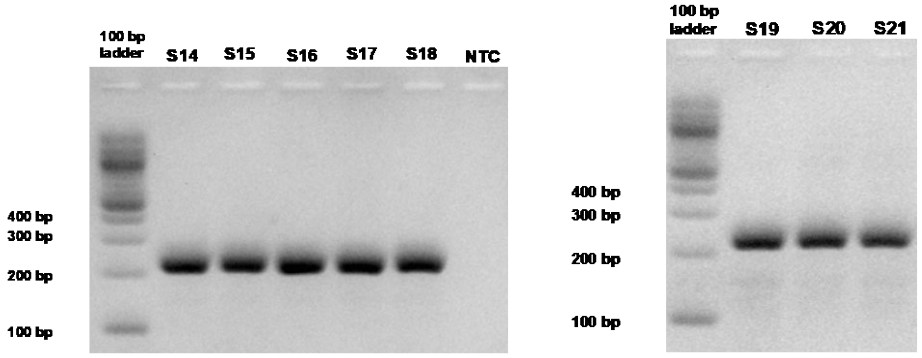


***Fragment sizes:* F1:** *321 bp;* **F2:** *627 bp;* **F3:** *236 bp;* **F4:** *334 bp;* **F5*:** *342 bp (before correct fragment was excised and reamplified);* **F6:** *353 bp; and* **F7:** *697 bp.*

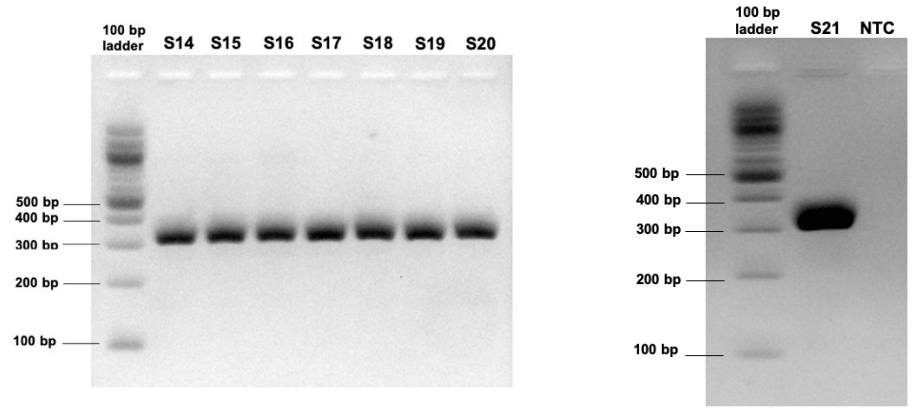
Correct* **F5 *fragment (342 bp) was excised and reamplified for S13.*

^*Successful reamplification of **F2** for S10, S12, and S13*

F3 (236 bp) (S14 – S21)

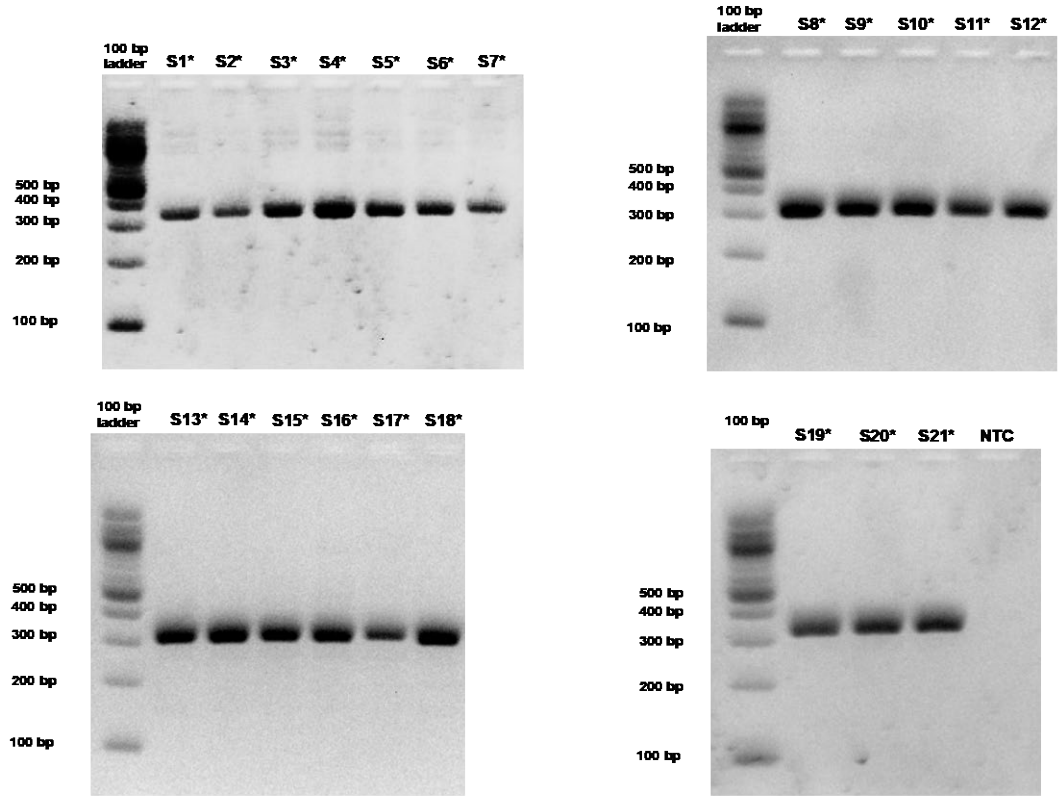


F4 (334 bp) (S14 – S21)

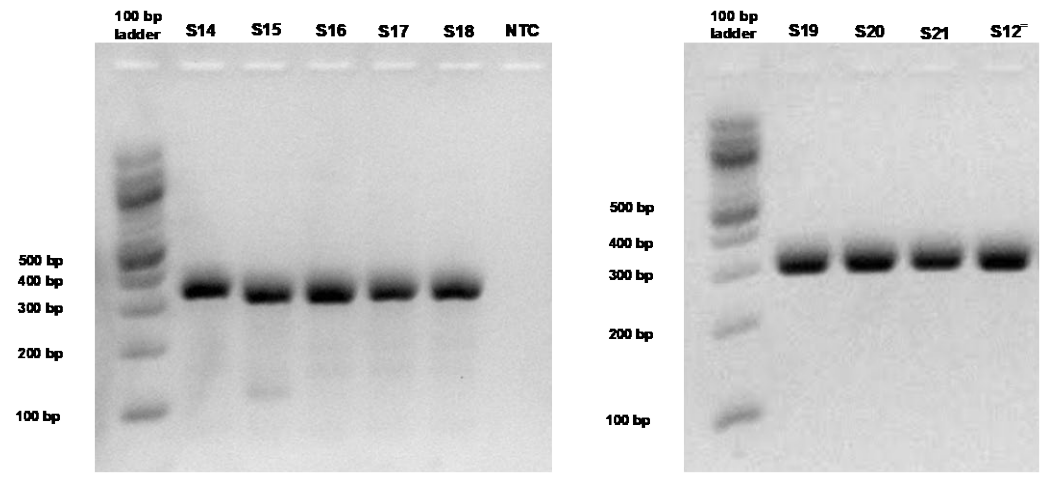


***Fragment sizes: F3:** 236 bp; **F4:** 334 bp*

F5 (342 bp) (S1 – S21)



F6 (353 bp) (S14 – S21)

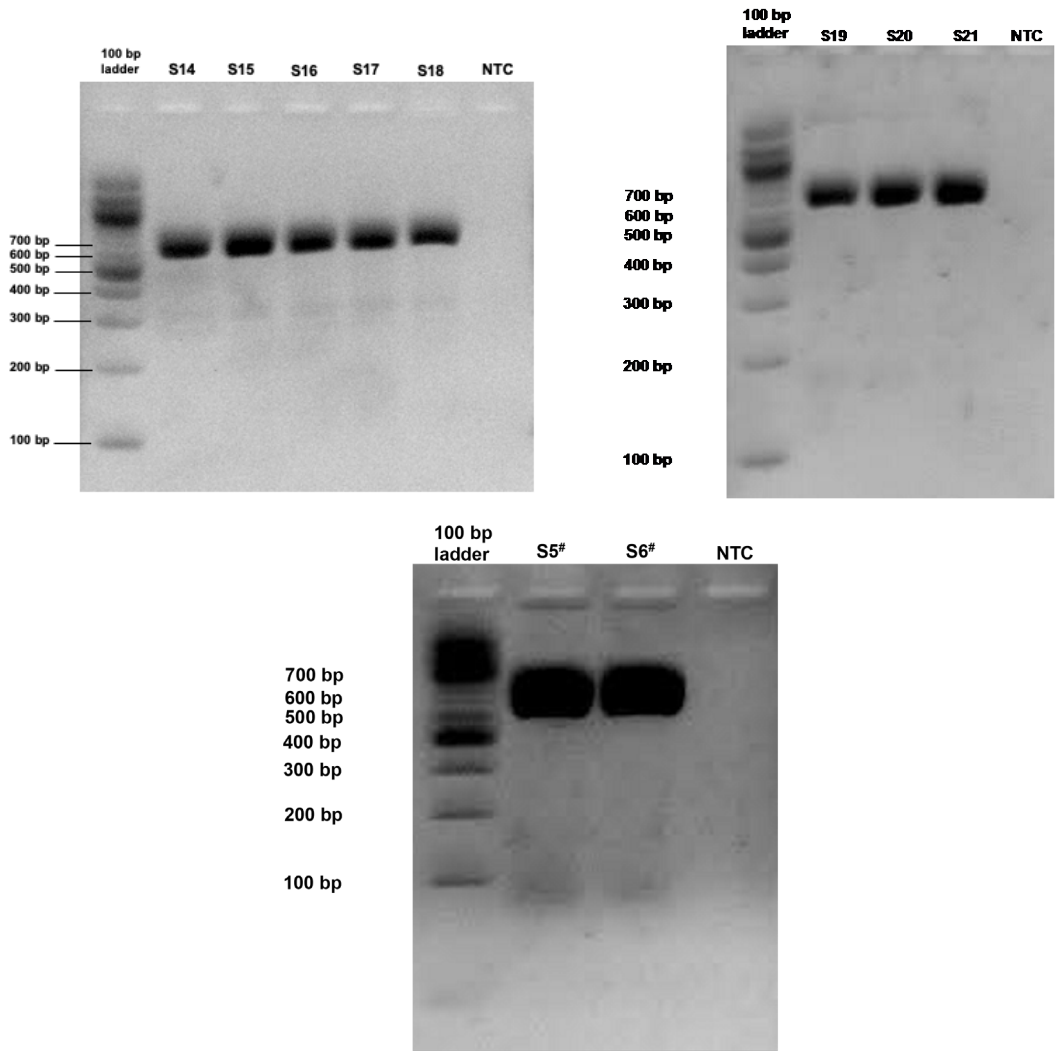


Fragment sizes: F5*: *342 bp (after correct fragment was excised and reamplified);* ***F6:*** *353 bp.*

Successful reamplification of* ***F5* *fragment (342 bp) for S1 – S21.*

=Successful reamplification of ***F6*** *for S12.*

F7 (697 bp) (S14 - S21)



Fragment sizes: F7: *697 bp*

*#Successful reamplification of **F7** for S5 and S6.*

Appendix M: Sanger sequencing analysis results for 21 study participants

Participant 1 (S1)

S1: *F9*_Exon 1

```
               10        20        30        40        50        60
S1Ex1  ATGCAGCGCGTGAACATGATCATGGCAGAATCACCAGGCCTCATCACCATCTGCCTTTTA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex1Ref ATGCAGCGCGTGAACATGATCATGGCAGAATCACCAGGCCTCATCACCATCTGCCTTTTA
               10        20        30        40        50        60

               70        80
S1Ex1  GGATATCTACTCAGTGCTGAATGTACAG
       ::::::::::::::::::::::::::::
Ex1Ref GGATATCTACTCAGTGCTGAATGTACAG
               70        80
```

S1: *F9*_Exon 2,3

```
                10        20        30        40        50        60
S1Ex2,3  TTTTTCTTGATCATGAAAACGCCAACAAAATTCTGAATCGGCCAAAGAGGTATAATTCAG
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref TTTTTCTTGATCATGAAAACGCCAACAAAATTCTGAATCGGCCAAAGAGGTATAATTCAG
                10        20        30        40        50        60

                70        80        90       100       110       120
S1Ex2,3  GTAAATTGGAAGAGTTTGTTCAAGGGAACCTTGAGAGAGAATGTATGGAAGAAAAGTGTA
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref GTAAATTGGAAGAGTTTGTTCAAGGGAACCTTGAGAGAGAATGTATGGAAGAAAAGTGTA
                70        80        90       100       110       120

               130       140       150       160       170       180
S1Ex2,3  GTTTTGAAGAAGCACGAGAAGTTTTTGAAAACACTGAAAGAACAGTGAGTATTTCCACAT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref GTTTTGAAGAAGCACGAGAAGTTTTTGAAAACACTGAAAGAACAGTGAGTATTTCCACAT
               130       140       150       160       170       180

               190       200       210       220       230       240
S1Ex2,3  AATACCCTTCAGATGCAGAGCATAGAATAGAAAATCTTTAAAAAGACACTTCTCTTTAAA
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref AATACCCTTCAGATGCAGAGCATAGAATAGAAAATCTTTAAAAAGACACTTCTCTTTAAA
               190       200       210       220       230       240

               250       260       270       280       290       300
S1Ex2,3  ATTTTAAAGCATCCATATATATTTATGTATGTTAAATGTTATAAAAGATAGGAAATCAAT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref ATTTTAAAGCATCCATATATATTTATGTATGTTAAATGTTATAAAAGATAGGAAATCAAT
               250       260       270       280       290       300

               310       320       330       340       350       360
S1Ex2,3  ACCAAAACACTTTAGATATTACCGTTAATTTGTCTTCTTTTATTCTTTATAGACTGAATT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref ACCAAAACACTTTAGATATTACCGTTAATTTGTCTTCTTTTATTCTTTATAGACTGAATT
               310       320       330       340       350       360

               370
S1Ex2,3  TTGGAAGCAGTATGTTG
         :::::::::::::::::
Ex2,3Ref TTGGAAGCAGTATGTTG
               370
```

S1: *F9*_Exon 4

```
               10        20        30        40        50        60
S1Ex4  ATGGAGATCAGTGTGAGTCCAATCCATGTTTAAATGGCGGCAGTTGCAAGGATGACATTA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex4Ref ATGGAGATCAGTGTGAGTCCAATCCATGTTTAAATGGCGGCAGTTGCAAGGATGACATTA
               10        20        30        40        50        60

               70        80        90       100       110
S1Ex4  ATTCCTATGAATGTTGGTGTCCCTTTGGATTTGAAGGAAAGAACTGTGAATTAG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex4Ref ATTCCTATGAATGTTGGTGTCCCTTTGGATTTGAAGGAAAGAACTGTGAATTAG
               70        80        90       100       110
```

S1: *F9*_Exon 5

```
             10        20        30        40        50        60
S1Ex5  ATGTAACATGTAACATTAAGAATGGCAGATGCGAGCAGTTTTGTAAAAATAGTGCTGATA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex5Ref ATGTAACATGTAACATTAAGAATGGCAGATGCGAGCAGTTTTGTAAAAATAGTGCTGATA
               10        20        30        40        50        60

               70        80        90       100       110       120
S1Ex5  ACAAGGTGGTTTGCTCCTGTACTGAGGGATATCGACTTGCAGAAAACCAGAAGTCCTGTG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex5Ref ACAAGGTGGTTTGCTCCTGTACTGAGGGATATCGACTTGCAGAAAACCAGAAGTCCTGTG
               70        80        90       100       110       120


S1Ex5  AACCAGCAG
       :::::::::
Ex5Ref AACCAGCAG
```

S1: *F9*_Exon 6

```
               10        20        30        40        50        60
S1Ex6  TGCCATTTCCATGTGGAAGAGTTTCTGTTTCACAAACTTCTAAGCTCACCCGTGCTGAGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex6Ref TGCCATTTCCATGTGGAAGAGTTTCTGTTTCACAAACTTCTAAGCTCACCCGTGCTGAGA
               10        20        30        40        50        60

               70        80        90       100       110       120
S1Ex6  CTGTTTTTCCTGATGTGGACTATGTAAATTCTACTGAAGCTGAAACCATTTTGGATAACA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex6Ref CTGTTTTTCCTGATGTGGACTATGTAAATTCTACTGAAGCTGAAACCATTTTGGATAACA
               70        80        90       100       110       120

              130       140       150       160       170       180
S1Ex6  TCACTCAAAGCACCCAATCATTTAATGACTTCACTCGGGTTGTTGGTGGAGAAGATGCCA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex6Ref TCACTCAAAGCACCCAATCATTTAATGACTTCACTCGGGTTGTTGGTGGAGAAGATGCCA
              130       140       150       160       170       180

              190       200
S1Ex6  AACCAGGTCAATTCCCTTGGCAG
       :::::::::::::::::::::::
Ex6Ref AACCAGGTCAATTCCCTTGGCAG
              190       200
```

S1: *F9*_Exon 7

```
               10        20        30        40        50        60
S1Ex7  GT-GTTTTGAATGGTAAAGTTGATGCATTCTGTGGAGGCTCTATCGTTAATGAAAAATGG
       :: ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex7Ref GTTGTTTTGAATGGTAAAGTTGATGCATTCTGTGGAGGCTCTATCGTTAATGAAAAATGG
               10        20        30        40        50        60

               70        80        90       100       110
S1Ex7  ATTGTAACTGCTGCCCACTGTGTTGAAACTGGTGTTAAAATTACAGTTGTCGCAG
       :::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex7Ref ATTGTAACTGCTGCCCACTGTGTTGAAACTGGTGTTAAAATTACAGTTGTCGCAG
               70        80        90       100       110
```

S1: *F9*_Exon 8

```
               10        20        30        40        50        60
S1Ex8  GTGAACATAATATTGAGGAGACAGAACATACAGAGCAAAAGCGAAATGTGATTCGAATTA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref GTGAACATAATATTGAGGAGACAGAACATACAGAGCAAAAGCGAAATGTGATTCGAATTA
               10        20        30        40        50        60

               70        80        90       100       110       120
S1Ex8  TTCCTCACCACAACTACAATGCAGCTATTAATAAGTACAACCATGACATTGCCCTTCTGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref TTCCTCACCACAACTACAATGCAGCTATTAATAAGTACAACCATGACATTGCCCTTCTGG
               70        80        90       100       110       120

              130       140       150       160       170       180
S1Ex8  AACTGGACGAACCCTTAGTGCTAAACAGCTACGTTACACCTATTTGCATTGCTGACAAGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref AACTGGACGAACCCTTAGTGCTAAACAGCTACGTTACACCTATTTGCATTGCTGACAAGG
              130       140       150       160       170       180

              190       200       210       220       230       240
S1Ex8  AATACACGAACATCTTCCTCAAATTTGGATCTGGCTATGTAAGTGGCTGGGGAAGAGTCT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref AATACACGAACATCTTCCTCAAATTTGGATCTGGCTATGTAAGTGGCTGGGGAAGAGTCT
              190       200       210       220       230       240

              250       260       270       280       290       300
S1Ex8  TCCACAAAGGGAGATCAGCTTTAGTTCTTCAGTACCTTAGAGTTCCACTTGTTGACCGAG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref TCCACAAAGGGAGATCAGCTTTAGTTCTTCAGTACCTTAGAGTTCCACTTGTTGACCGAG
              250       260       270       280       290       300

              310       320       330       340       350       360
S1Ex8  CCACATGTCTTCGATCTACAAAGTTCACCATCTATAACAACATGTTCTGTGCTGGCTTCC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref CCACATGTCTTCGATCTACAAAGTTCACCATCTATAACAACATGTTCTGTGCTGGCTTCC
              310       320       330       340       350       360

              370       380       390       400       410       420
S1Ex8  ATGAAGGAGGTAGAGATTCATGTCAAGGAGATAGTGGGGGACCCCATGTTACTGAAGTGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref ATGAAGGAGGTAGAGATTCATGTCAAGGAGATAGTGGGGGACCCCATGTTACTGAAGTGG
              370       380       390       400       410       420

              430       440       450       460       470       480
S1Ex8  AAGGGACCAGTTTCTTAACTGGAATTATTAGCTGGGGTGAAGAGTGTGCAATGAAAGGCA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref AAGGGACCAGTTTCTTAACTGGAATTATTAGCTGGGGTGAAGAGTGTGCAATGAAAGGCA
              430       440       450       460       470       480

              490       500       510       520       530       540
S1Ex8  AATATGGAATATATACCAAGGTATCCCGGTATGTCAACTGGATTAAGGAAAAAACAAAGC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref AATATGGAATATATACCAAGGTATCCCGGTATGTCAACTGGATTAAGGAAAAAACAAAGC
              490       500       510       520       530       540


S1Ex8  TCACTTAA
       ::::::::
Ex8Ref TCACTTAA
```

Participant 2 (S2)

S2: *F9*_Exon 1

```
               10        20        30        40        50        60
S2Ex1  ATGCAGCGCGTGAACATGATCATGGCAGAATCACCAGGCCTCATCACCATCTGCCTTTTA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex1Ref ATGCAGCGCGTGAACATGATCATGGCAGAATCACCAGGCCTCATCACCATCTGCCTTTTA
               10        20        30        40        50        60

               70        80
S2Ex1  GGATATCTACTCAGTGCTGAATGTACAG
       ::::::::::::::::::::::::::::
Ex1Ref GGATATCTACTCAGTGCTGAATGTACAG
               70        80
```

S2: *F9*_Exon 2,3

```
                 10        20        30        40        50        60
S2Ex2,3  TTTTTCTTGATCATGAAAACGCCAACAAAATTCTGAATCGGCCAAAGAGGTATAATTCAG
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref TTTTTCTTGATCATGAAAACGCCAACAAAATTCTGAATCGGCCAAAGAGGTATAATTCAG
                 10        20        30        40        50        60

                 70        80        90       100       110       120
S2Ex2,3  GTAAATTGGAAGAGTTTGTTCAAGGGAACCTTGAGAGAGAATGTATGGAAGAAAAGTGTA
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref GTAAATTGGAAGAGTTTGTTCAAGGGAACCTTGAGAGAGAATGTATGGAAGAAAAGTGTA
                 70        80        90       100       110       120

                130       140       150       160       170       180
S2Ex2,3  GTTTTGAAGAAGCACGAGAAGTTTTTGAAAACACTGAAAGAACAGTGAGTATTTCCACAT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref GTTTTGAAGAAGCACGAGAAGTTTTTGAAAACACTGAAAGAACAGTGAGTATTTCCACAT
                130       140       150       160       170       180

                190       200       210       220       230       240
S2Ex2,3  AATACCCTTCAGATGCAGAGCATAGAATAGAAAATCTTTAAAAAGACACTTCTCTTTAAA
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref AATACCCTTCAGATGCAGAGCATAGAATAGAAAATCTTTAAAAAGACACTTCTCTTTAAA
                190       200       210       220       230       240

                250       260       270       280       290       300
S2Ex2,3  ATTTTAAAGCATCCATATATATTTATGTATGTTAAATGTTATAAAAGATAGGAAATCAAT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref ATTTTAAAGCATCCATATATATTTATGTATGTTAAATGTTATAAAAGATAGGAAATCAAT
                250       260       270       280       290       300

                310       320       330       340       350       360
S2Ex2,3  ACCAAAACACTTTAGATATTACCGTTAATTTGTCTTCTTTTATTCTTTATAGACTGAATT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref ACCAAAACACTTTAGATATTACCGTTAATTTGTCTTCTTTTATTCTTTATAGACTGAATT
                310       320       330       340       350       360

                370
S2Ex2,3  TTGGAAGCAGTATGTTG
         :::::::::::::::::
Ex2,3Ref TTGGAAGCAGTATGTTG
                370
```

S2: *F9*_Exon 4

```
               10        20        30        40        50        60
S2Ex4  ATGGAGATCAGTGTGAGTCCAATCCATGTTTAAATGGCGGCAGTTGCAAGGATGACATTA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex4Ref ATGGAGATCAGTGTGAGTCCAATCCATGTTTAAATGGCGGCAGTTGCAAGGATGACATTA
               10        20        30        40        50        60

               70        80        90       100       110
S2Ex4  ATTCCTATGAATGTTGGTGTCCCTTTGGATTTGAAGGAAAGAACTGTGAATTAG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex4Ref ATTCCTATGAATGTTGGTGTCCCTTTGGATTTGAAGGAAAGAACTGTGAATTAG
               70        80        90       100       110
```

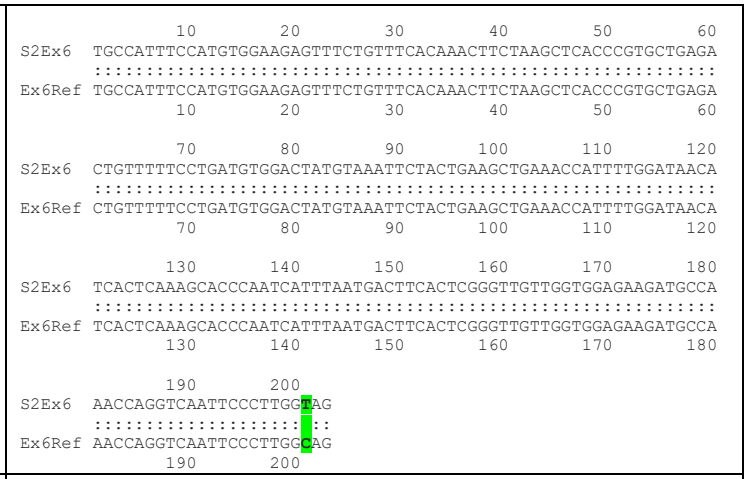
S2: *F9*_Exon 5

```
               10        20        30        40        50        60
S2Ex5  ATGTAACATGTAACATTAAGAATGGCAGATGCGAGCAGTTTTGTAAAAATAGTGCTGATA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex5Ref ATGTAACATGTAACATTAAGAATGGCAGATGCGAGCAGTTTTGTAAAAATAGTGCTGATA
               10        20        30        40        50        60

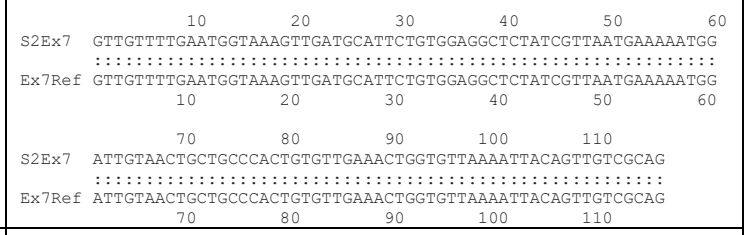
               70        80        90       100       110       120
S2Ex5  ACAAGGTGGTTTGCTCCTGTACTGAGGGATATCGACTTGCAGAAAACCAGAAGTCCTGTG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex5Ref ACAAGGTGGTTTGCTCCTGTACTGAGGGATATCGACTTGCAGAAAACCAGAAGTCCTGTG
               70        80        90       100       110       120


2_Ex5  AACCAGCAG
       :::::::::
Ex5Ref AACCAGCAG
```

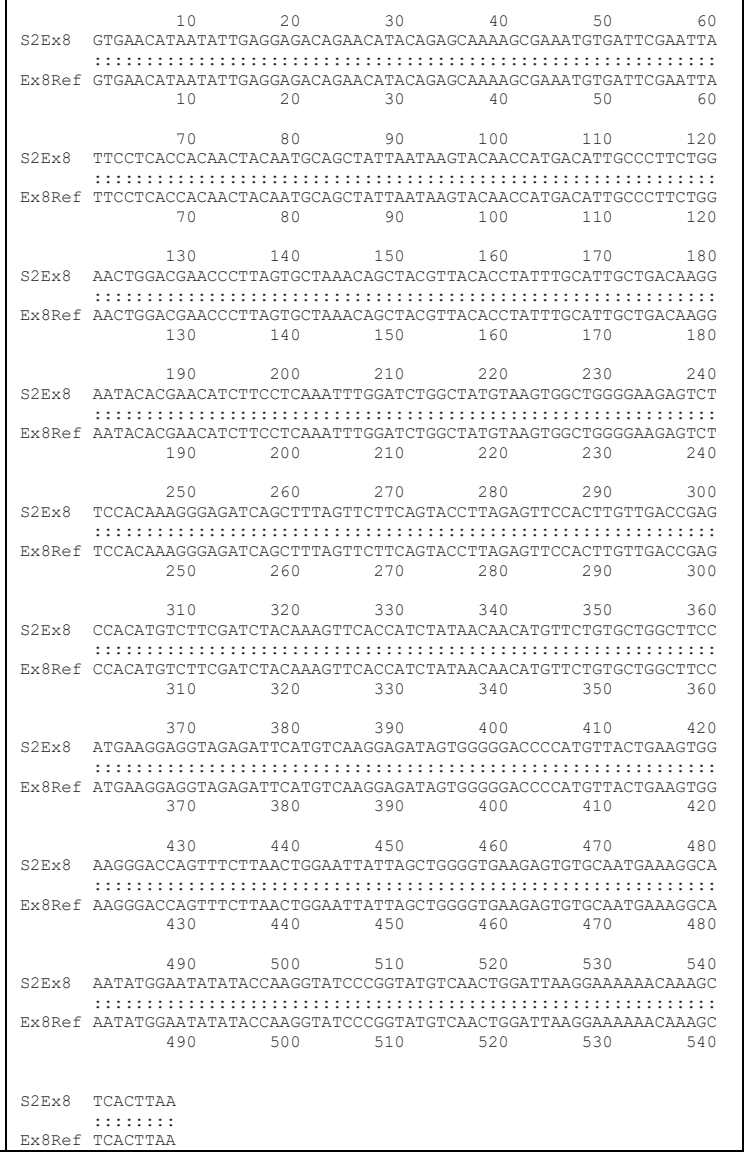
S2: *F9*_Exon 6



S2: *F9*_Exon 7



S2: *F9*_Exon 8



Participant 3 (S3)

S3: *F9*_Exon 1

```
                10        20        30        40        50        60
S3Ex1  ATGCAGCGCGTGAACATGATCATGGCAGAATCACCAGGCCTCATCACCATCTGCCTTTTA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex1Ref ATGCAGCGCGTGAACATGATCATGGCAGAATCACCAGGCCTCATCACCATCTGCCTTTTA
                10        20        30        40        50        60

                70        80
S3Ex1  GGATATCTACTCAGTGCTGAATGTACAG
       :::::::::::::::::::::::::::
Ex1Ref GGATATCTACTCAGTGCTGAATGTACAG
                70        80
```

S3: *F9*_Exon 2,3

```
                 10        20        30        40        50        60
S3Ex2,3  TTTTTCTTGATCATGAAAACGCCAACAAAATTCTGAATCGGCCAAAGAGGTATAATTCAG
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref TTTTTCTTGATCATGAAAACGCCAACAAAATTCTGAATCGGCCAAAGAGGTATAATTCAG
                 10        20        30        40        50        60

                 70        80        90       100       110       120
S3Ex2,3  GTAAATTGGAAGAGTTTGTTCAAGGGAACCTTGAGAGAGAATGTATGGAAGAAAAGTGTA
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref GTAAATTGGAAGAGTTTGTTCAAGGGAACCTTGAGAGAGAATGTATGGAAGAAAAGTGTA
                 70        80        90       100       110       120

                130       140       150       160       170       180
S3Ex2,3  GTTTTGAAGAAGCACGAGAAGTTTTTGAAAACACTGAAAGAACAGTGAGTATTTCCACAT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref GTTTTGAAGAAGCACGAGAAGTTTTTGAAAACACTGAAAGAACAGTGAGTATTTCCACAT
                130       140       150       160       170       180

                190       200       210       220       230       240
S3Ex2,3  AATACCCTTCAGATGCAGAGCATAGAATAGAAAATCTTTAAAAAGACACTTCTCTTTAAA
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref AATACCCTTCAGATGCAGAGCATAGAATAGAAAATCTTTAAAAAGACACTTCTCTTTAAA
                190       200       210       220       230       240

                250       260       270       280       290       300
S3Ex2,3  ATTTTAAAGCATCCATATATATTTATGTATGTTAAATGTTATAAAAGATAGGAAATCAAT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref ATTTTAAAGCATCCATATATATTTATGTATGTTAAATGTTATAAAAGATAGGAAATCAAT
                250       260       270       280       290       300

                310       320       330       340       350       360
S3Ex2,3  ACCAAAACACTTTAGATATTACCGTTAATTTGTCTTCTTTTATTCTTTATAGACTGAATT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref ACCAAAACACTTTAGATATTACCGTTAATTTGTCTTCTTTTATTCTTTATAGACTGAATT
                310       320       330       340       350       360

                370
S3Ex2,3  TTGGAAGCAGTATGTTG
         :::::::::::::::::
Ex2,3Ref TTGGAAGCAGTATGTTG
                370
```

S3: *F9*_Exon 4

```
                10        20        30        40        50        60
S3Ex4  ATGGAGATCAGTGTGAGTCCAATCCATGTTTAAATGGCGGCAGTTGCAAGGATGACATTA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex4Ref ATGGAGATCAGTGTGAGTCCAATCCATGTTTAAATGGCGGCAGTTGCAAGGATGACATTA
                10        20        30        40        50        60

                70        80        90       100       110
S3Ex4  ATTCCTATGAATGTTGGTGTCCCTTTGGATTTGAAGGAAAGAACTGTGAATTAG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex4Ref ATTCCTATGAATGTTGGTGTCCCTTTGGATTTGAAGGAAAGAACTGTGAATTAG
                70        80        90       100       110
```

S3: *F9*_Exon 5

```
                10        20        30        40        50        60
S3Ex5  ATGTAACATGTAACATTAAGAATGGCAGATGCGAGCAGTTTTGTAAAAATAGTGCTGATA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex5Ref ATGTAACATGTAACATTAAGAATGGCAGATGCGAGCAGTTTTGTAAAAATAGTGCTGATA
                10        20        30        40        50        60

                70        80        90       100       110       120
S3Ex5  ACAAGGTGGTTTGCTCCTGTACTGAGGGATATCGACTTGCAGAAAACCAGAAGTCCTGTG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex5Ref ACAAGGTGGTTTGCTCCTGTACTGAGGGATATCGACTTGCAGAAAACCAGAAGTCCTGTG
                70        80        90       100       110       120


S3Ex5  AACCAGCAG
       :::::::::
Ex5Ref AACCAGCAG
```

S3: *F9*_Exon 6

```
               10        20        30        40        50        60
S3Ex6  TGCCATTTCCATGTGGAAGAGTTTCTGTTTCACAAACTTCTAAGCTCACCCGTGCTGAGA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex6Ref TGCCATTTCCATGTGGAAGAGTTTCTGTTTCACAAACTTCTAAGCTCACCCGTGCTGAGA
               10        20        30        40        50        60

               70        80        90       100       110       120
S3Ex6  CTGTTTTTCCTGATGTGGACTATGTAAATTCTACTGAAGCTGAAACCATTTTGGATAACA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex6Ref CTGTTTTTCCTGATGTGGACTATGTAAATTCTACTGAAGCTGAAACCATTTTGGATAACA
               70        80        90       100       110       120

              130       140       150       160       170       180
S3Ex6  TCACTCAAAGCACCCAATCATTTAATGACTTCACTCGGGTTGTTGGTGGAGAAGATGCCA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex6Ref TCACTCAAAGCACCCAATCATTTAATGACTTCACTCGGGTTGTTGGTGGAGAAGATGCCA
              130       140       150       160       170       180

              190       200
S3Ex6  AACCAGGTCAATTCCCTTGGCAG
       :::::::::::::::::::::::
Ex6Ref AACCAGGTCAATTCCCTTGGCAG
              190       200
```

S3: *F9*_Exon 7

```
               10        20        30        40        50        60
S3Ex7  GTTGTTTTGAATGGTAAAGTTGATGCATTCTGTGGAGGCTCTATCGTTAATGAAAAATGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex7Ref GTTGTTTTGAATGGTAAAGTTGATGCATTCTGTGGAGGCTCTATCGTTAATGAAAAATGG
               10        20        30        40        50        60

               70        80        90       100       110
S3Ex7  ATTGTAACTGCTGCCCACTGTGTTGAAACTGGTGTTAAAATTACAGTTGTCGCAG
       :::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex7Ref ATTGTAACTGCTGCCCACTGTGTTGAAACTGGTGTTAAAATTACAGTTGTCGCAG
               70        80        90       100       110
```

S3: *F9*_Exon 8

```
               10        20        30        40        50        60
S3Ex8  GTGAACATAATATTGAGGAGACAGAACATACAGAGCAAAAGCGAAATGTGATTCGAATTA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref GTGAACATAATATTGAGGAGACAGAACATACAGAGCAAAAGCGAAATGTGATTCGAATTA
               10        20        30        40        50        60

               70        80        90       100       110       120
S3Ex8  TTCCTCACCACAACTACAATGCAGCTATTAATAAGTACAACCATGACATTGCCCTTCTGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref TTCCTCACCACAACTACAATGCAGCTATTAATAAGTACAACCATGACATTGCCCTTCTGG
               70        80        90       100       110       120

              130       140       150       160       170       180
S3Ex8  AACTGGACGAACCCTTAGTGCTAAACAGCTACGTTACACCTATTTGCATTGCTGACAAGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref AACTGGACGAACCCTTAGTGCTAAACAGCTACGTTACACCTATTTGCATTGCTGACAAGG
              130       140       150       160       170       180

              190       200       210       220       230       240
S3Ex8  AATACACGAACATCTTCCTCAAATTTGGATCTGGCTATGTAAGTGGCTGGGGAAGAGTCT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref AATACACGAACATCTTCCTCAAATTTGGATCTGGCTATGTAAGTGGCTGGGGAAGAGTCT
              190       200       210       220       230       240

              250       260       270       280       290       300
S3Ex8  TCCACAAAGGGAGATCAGCTTTAGTTCTTCAGTACCTTAGAGTTCCACTTGTTGACCGAG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref TCCACAAAGGGAGATCAGCTTTAGTTCTTCAGTACCTTAGAGTTCCACTTGTTGACCGAG
              250       260       270       280       290       300

              310       320       330       340       350       360
S3Ex8  CCACATGTCTTCGATCTACAAAGTTCACCATCTATAACAACATGTTCTGTGCTGGCTTCC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref CCACATGTCTTCGATCTACAAAGTTCACCATCTATAACAACATGTTCTGTGCTGGCTTCC
              310       320       330       340       350       360

              370       380       390       400       410       420
S3Ex8  ATGAAGGAGGTAGAGATTCATGTCAAGGAGATAGTGGGGGACCCCATGTTACTGAAGTGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref ATGAAGGAGGTAGAGATTCATGTCAAGGAGATAGTGGGGGACCCCATGTTACTGAAGTGG
              370       380       390       400       410       420

              430       440       450       460       470       480
S3Ex8  AAGGGACCAGTTTCTTAACTGGAATTATTAGCTGGGGTGAAGAGTGTGCAATGAAAGGCA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref AAGGGACCAGTTTCTTAACTGGAATTATTAGCTGGGGTGAAGAGTGTGCAATGAAAGGCA
              430       440       450       460       470       480

              490       500       510       520       530       540
S3Ex8  AATATGGAATATATACCAAGGTATCCCGGTATGTCAACTGGATTAAGGAAAAAACAAAGC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref AATATGGAATATATACCAAGGTATCCCGGTATGTCAACTGGATTAAGGAAAAAACAAAGC
              490       500       510       520       530       540


S3Ex8  TCACTTAA
       ::::::::
Ex8Ref TCACTTAA
```

Participant 4 (S4)

S4: *F9*_Exon 1

```
               10        20        30        40        50        60
S4Ex1  ATGCAGCGCGTGAACATGATCATGGCAGAATCACCAGGCCTCATCACCATCTGCCTTTTA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex1Ref ATGCAGCGCGTGAACATGATCATGGCAGAATCACCAGGCCTCATCACCATCTGCCTTTTA
               10        20        30        40        50        60

               70        80
S4Ex1  GGATATCTACTCAGTGCTGAATGTACAG
       ::::::::::::::::::::::::::::
Ex1Ref GGATATCTACTCAGTGCTGAATGTACAG
               70        80
```

S4: *F9*_Exon 2,3

```
                10        20        30        40        50        60
S4Ex2,3  TTTTTCTTGATCATGAAAACGCCAACAAAATTCTGAATCGGCCAAAGAGGTATAATTCAG
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref TTTTTCTTGATCATGAAAACGCCAACAAAATTCTGAATCGGCCAAAGAGGTATAATTCAG
                10        20        30        40        50        60

                70        80        90       100       110       120
S4Ex2,3  GTAAATTGGAAGAGTTTGTTCAAGGGAACCTTGAGAGAGAATGTATGGAAGAAAAGTGTA
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref GTAAATTGGAAGAGTTTGTTCAAGGGAACCTTGAGAGAGAATGTATGGAAGAAAAGTGTA
                70        80        90       100       110       120

               130       140       150       160       170
S4Ex2,3  GTTTTGAAGAAGCACGAGAAGTTTTTGAAAACACTGAAAGAG-AGTGAGTATTTCCACAT
         ::::::::::::::::::::::::::::::::::::::::::: ::::::::::::::::
Ex2,3Ref GTTTTGAAGAAGCACGAGAAGTTTTTGAAAACACTGAAAGAACAGTGAGTATTTCCACAT
               130       140       150       160       170       180

       180       190       200       210       220       230
S4Ex2,3  AATACCCTTCAGATGCAGAGCATAGAATAGAAAATCTTTAAAAAGACACTTCTCTTTAAA
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref AATACCCTTCAGATGCAGAGCATAGAATAGAAAATCTTTAAAAAGACACTTCTCTTTAAA
               190       200       210       220       230       240

       240       250       260       270       280       290
S4Ex2,3  ATTTTAAAGCATCCATATATATTTATGTATGTTAAATGTTATAAAAGATAGGAAATCAAT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref ATTTTAAAGCATCCATATATATTTATGTATGTTAAATGTTATAAAAGATAGGAAATCAAT
              250       260       270       280       290       300

       300       310       320       330       340       350
S4Ex2,3  ACCAAAACACTTTAGATATTACCGTTAATTTGTCTTCTTTTATTCTTTATAGACTGAATT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref ACCAAAACACTTTAGATATTACCGTTAATTTGTCTTCTTTTATTCTTTATAGACTGAATT
               310       320       330       340       350       360

       360       370
S4Ex2,3  TTGGAAGCAGTATGTTG
         :::::::::::::::::
Ex2,3Ref TTGGAAGCAGTATGTTG
               370
```

S4: *F9*_Exon 4

```
               10        20        30        40        50        60
S4Ex4  ATGGAGATCAGTGTGAGTCCAATCCATGTTTAAATGGCGGCAGTTGCAAGGATGACATTA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex4Ref ATGGAGATCAGTGTGAGTCCAATCCATGTTTAAATGGCGGCAGTTGCAAGGATGACATTA
               10        20        30        40        50        60

               70        80        90       100       110
S4Ex4  ATTCCTATGAATGTTGGTGTCCCTTTGGATTTGAAGGAAAGAACTGTGAATTAG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex4Ref ATTCCTATGAATGTTGGTGTCCCTTTGGATTTGAAGGAAAGAACTGTGAATTAG
               70        80        90       100       110
```

S4: *F9*_Exon 5

```
               10        20        30        40        50        60
S4Ex5  ATGTAACATGTAACATTAAGAATGGCAGATGCGAGCAGTTTTGTAAAAATAGTGCTGATA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex5Ref ATGTAACATGTAACATTAAGAATGGCAGATGCGAGCAGTTTTGTAAAAATAGTGCTGATA
               10        20        30        40        50        60

               70        80        90       100       110       120
S4Ex5  ACAAGGTGGTTTGCTCCTGTACTGAGGGATATCGACTTGCAGAAAACCAGAAGTCCTGTG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex5Ref ACAAGGTGGTTTGCTCCTGTACTGAGGGATATCGACTTGCAGAAAACCAGAAGTCCTGTG
               70        80        90       100       110       120


S4Ex5  AACCAGCAG
       :::::::::
Ex5Ref AACCAGCAG
```

S4: *F9*_Exon 6

```
                10        20        30        40        50        60
S4Ex6   TGCCATTTCCATGTGGAAGAGTTTCTGTTTCACAAACTTCTAAGCTCACCCGTGCTGAGA
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex6Ref  TGCCATTTCCATGTGGAAGAGTTTCTGTTTCACAAACTTCTAAGCTCACCCGTGCTGAGA
                10        20        30        40        50        60

                70        80        90       100       110       120
S4Ex6   CTGTTTTTCCTGATGTGGACTATGTAAATTCTACTGAAGCTGAAACCATTTTGGATAACA
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex6Ref  CTGTTTTTCCTGATGTGGACTATGTAAATTCTACTGAAGCTGAAACCATTTTGGATAACA
                70        80        90       100       110       120

               130       140       150       160       170       180
S4Ex6   TCACTCAAAGCACCCAATCATTTAATGACTTCACTCGGGTTGTTGGTGGAGAAGATGCCA
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex6Ref  TCACTCAAAGCACCCAATCATTTAATGACTTCACTCGGGTTGTTGGTGGAGAAGATGCCA
               130       140       150       160       170       180

               190       200
S4Ex6   AACCAGGTCAATTCCCTTGGCAG
        :::::::::::::::::::::::
Ex6Ref  AACCAGGTCAATTCCCTTGGCAG
               190       200
```

S4: *F9*_Exon 7

```
                10        20        30        40        50        60
S4Ex7   GTTGTTTTGAATGGTAAAGTTGATGCATTCTGTGGAGGCTCTATCGTTAATGAAAAATGG
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex7Ref  GTTGTTTTGAATGGTAAAGTTGATGCATTCTGTGGAGGCTCTATCGTTAATGAAAAATGG
                10        20        30        40        50        60

                70        80        90       100       110
S4Ex7   ATTGTAACTGCTGCCCACTGTGTTGAAACTGGTGTTAAAATTACAGTTGTCGCAG
        :::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex7Ref  ATTGTAACTGCTGCCCACTGTGTTGAAACTGGTGTTAAAATTACAGTTGTCGCAG
                70        80        90       100       110
```

S4: *F9*_Exon 8

```
                10        20        30        40        50        60
S4Ex8   GTGAACATAATATTGAGGAGACAGAACATACAGAGCAAAAGCGAAATGTGATTCGAATTA
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref  GTGAACATAATATTGAGGAGACAGAACATACAGAGCAAAAGCGAAATGTGATTCGAATTA
                10        20        30        40        50        60

                70        80        90       100       110       120
S4Ex8   TTCCTCACCACAACTACAATGCAGCTATTAATAAGTACAACCATGACATTGCCCTTCTGG
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref  TTCCTCACCACAACTACAATGCAGCTATTAATAAGTACAACCATGACATTGCCCTTCTGG
                70        80        90       100       110       120

               130       140       150       160       170       180
S4Ex8   AACTGGACGAACCCTTAGTGCTAAACAGCTACGTTACACCTATTTGCATTGCTGACAAGG
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref  AACTGGACGAACCCTTAGTGCTAAACAGCTACGTTACACCTATTTGCATTGCTGACAAGG
               130       140       150       160       170       180

               190       200       210       220       230       240
S4Ex8   AATACACGAACATCTTCCTCAAATTTGGATCTGGCTATGTAAGTGGCTGGGGAAGAGTCT
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref  AATACACGAACATCTTCCTCAAATTTGGATCTGGCTATGTAAGTGGCTGGGGAAGAGTCT
               190       200       210       220       230       240

               250       260       270       280       290       300
S4Ex8   TCCACAAAGGGAGATCAGCTTTAGTTCTTCAGTACCTTAGAGTTCCACTTGTTGACCGAG
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref  TCCACAAAGGGAGATCAGCTTTAGTTCTTCAGTACCTTAGAGTTCCACTTGTTGACCGAG
               250       260       270       280       290       300

               310       320       330       340       350       360
S4Ex8   CCACATGTCTTCGATCTACAAAGTTCACCATCTATAACAACATGTTCTGTGCTGGCTTCC
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref  CCACATGTCTTCGATCTACAAAGTTCACCATCTATAACAACATGTTCTGTGCTGGCTTCC
               310       320       330       340       350       360

               370       380       390       400       410       420
S4Ex8   ATGAAGGAGGTAGAGATTCATGTCAAGGAGATAGTGGGGGACCCCATGTTACTGAAGTGG
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref  ATGAAGGAGGTAGAGATTCATGTCAAGGAGATAGTGGGGGACCCCATGTTACTGAAGTGG
               370       380       390       400       410       420

               430       440       450       460       470       480
S4Ex8   AAGGGACCAGTTTCTTAACTGGAATTATTAGCTGGGGTGAAGAGTGTGCAATGAAAGGCA
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref  AAGGGACCAGTTTCTTAACTGGAATTATTAGCTGGGGTGAAGAGTGTGCAATGAAAGGCA
               430       440       450       460       470       480

               490       500       510       520       530       540
S4Ex8   AATATGGAATATATACCAAGGTATCCCGGTATGTCAACTGGATTAAGGAAAAAACAAAGC
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref  AATATGGAATATATACCAAGGTATCCCGGTATGTCAACTGGATTAAGGAAAAAACAAAGC
               490       500       510       520       530       540


S4Ex8   TCACTTAA
        ::::::::
Ex8Ref  TCACTTAA
```

Participant 5 (S5)

S5: *F9*_Exon 1

```
               10        20        30        40        50        60
S5Ex1  ATGCAGCGCGTGAACATGATCATGGCAGAATCACCAGGCCTCATCACCATCTGCCTTTTA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex1Ref ATGCAGCGCGTGAACATGATCATGGCAGAATCACCAGGCCTCATCACCATCTGCCTTTTA
               10        20        30        40        50        60

               70        80
S5Ex1  GGATATCTACTCAGTGCTGAATGTACAG
       ::::::::::::::::::::::::::::
Ex1Ref GGATATCTACTCAGTGCTGAATGTACAG
               70        80
```

S5: *F9*_Exon 2,3

```
                 10        20        30        40        50        60
S5Ex2,3  TTTTTCTTGATCATGAAAACGCCAACAAAATTCTGAATCGGCCAAAGAGGTATAATTCAG
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref TTTTTCTTGATCATGAAAACGCCAACAAAATTCTGAATCGGCCAAAGAGGTATAATTCAG
                 10        20        30        40        50        60

                 70        80        90       100       110       120
S5Ex2,3  GTAAATTGGAAGAGTTTGTTCAAGGGAACCTTGAGAGAGAATGTATGGAAGAAAAGTGTA
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref GTAAATTGGAAGAGTTTGTTCAAGGGAACCTTGAGAGAGAATGTATGGAAGAAAAGTGTA
                 70        80        90       100       110       120

                130       140       150       160       170       180
S5Ex2,3  GTTTTGAAGAAGCACGAGAAGTTTTTGAAAACACTGAAAGAACAGTGAGTATTTCCACAT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref GTTTTGAAGAAGCACGAGAAGTTTTTGAAAACACTGAAAGAACAGTGAGTATTTCCACAT
                130       140       150       160       170       180

                190       200       210       220       230       240
S5Ex2,3  AATACCCTTCAGATGCAGAGCATAGAATAGAAAATCTTTAAAAAGACACTTCTCTTTAAA
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref AATACCCTTCAGATGCAGAGCATAGAATAGAAAATCTTTAAAAAGACACTTCTCTTTAAA
                190       200       210       220       230       240

                250       260       270       280       290       300
S5Ex2,3  ATTTTAAAGCATCCATATATATTTATGTATGTTAAATGTTATAAAAGATAGGAAATCAAT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref ATTTTAAAGCATCCATATATATTTATGTATGTTAAATGTTATAAAAGATAGGAAATCAAT
                250       260       270       280       290       300

                310       320       330       340       350       360
S5Ex2,3  ACCAAAACACTTTAGATATTACCGTTAATTTGTCTTCTTTTATTCTTTATAGACTGAATT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref ACCAAAACACTTTAGATATTACCGTTAATTTGTCTTCTTTTATTCTTTATAGACTGAATT
                310       320       330       340       350       360

                370
S5Ex2,3  TTGGAAGCAGTATGTTG
         :::::::::::::::::
Ex2,3Ref TTGGAAGCAGTATGTTG
                370
```

S5: *F9*_Exon 4

```
               10        20        30        40        50        60
S5Ex4  ATGGAGATCAGTGTGAGTCCAATCCATGTTTAAATGGCGGCAGTTGCAAGGATGACATTA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex4Ref ATGGAGATCAGTGTGAGTCCAATCCATGTTTAAATGGCGGCAGTTGCAAGGATGACATTA
               10        20        30        40        50        60

               70        80        90       100       110
S5Ex4  ATTCCTATGAATGTTGGTGTCCCTTTGGATTTGAAGGAAAGAACTGTGAATTAG
       :::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex4Ref ATTCCTATGAATGTTGGTGTCCCTTTGGATTTGAAGGAAAGAACTGTGAATTAG
               70        80        90       100       110
```

S5: *F9*_Exon 5

```
               10        20        30        40        50        60
S5Ex5  ATGTAACATGTAACATTAAGAATGGCAGATGCGAGCAGTTTTGTAAAAATAGTGCTGATA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex5Ref ATGTAACATGTAACATTAAGAATGGCAGATGCGAGCAGTTTTGTAAAAATAGTGCTGATA
               10        20        30        40        50        60

               70        80        90       100       110       120
S5Ex5  ACAAGGTGGTTTGCTCCTGTACTGAGGGATATCGACTTGCAGAAAACCAGAAGTCCTGTG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex5Ref ACAAGGTGGTTTGCTCCTGTACTGAGGGATATCGACTTGCAGAAAACCAGAAGTCCTGTG
               70        80        90       100       110       120


S5Ex5  AACCAGCAG
       :::::::::
Ex5Ref AACCAGCAG
```

S5: *F9*_Exon 6

```
                10        20        30        40        50        60
S5Ex6   TGCCATTTCCATGTGGAAGAGTTTCTGTTTCACAAACTTCTAAGCTCACCCGTGCTGAGA
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex6Ref  TGCCATTTCCATGTGGAAGAGTTTCTGTTTCACAAACTTCTAAGCTCACCCGTGCTGAGA
                10        20        30        40        50        60

                70        80        90       100       110       120
S5Ex6   CTGTTTTTCCTGATGTGGACTATGTAAATTCTACTGAAGCTGAAACCATTTTGGATAACA
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex6Ref  CTGTTTTTCCTGATGTGGACTATGTAAATTCTACTGAAGCTGAAACCATTTTGGATAACA
                70        80        90       100       110       120

               130       140       150       160       170       180
S5Ex6   TCACTCAAAGCACCCAATCATTTAATGACTTCACTCGGGTTGTTGGTGGAGAAGATGCCA
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex6Ref  TCACTCAAAGCACCCAATCATTTAATGACTTCACTCGGGTTGTTGGTGGAGAAGATGCCA
               130       140       150       160       170       180

               190       200
S5Ex6   AACCAGGTCAATTCCCTTGGCAG
        :::::::::::::::::::::::
Ex6Ref  AACCAGGTCAATTCCCTTGGCAG
               190       200
```

S5: *F9*_Exon 7

```
                10        20        30        40        50        60
S5Ex7   GTTGTTTTGAATGGTAAAGTTGATGCATTCTGTGGAGGCTCTATCGTTAATGAAAAATGG
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex7Ref  GTTGTTTTGAATGGTAAAGTTGATGCATTCTGTGGAGGCTCTATCGTTAATGAAAAATGG
                10        20        30        40        50        60

                70        80        90       100       110
S5Ex7   ATTGTAACTGCTGCCCACTGTGTTGAAACTGGTGTTAAAATTACAGTTGTCGCAG
        :::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex7Ref  ATTGTAACTGCTGCCCACTGTGTTGAAACTGGTGTTAAAATTACAGTTGTCGCAG
                70        80        90       100       110
```

S5: *F9*_Exon 8

```
                10        20        30        40        50        60
S5Ex8   GTGAACATAATATTGAGGAGACAGAACATACAGAGCAAAAGCGAAATGTGATTCGAATTA
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref  GTGAACATAATATTGAGGAGACAGAACATACAGAGCAAAAGCGAAATGTGATTCGAATTA
                10        20        30        40        50        60

                70        80        90       100       110       120
S5Ex8   TTCCTCACCACAACTACAATGCAGCTATTAATAAGTACAACCATGACATTGCCCTTCTGG
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref  TTCCTCACCACAACTACAATGCAGCTATTAATAAGTACAACCATGACATTGCCCTTCTGG
                70        80        90       100       110       120

               130       140       150       160       170       180
S5Ex8   AACTGGACGAACCCTTAGTGCTAAACAGCTACGTTACACCTATTTGCATTGCTGACAAGG
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref  AACTGGACGAACCCTTAGTGCTAAACAGCTACGTTACACCTATTTGCATTGCTGACAAGG
               130       140       150       160       170       180

               190       200       210       220       230       240
S5Ex8   AATACACGAACATCTTCCTCAAATTTGGATCTGGCTATGTAAGTGGCTGGGGAAGAGTCT
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref  AATACACGAACATCTTCCTCAAATTTGGATCTGGCTATGTAAGTGGCTGGGGAAGAGTCT
               190       200       210       220       230       240

               250       260       270       280       290       300
S5Ex8   TCCACAAAGGGAGATCAGCTTTAGTTCTTCAGTACCTTAGAGTTCCACTTGTTGACCGAG
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref  TCCACAAAGGGAGATCAGCTTTAGTTCTTCAGTACCTTAGAGTTCCACTTGTTGACCGAG
               250       260       270       280       290       300

               310       320       330       340       350       360
S5Ex8   CCACATGTCTTCGATCTACAAAGTTCACCATCTATAACAACATGTTCTGTGCTGGCTTCC
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref  CCACATGTCTTCGATCTACAAAGTTCACCATCTATAACAACATGTTCTGTGCTGGCTTCC
               310       320       330       340       350       360

               370       380       390       400       410       420
S5Ex8   ATGAAGGAGGTAGAGATTCATGTCAAGGAGATAGTGGGGGACCCCATGTTACTGAAGTGG
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref  ATGAAGGAGGTAGAGATTCATGTCAAGGAGATAGTGGGGGACCCCATGTTACTGAAGTGG
               370       380       390       400       410       420

               430       440       450       460       470       480
S5Ex8   AAGGGACCAGTTTCTTAACTGGAATTATTAGCTGGGGTGAAGAGTGTGCAATGAAAGGCA
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref  AAGGGACCAGTTTCTTAACTGGAATTATTAGCTGGGGTGAAGAGTGTGCAATGAAAGGCA
               430       440       450       460       470       480

               490       500       510       520       530       540
S5Ex8   AATATGGAATATATACCAAGGTATCCCGGTATGTCAACTGGATTAAGGAAAAAACAAAGC
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref  AATATGGAATATATACCAAGGTATCCCGGTATGTCAACTGGATTAAGGAAAAAACAAAGC
               490       500       510       520       530       540


S5Ex8   TCACTTAA
        ::::::::
Ex8Ref  TCACTTAA
```

Participant 6 (S6)

S6: *F9*_Exon 1

```
               10        20        30        40        50        60
S6Ex1   ATGCAGCGCGTGAACATGATCATGGCAGAATCACCAGGCCTCATCACCATCTGCCTTTTA
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex1Ref ATGCAGCGCGTGAACATGATCATGGCAGAATCACCAGGCCTCATCACCATCTGCCTTTTA
               10        20        30        40        50        60

               70        80
S6Ex1   GGATATCTACTCAGTGCTGAATGTACAG
        ::::::::::::::::::::::::::::
Ex1Ref GGATATCTACTCAGTGCTGAATGTACAG
               70        80
```

S6: *F9*_Exon 2,3

```
                 10        20        30        40        50        60
S6Ex2,3  TTTTTCTTGATCATGAAAACGCCAACAAAATTCTGAATCGGCCAAAGAGGTATAATTCAG
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref TTTTTCTTGATCATGAAAACGCCAACAAAATTCTGAATCGGCCAAAGAGGTATAATTCAG
                 10        20        30        40        50        60

                 70        80        90       100       110       120
S6Ex2,3  GTAAATTGGAAGAGTTTGTTCAAGGGAACCTTGAGAGAGAATGTATGGAAGAAAAGTGTA
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref GTAAATTGGAAGAGTTTGTTCAAGGGAACCTTGAGAGAGAATGTATGGAAGAAAAGTGTA
                 70        80        90       100       110       120

                130       140       150       160       170       180
S6Ex2,3  GTTTTGAAGAAGCACGAGAAGTTTTTGAAAACACTGAAAGAACAGTGAGTATTTCCACAT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref GTTTTGAAGAAGCACGAGAAGTTTTTGAAAACACTGAAAGAACAGTGAGTATTTCCACAT
                130       140       150       160       170       180

                190       200       210       220       230       240
S6Ex2,3  AATACCCTTCAGATGCAGAGCATAGAATAGAAAATCTTTAAAAAGACACTTCTCTTTAAA
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref AATACCCTTCAGATGCAGAGCATAGAATAGAAAATCTTTAAAAAGACACTTCTCTTTAAA
                190       200       210       220       230       240

                250       260       270       280       290       300
S6Ex2,3  ATTTTAAAGCATCCATATATATTTATGTATGTTAAATGTTATAAAAGATAGGAAATCAAT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref ATTTTAAAGCATCCATATATATTTATGTATGTTAAATGTTATAAAAGATAGGAAATCAAT
                250       260       270       280       290       300

                310       320       330       340       350       360
S6Ex2,3  ACCAAAACACTTTAGATATTACCGTTAATTTGTCTTCTTTTATTCTTTATAGACTGAATT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref ACCAAAACACTTTAGATATTACCGTTAATTTGTCTTCTTTTATTCTTTATAGACTGAATT
                310       320       330       340       350       360

                370
S6Ex2,3  TTGGAAGCAGTATGTTG
         :::::::::::::::::
Ex2,3Ref TTGGAAGCAGTATGTTG
                370
```

S6: *F9*_Exon 4

```
              10        20        30        40        50
S6Ex4   ATGGAGATCAGTGTGAGTCCAATCCATGTTTAA-TGGCGGCAGTTGCAAGGATGACATTA
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex4Ref ATGGAGATCAGTGTGAGTCCAATCCATGTTTAAATGGCGGCAGTTGCAAGGATGACATTA
               10        20        30        40        50        60

      60        70        80        90       100       110
S6Ex4   ATTCCTATGAATGTTGGTGTCCCTTTGGATTTGAAGGAAAGAACTGTGAATTAG
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex4Ref ATTCCTATGAATGTTGGTGTCCCTTTGGATTTGAAGGAAAGAACTGTGAATTAG
               70        80        90       100       110
```

S6: *F9*_Exon 5

```
               10        20        30        40        50        60
S6Ex5   ATGTAACATGTAACATTAAGAATGGCAGATGCGAGCAGTTTTGTAAAAATAGTGCTGATA
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex5Ref ATGTAACATGTAACATTAAGAATGGCAGATGCGAGCAGTTTTGTAAAAATAGTGCTGATA
               10        20        30        40        50        60

               70        80        90       100       110       120
S6Ex5   ACAAGGTGGTTTGCTCCTGTACTGAGGGATATCGACTTGCAGAAAACCAGAAGTCCTGTG
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex5Ref ACAAGGTGGTTTGCTCCTGTACTGAGGGATATCGACTTGCAGAAAACCAGAAGTCCTGTG
               70        80        90       100       110       120


S6Ex5   AACCAGCAG
        :::::::::
Ex5Ref AACCAGCAG
```

S6: *F9*_Exon 6

```
                10        20        30        40        50        60
S6Ex6   TGCCATTTCCATGTGGAAGAGTTTCTGTTTCACAAACTTCTAAGCTCACCCGTGCTGAGA
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex6Ref  TGCCATTTCCATGTGGAAGAGTTTCTGTTTCACAAACTTCTAAGCTCACCCGTGCTGAGA
                10        20        30        40        50        60

                70        80        90       100       110       120
S6Ex6   CTGTTTTTCCTGATGTGGACTATGTAAATTCTACTGAAGCTGAAACCATTTTGGATAACA
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex6Ref  CTGTTTTTCCTGATGTGGACTATGTAAATTCTACTGAAGCTGAAACCATTTTGGATAACA
                70        80        90       100       110       120

               130       140       150       160       170       180
S6Ex6   TCACTCAAAGCACCCAATCATTTAATGACTTCACTCGGGTTGTTGGTGGAGAAGATGCCA
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex6Ref  TCACTCAAAGCACCCAATCATTTAATGACTTCACTCGGGTTGTTGGTGGAGAAGATGCCA
               130       140       150       160       170       180

               190       200
S6Ex6   AACCAGGTCAATTCCCTTGGCAG
        :::::::::::::::::::::::
Ex6Ref  AACCAGGTCAATTCCCTTGGCAG
               190       200
```

S6: *F9*_Exon 7

```
                10        20        30        40        50        60
S6Ex7   GTTGTTTTGAATGGTAAAGTTGATGCATTCTGTGGAGGCTCTATCGTTAATGAAAAATGG
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex7Ref  GTTGTTTTGAATGGTAAAGTTGATGCATTCTGTGGAGGCTCTATCGTTAATGAAAAATGG
                10        20        30        40        50        60

                70        80        90       100       110
S6Ex7   ATTGTAACTGCTGCCCACTGTGTTGAAACTGGTGTTAAAATTACAGTTGTCGCAG
        :::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex7Ref  ATTGTAACTGCTGCCCACTGTGTTGAAACTGGTGTTAAAATTACAGTTGTCGCAG
                70        80        90       100       110
```

S6: *F9*_Exon 8

```
                10        20        30        40        50        60
S6Ex8   GTGAACATAATATTGAGGAGACAGAACATACAGAGCAAAAGCGAAATGTGATTCGAATTA
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref  GTGAACATAATATTGAGGAGACAGAACATACAGAGCAAAAGCGAAATGTGATTCGAATTA
                10        20        30        40        50        60

                70        80        90       100       110       120
S6Ex8   TTCCTCACCACAACTACAATGCAGCTATTAATAAGTACAACCATGACATTGCCCTTCTGG
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref  TTCCTCACCACAACTACAATGCAGCTATTAATAAGTACAACCATGACATTGCCCTTCTGG
                70        80        90       100       110       120

               130       140       150       160       170       180
S6Ex8   AACTGGACGAACCCTTAGTGCTAAACAGCTACGTTACACCTATTTGCATTGCTGACAAGG
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref  AACTGGACGAACCCTTAGTGCTAAACAGCTACGTTACACCTATTTGCATTGCTGACAAGG
               130       140       150       160       170       180

               190       200       210       220       230       240
S6Ex8   AATACACGAACATCTTCCTCAAATTTGGATCTGGCTATGTAAGTGGCTGGGGAAGAGTCT
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref  AATACACGAACATCTTCCTCAAATTTGGATCTGGCTATGTAAGTGGCTGGGGAAGAGTCT
               190       200       210       220       230       240

               250       260       270       280       290       300
S6Ex8   TCCACAAAGGGAGATCAGCTTTAGTTCTTCAGTACCTTAGAGTTCCACTTGTTGACCGAG
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref  TCCACAAAGGGAGATCAGCTTTAGTTCTTCAGTACCTTAGAGTTCCACTTGTTGACCGAG
               250       260       270       280       290       300

               310       320       330       340       350       360
S6Ex8   CCACATGTCTTCGATCTACAAAGTTCACCATCTATAACAACATGTTCTGTGCTGGCTTCC
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref  CCACATGTCTTCGATCTACAAAGTTCACCATCTATAACAACATGTTCTGTGCTGGCTTCC
               310       320       330       340       350       360

               370       380       390       400       410       420
S6Ex8   ATGAAGGAGGTAGAGATTCATGTCAAGGAGATAGTGGGGGACCCCATGTTACTGAAGTGG
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref  ATGAAGGAGGTAGAGATTCATGTCAAGGAGATAGTGGGGGACCCCATGTTACTGAAGTGG
               370       380       390       400       410       420

               430       440       450       460       470       480
S6Ex8   AAGGGACCAGTTTCTTAACTGGAATTATTAGCTGGGGTGAAGAGTGTGCAATGAAAGGCA
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref  AAGGGACCAGTTTCTTAACTGGAATTATTAGCTGGGGTGAAGAGTGTGCAATGAAAGGCA
               430       440       450       460       470       480

               490       500       510       520       530       540
S6Ex8   AATATGGAATATATACCAAGGTATCCCGGTATGTCAACTGGATTAAGGAAAAAACAAAGC
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref  AATATGGAATATATACCAAGGTATCCCGGTATGTCAACTGGATTAAGGAAAAAACAAAGC
               490       500       510       520       530       540


S6Ex8   TCACTTAA
        ::::::::
Ex8Ref  TCACTTAA
```

Participant 7 (S7)

S7: *F9*_Exon 1

```
               10        20        30        40        50        60
S7Ex1  ATGCAGCGCGTGAACATGATCATGGCAGAATCACCAGGCCTCATCACCATCTGCCTTTTA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex1Ref ATGCAGCGCGTGAACATGATCATGGCAGAATCACCAGGCCTCATCACCATCTGCCTTTTA
               10        20        30        40        50        60

               70        80
S7Ex1  GGATATCTACTCAGTGCTGAATGTACAG
       :::::::::::::::::::::::::::
Ex1Ref GGATATCTACTCAGTGCTGAATGTACAG
               70        80
```

S7: *F9*_Exon 2,3

```
                 10        20        30        40        50        60
S7Ex2,3  TTTTTCTTGATCATGAAAACGCCAACAAAATTCTGAATCGGCCAAAGAGGTATAATTCAG
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref TTTTTCTTGATCATGAAAACGCCAACAAAATTCTGAATCGGCCAAAGAGGTATAATTCAG
                 10        20        30        40        50        60

                 70        80        90       100       110       120
S7Ex2,3  GTAAATTGGAAGAGTTTGTTCAAGGGAACCTTGAGAGAGAATGTATGGAAGAAAAGTGTA
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref GTAAATTGGAAGAGTTTGTTCAAGGGAACCTTGAGAGAGAATGTATGGAAGAAAAGTGTA
                 70        80        90       100       110       120

                130       140       150       160       170       180
S7Ex2,3  GTTTTGAAGAAGCACGAGAAGTTTTTGAAAACACTGAAAGAACAGTGAGTATTTCCACAT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref GTTTTGAAGAAGCACGAGAAGTTTTTGAAAACACTGAAAGAACAGTGAGTATTTCCACAT
                130       140       150       160       170       180

                190       200       210       220       230       240
S7Ex2,3  AATACCCTTCAGATGCAGAGCATAGAATAGAAAATCTTTAAAAAGACACTTCTCTTTAAA
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref AATACCCTTCAGATGCAGAGCATAGAATAGAAAATCTTTAAAAAGACACTTCTCTTTAAA
                190       200       210       220       230       240

                250       260       270       280       290       300
S7Ex2,3  ATTTTAAAGCATCCATATATATTTATGTATGTTAAATGTTATAAAAGATAGGAAATCAAT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref ATTTTAAAGCATCCATATATATTTATGTATGTTAAATGTTATAAAAGATAGGAAATCAAT
                250       260       270       280       290       300

                310       320       330       340       350       360
S7Ex2,3  ACCAAAACACTTTAGATATTACCGTTAATTTGTCTTCTTTTATTCTTTATAGACTGAATT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref ACCAAAACACTTTAGATATTACCGTTAATTTGTCTTCTTTTATTCTTTATAGACTGAATT
                310       320       330       340       350       360

                370
S7Ex2,3  TTGGAAGCAGTATGTTG
         :::::::::::::::::
Ex2,3Ref TTGGAAGCAGTATGTTG
                370
```

S7: *F9*_Exon 4

```
               10        20        30        40        50        60
S7Ex4  ATGGAGATCAGTGTGAGTCCAATCCATGTTTAAATGGCGGCAGTTGCAAGGATGACATTA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex4Ref ATGGAGATCAGTGTGAGTCCAATCCATGTTTAAATGGCGGCAGTTGCAAGGATGACATTA
               10        20        30        40        50        60

               70        80        90       100       110
S7Ex4  ATTCCTATGAATGTTGGTGTCCCTTTGGATTTGAAGGAAAGAACTGTGAATTAG
       :::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex4Ref ATTCCTATGAATGTTGGTGTCCCTTTGGATTTGAAGGAAAGAACTGTGAATTAG
               70        80        90       100       110
```

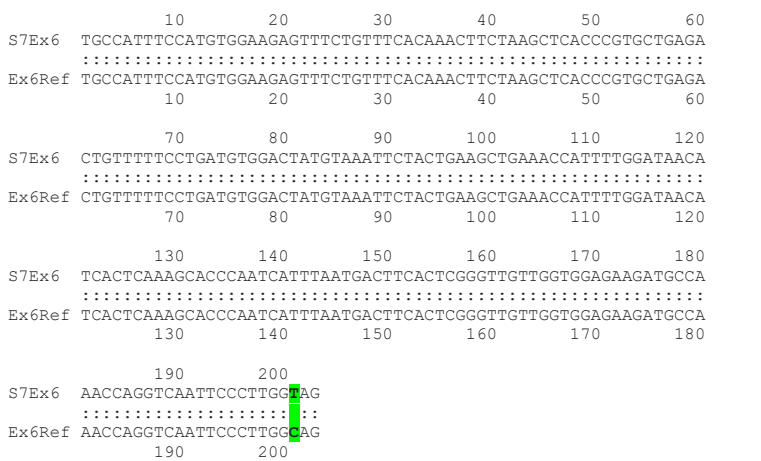
S7: *F9*_Exon 5

```
               10        20        30        40        50        60
S7Ex5  ATGTAACATGTAACATTAAGAATGGCAGATGCGAGCAGTTTTGTAAAAATAGTGCTGATA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex5Ref ATGTAACATGTAACATTAAGAATGGCAGATGCGAGCAGTTTTGTAAAAATAGTGCTGATA
               10        20        30        40        50        60

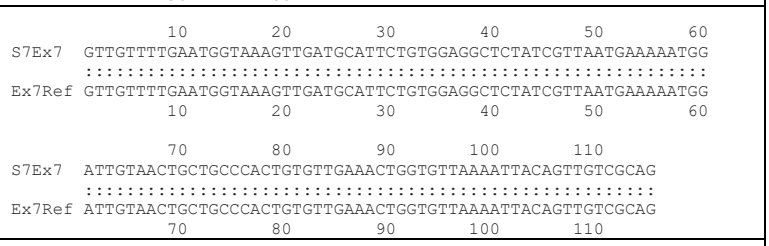
               70        80        90       100       110       120
S7Ex5  ACAAGGTGGTTTGCTCCTGTACTGAGGGATATCGACTTGCAGAAAACCAGAAGTCCTGTG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex5Ref ACAAGGTGGTTTGCTCCTGTACTGAGGGATATCGACTTGCAGAAAACCAGAAGTCCTGTG
               70        80        90       100       110       120


S7Ex5  AACCAGCAG
       :::::::::
Ex5Ref AACCAGCAG
```

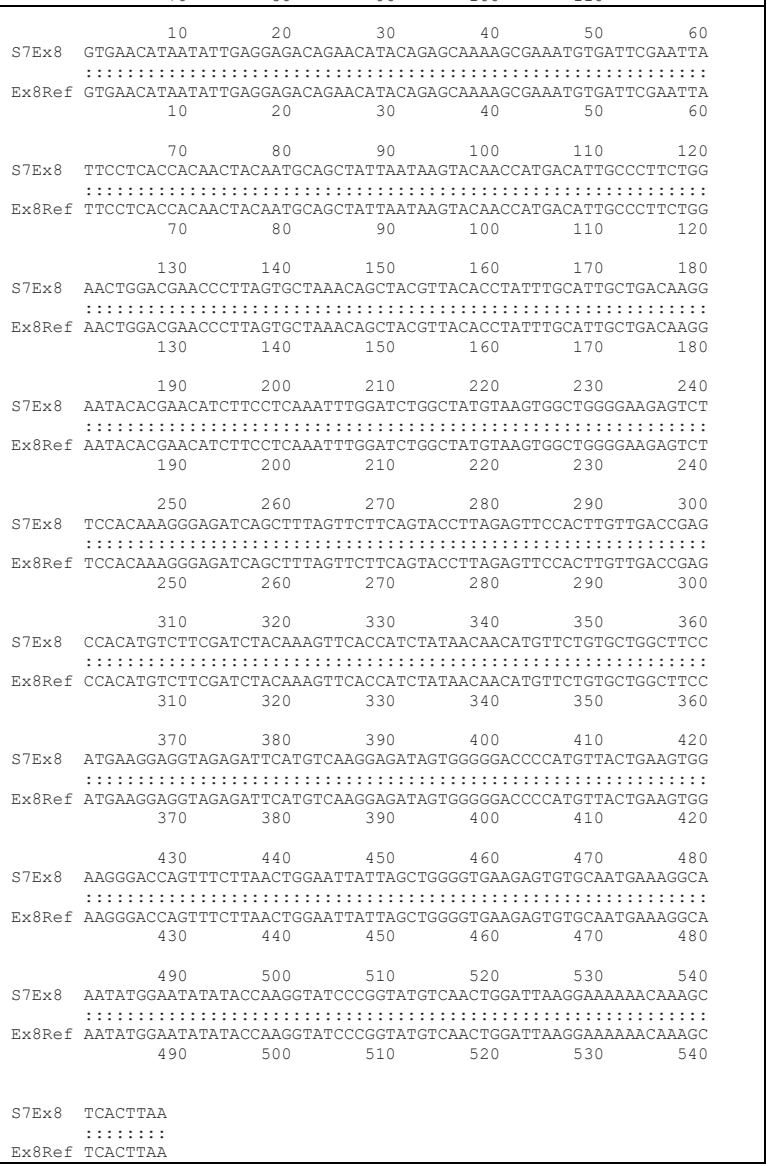
S7: *F9*_Exon 6



S7: *F9*_Exon 7



S7: *F9*_Exon 8



Participant 8 (S8)

S8: *F9*_Exon 1

```
               10        20        30        40        50        60
S8Ex1  ATGCAGCGCGTGAACATGATCATGGCAGAATCACCAGGCCTCATCACCATCTGCCTTTTA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex1Ref ATGCAGCGCGTGAACATGATCATGGCAGAATCACCAGGCCTCATCACCATCTGCCTTTTA
               10        20        30        40        50        60

               70        80
S8Ex1  GGATATCTACTCAGTGCTGAATGTACAG
       :::::::::::::::::::::::::::
Ex1Ref GGATATCTACTCAGTGCTGAATGTACAG
               70        80
```

S8: *F9*_Exon 2,3

```
                10        20        30        40        50        60
S8Ex2,3  TTTTTCTTGATCATGAAAACGCCAACAAAATTCTGAATCGGCCAAAGAGGTATAATTCAG
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref TTTTTCTTGATCATGAAAACGCCAACAAAATTCTGAATCGGCCAAAGAGGTATAATTCAG
                10        20        30        40        50        60

                70        80        90       100       110       120
S8Ex2,3  GTAAATTGGAAGAGTTTGTTCAAGGGAACCTTGAGAGAGAATGTATGGAAGAAAAGTGTA
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref GTAAATTGGAAGAGTTTGTTCAAGGGAACCTTGAGAGAGAATGTATGGAAGAAAAGTGTA
                70        80        90       100       110       120

               130       140       150       160       170       180
S8Ex2,3  GTTTTGAAGAAGCACGAGAAGTTTTTGAAAACACTGAAAGAACAGTGAGTATTTCCACAT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref GTTTTGAAGAAGCACGAGAAGTTTTTGAAAACACTGAAAGAACAGTGAGTATTTCCACAT
               130       140       150       160       170       180

               190       200       210       220       230       240
S8Ex2,3  AATACCCTTCAGATGCAGAGCATAGAATAGAAAATCTTTAAAAAGACACTTCTCTTTAAA
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref AATACCCTTCAGATGCAGAGCATAGAATAGAAAATCTTTAAAAAGACACTTCTCTTTAAA
               190       200       210       220       230       240

               250       260       270       280       290       300
S8Ex2,3  ATTTTAAAGCATCCATATATATTTATGTATGTTAAATGTTATAAAAGATAGGAAATCAAT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref ATTTTAAAGCATCCATATATATTTATGTATGTTAAATGTTATAAAAGATAGGAAATCAAT
               250       260       270       280       290       300

               310       320       330       340       350       360
S8Ex2,3  ACCAAAACACTTTAGATATTACCGTTAATTTGTCTTCTTTTATTCTTTATAGACTGAATT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref ACCAAAACACTTTAGATATTACCGTTAATTTGTCTTCTTTTATTCTTTATAGACTGAATT
               310       320       330       340       350       360

               370
SEx2,3   TTGGAAGCAGTATGTTG
         :::::::::::::::::
Ex2,3Ref TTGGAAGCAGTATGTTG
               370
```

S8: *F9*_Exon 4

```
               10        20        30        40        50        60
S8Ex4  ATGGAGATCAGTGTGAGTCCAATCCATGTTTAAATGGCGGCAGTTGCAAGGATGACATTA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex4Ref ATGGAGATCAGTGTGAGTCCAATCCATGTTTAAATGGCGGCAGTTGCAAGGATGACATTA
               10        20        30        40        50        60

               70        80        90       100       110
S8Ex4  ATTCCTATGAATGTTGGTGTCCCTTTGGATTTGAAGGAAAGAACTGTGAATTAG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex4Ref ATTCCTATGAATGTTGGTGTCCCTTTGGATTTGAAGGAAAGAACTGTGAATTAG
               70        80        90       100       110
```

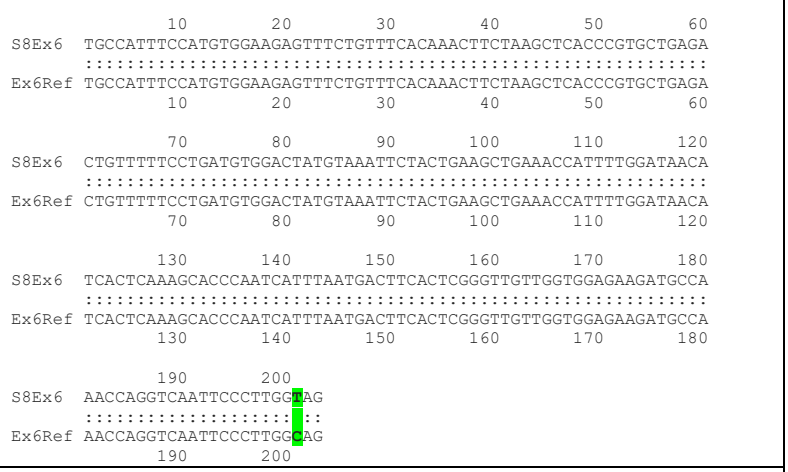
S8: *F9*_Exon 5

```
               10        20        30        40        50        60
S8Ex5  ATGTAACATGTAACATTAAGAATGGCAGATGCGAGCAGTTTTGTAAAAATAGTGCTGATA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex5Ref ATGTAACATGTAACATTAAGAATGGCAGATGCGAGCAGTTTTGTAAAAATAGTGCTGATA
               10        20        30        40        50        60

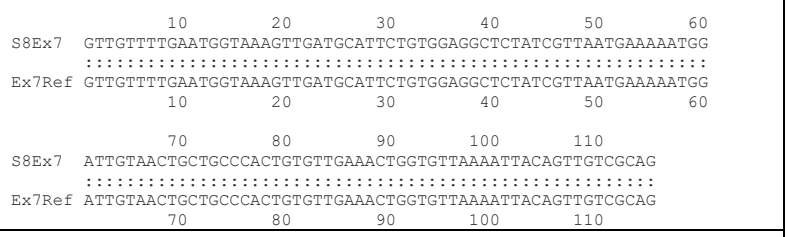
               70        80        90       100       110       120
S8Ex5  ACAAGGTGGTTTGCTCCTGTACTGAGGGATATCGACTTGCAGAAAACCAGAAGTCCTGTG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex5Ref ACAAGGTGGTTTGCTCCTGTACTGAGGGATATCGACTTGCAGAAAACCAGAAGTCCTGTG
               70        80        90       100       110       120


S8Ex5  AACCAGCAG
       :::::::::
Ex5Ref AACCAGCAG
```

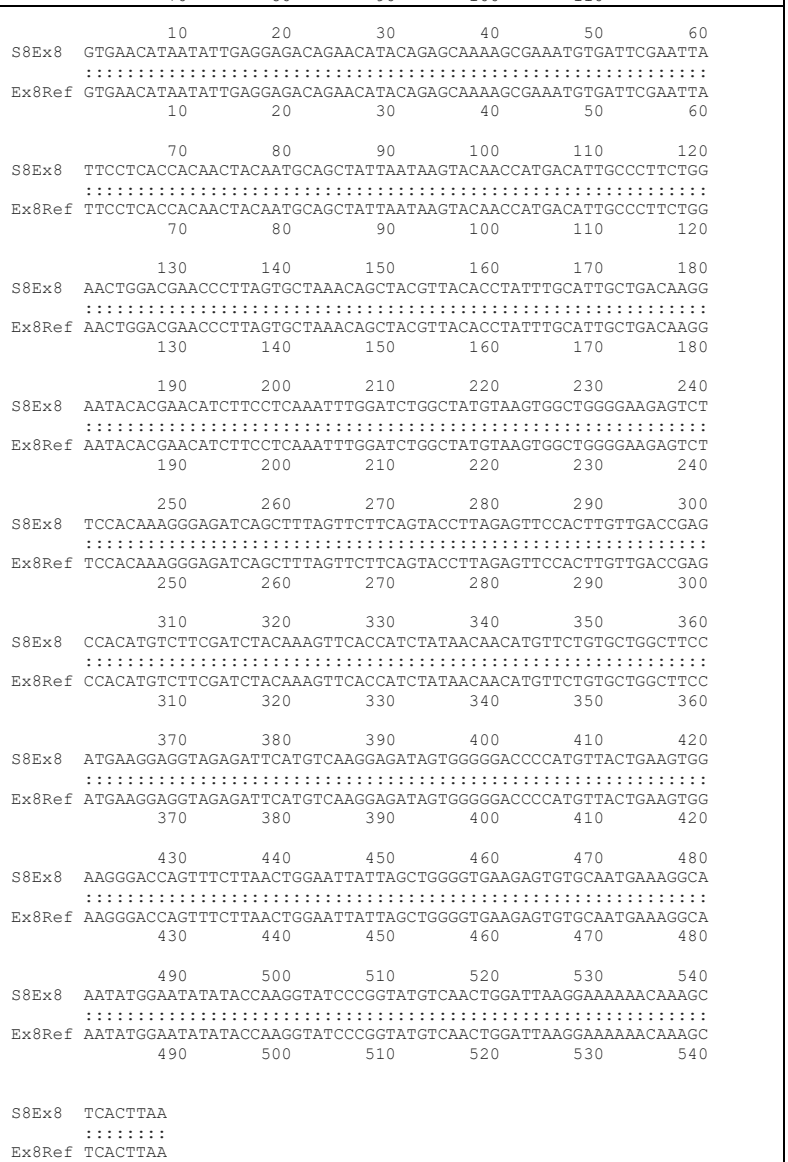
S8: *F9*_Exon 6



S8: *F9*_Exon 7



S8: *F9*_Exon 8



Participant 9 (S9)

S9: *F9*_Exon 1

```
               10        20        30        40        50        60
S9Ex1  ATGCAGCGCGTGAACATGATCATGGCAGAATCACCAGGCCTCATCACCATCTGCCTTTTA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex1Ref ATGCAGCGCGTGAACATGATCATGGCAGAATCACCAGGCCTCATCACCATCTGCCTTTTA
               10        20        30        40        50        60

               70        80
S9Ex1  GGATATCTACTCAGTGCTGAATGTACAG
       :::::::::::::::::::::::::::
Ex1Ref GGATATCTACTCAGTGCTGAATGTACAG
               70        80
```

S9: *F9*_Exon 2,3

```
                10        20        30        40        50        60
S9Ex2,3  TTTTTCTTGATCATGAAAACGCCAACAAAATTCTGAATCGGCCAAAGAGGTATAATTCAG
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref TTTTTCTTGATCATGAAAACGCCAACAAAATTCTGAATCGGCCAAAGAGGTATAATTCAG
                10        20        30        40        50        60

                70        80        90       100       110       120
S9Ex2,3  GTAAATTGGAAGAGTTTGTTCAAGGGAACCTTGAGAGAGAATGTATGGAAGAAAAGTGTA
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref GTAAATTGGAAGAGTTTGTTCAAGGGAACCTTGAGAGAGAATGTATGGAAGAAAAGTGTA
                70        80        90       100       110       120

               130       140       150       160       170       180
S9Ex2,3  GTTTTGAAGAAGCACGAGAAGTTTTTGAAAACACTGAAAGAACAGTGAGTATTTCCACAT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref GTTTTGAAGAAGCACGAGAAGTTTTTGAAAACACTGAAAGAACAGTGAGTATTTCCACAT
               130       140       150       160       170       180

               190       200       210       220       230       240
S9Ex2,3  AATACCCTTCAGATGCAGAGCATAGAATAGAAAATCTTTAAAAAGACACTTCTCTTTAAA
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref AATACCCTTCAGATGCAGAGCATAGAATAGAAAATCTTTAAAAAGACACTTCTCTTTAAA
               190       200       210       220       230       240

               250       260       270       280       290       300
S9Ex2,3  ATTTTAAAGCATCCATATATATTTATGTATGTTAAATGTTATAAAAGATAGGAAATCAAT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref ATTTTAAAGCATCCATATATATTTATGTATGTTAAATGTTATAAAAGATAGGAAATCAAT
               250       260       270       280       290       300

               310       320       330       340       350       360
S9Ex2,3  ACCAAAACACTTTAGATATTACCGTTAATTTGTCTTCTTTTATTCTTTATAGACTGAATT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref ACCAAAACACTTTAGATATTACCGTTAATTTGTCTTCTTTTATTCTTTATAGACTGAATT
               310       320       330       340       350       360

               370
S9Ex2,3  TTGGAAGCAGTATGTTG
         :::::::::::::::::
Ex2,3Ref TTGGAAGCAGTATGTTG
               370
```

S9: *F9*_Exon 4

```
              10        20        30        40        50        60
S9Ex4  ATGGAGATCAGTGTGAGTCCAATCCATGTTTAAATGGCGGCAGTTGCAAGGATGACATTA
      ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex4Ref ATGGAGATCAGTGTGAGTCCAATCCATGTTTAAATGGCGGCAGTTGCAAGGATGACATTA
              10        20        30        40        50        60

              70        80        90       100       110
S9Ex4  ATTCCTATGAATGTTGGTGTCCCTTTGGATTTGAAGGAAAGAACTGTGAATTAG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex4Ref ATTCCTATGAATGTTGGTGTCCCTTTGGATTTGAAGGAAAGAACTGTGAATTAG
              70        80        90       100       110
```

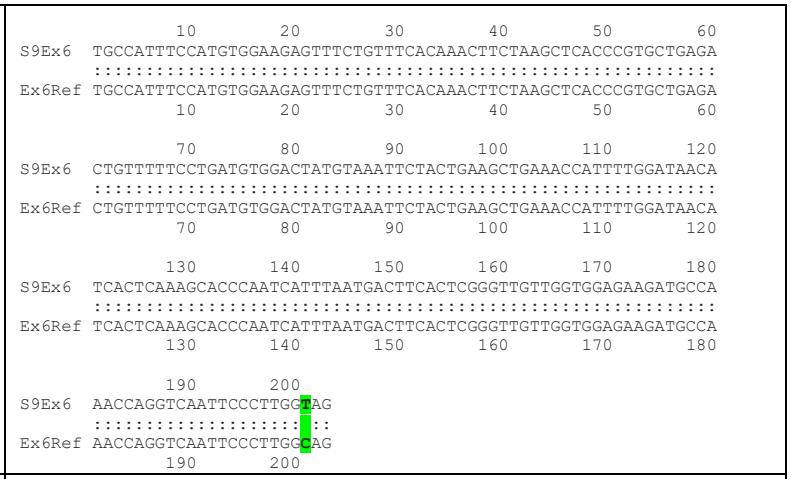
S9: *F9*_Exon 5

```
               10        20        30        40        50        60
S9Ex5  ATGTAACATGTAACATTAAGAATGGCAGATGCGAGCAGTTTTGTAAAAATAGTGCTGATA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex5Ref ATGTAACATGTAACATTAAGAATGGCAGATGCGAGCAGTTTTGTAAAAATAGTGCTGATA
               10        20        30        40        50        60

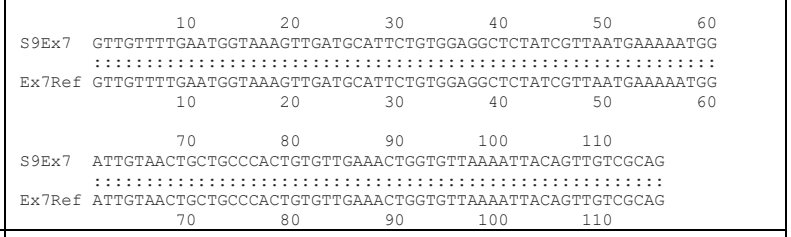
               70        80        90       100       110       120
S9Ex5  ACAAGGTGGTTTGCTCCTGTACTGAGGGATATCGACTTGCAGAAAACCAGAAGTCCTGTG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex5Ref ACAAGGTGGTTTGCTCCTGTACTGAGGGATATCGACTTGCAGAAAACCAGAAGTCCTGTG
               70        80        90       100       110       120


S9Ex5  AACCAGCAG
       :::::::::
Ex5Ref AACCAGCAG
```

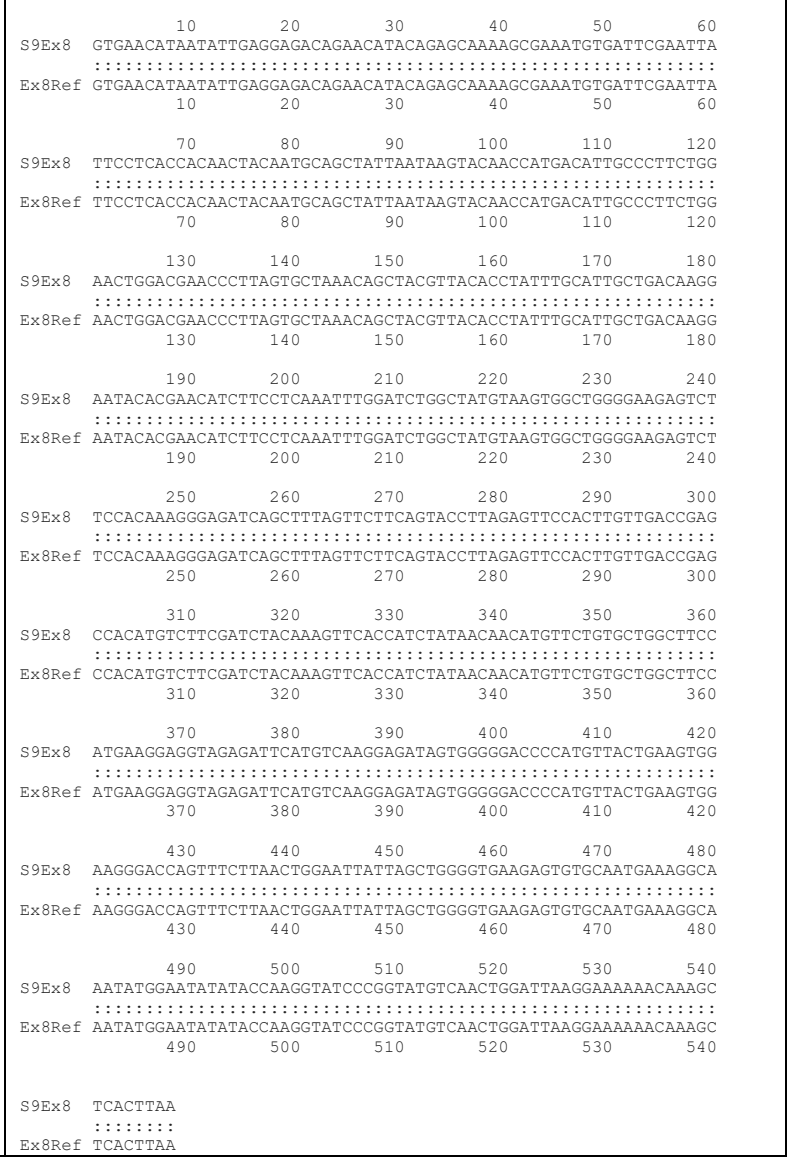
S9: *F9*_Exon 6



S9: *F9*_Exon 7



S9: *F9*_Exon 8



Participant 10 (S10)

S10: *F9*_Exon 1

```
               10        20        30        40        50        60
S10Ex1 ATGCAGCGCGTGAACATGATCATGGCAGAATCACCAGGCCTCATCACCATCTGCCTTTTA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex1Ref ATGCAGCGCGTGAACATGATCATGGCAGAATCACCAGGCCTCATCACCATCTGCCTTTTA
               10        20        30        40        50        60

               70        80
S10Ex1 GGATATCTACTCAGTGCTGAATGTACAG
       ::::::::::::::::::::::::::::
Ex1Ref GGATATCTACTCAGTGCTGAATGTACAG
               70        80
```

S10: *F9*_Exon 2,3

```
                 10        20        30        40        50        60
S10Ex2,3 TTTTTCTTGATCATGAAAACGCCAACAAAATTCTGAATCGGCCAAAGAGGTATAATTCAG
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref TTTTTCTTGATCATGAAAACGCCAACAAAATTCTGAATCGGCCAAAGAGGTATAATTCAG
                 10        20        30        40        50        60

                 70        80        90       100       110       120
S10Ex2,3 GTAAATTGGAAGAGTTTGTTCAAGGGAACCTTGAGAGAGAATGTATGGAAGAAAAGTGTA
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref GTAAATTGGAAGAGTTTGTTCAAGGGAACCTTGAGAGAGAATGTATGGAAGAAAAGTGTA
                 70        80        90       100       110       120

                130       140       150       160       170       180
S10Ex2,3 GTTTTGAAGAAGCACGAGAAGTTTTTGAAAACACTGAAAGAACAGTGAGTATTTCCACAT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref GTTTTGAAGAAGCACGAGAAGTTTTTGAAAACACTGAAAGAACAGTGAGTATTTCCACAT
                130       140       150       160       170       180

                190       200       210       220       230       240
S10Ex2,3 AATACCCTTCAGATGCAGAGCATAGAATAGAAAATCTTTAAAAAGACACTTCTCTTTAAA
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref AATACCCTTCAGATGCAGAGCATAGAATAGAAAATCTTTAAAAAGACACTTCTCTTTAAA
                190       200       210       220       230       240

                250       260       270       280       290       300
S10Ex2,3 ATTTTAAAGCATCCATATATATTTATGTATGTTAAATGTTATAAAAGATAGGAAATCAAT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref ATTTTAAAGCATCCATATATATTTATGTATGTTAAATGTTATAAAAGATAGGAAATCAAT
                250       260       270       280       290       300

                310       320       330       340       350       360
S10Ex2,3 ACCAAAACACTTTAGATATTACCGTTAATTTGTCTTCTTTTATTCTTTATAGACTGAATT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref ACCAAAACACTTTAGATATTACCGTTAATTTGTCTTCTTTTATTCTTTATAGACTGAATT
                310       320       330       340       350       360

                370
S10Ex2,3 TTGGAAGCAGTATGTTG
         :::::::::::::::::
Ex2,3Ref TTGGAAGCAGTATGTTG
                370
```

S10: *F9*_Exon 4

```
               10        20        30        40        50        60
S10Ex4 ATGGAGATCAGTGTGAGTCCAATCCATGTTTAAATGGCGGCAGTTGCAAGGATGACATTA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex4Ref ATGGAGATCAGTGTGAGTCCAATCCATGTTTAAATGGCGGCAGTTGCAAGGATGACATTA
               10        20        30        40        50        60

               70        80        90       100       110
S10Ex4 ATTCCTATGAATGTTGGTGTCCCTTTGGATTTGAAGGAAAGAACTGTGAATTAG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex4Ref ATTCCTATGAATGTTGGTGTCCCTTTGGATTTGAAGGAAAGAACTGTGAATTAG
               70        80        90       100       110
```

S10: *F9*_Exon 5

```
               10        20        30        40        50        60
S10Ex5 ATGTAACATGTAACATTAAGAATGGCAGATGCGAGCAGTTTTGTAAAAATAGTGCTGATA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex5Ref ATGTAACATGTAACATTAAGAATGGCAGATGCGAGCAGTTTTGTAAAAATAGTGCTGATA
               10        20        30        40        50        60

               70        80        90       100       110       120
S10Ex5 ACAAGGTGGTTTGCTCCTGTACTGAGGGATATCGACTTGCAGAAAACCAGAAGTCCTGTG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex5Ref ACAAGGTGGTTTGCTCCTGTACTGAGGGATATCGACTTGCAGAAAACCAGAAGTCCTGTG
               70        80        90       100       110       120


S10Ex5 AACCAGCAG
       :::::::::
Ex5Ref AACCAGCAG
```

S10: *F9*_Exon 6

```
               10        20        30        40        50        60
S10Ex6 TGCCATTTCCATGTGGAAGAGTTTCTGTTTCACAAACTTCTAAGCTCACCCGTGCTGAGA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex6Ref TGCCATTTCCATGTGGAAGAGTTTCTGTTTCACAAACTTCTAAGCTCACCCGTGCTGAGA
               10        20        30        40        50        60

               70        80        90       100       110       120
S10Ex6 CTGTTTTTCCTGATGTGGACTATGTAAATTCTACTGAAGCTGAAACCATTTTGGATAACA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex6Ref CTGTTTTTCCTGATGTGGACTATGTAAATTCTACTGAAGCTGAAACCATTTTGGATAACA
               70        80        90       100       110       120

              130       140       150       160       170       180
S10Ex6 TCACTCAAAGCACCCAATCATTTAATGACTTCACTCGGGTTGTTGGTGGAGAAGATGCCA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex6Ref TCACTCAAAGCACCCAATCATTTAATGACTTCACTCGGGTTGTTGGTGGAGAAGATGCCA
              130       140       150       160       170       180

              190       200
S10Ex6 AACCAGGTCAATTCCCTTGGCAG
       :::::::::::::::::::::::
Ex6Ref AACCAGGTCAATTCCCTTGGCAG
              190       200
```

S10: *F9*_Exon 7

```
               10        20        30        40        50        60
S10Ex7 GTTGTTTTGAATGGTAAAGTTGATGCATTCTGTGGAGGCTCTATCGTTAATGAAAAATGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex7Ref GTTGTTTTGAATGGTAAAGTTGATGCATTCTGTGGAGGCTCTATCGTTAATGAAAAATGG
               10        20        30        40        50        60

               70        80        90       100       110
S10Ex7 ATTGTAACTGCTGCCCACTGTGTTGAAACTGGTGTTAAAATTACAGTTGTCGCAG
       :::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex7Ref ATTGTAACTGCTGCCCACTGTGTTGAAACTGGTGTTAAAATTACAGTTGTCGCAG
               70        80        90       100       110
```

S10: *F9*_Exon 8

```
               10        20        30        40        50        60
S10Ex8 GTGAACATAATATTGAGGAGACAGAACATACAGAGCAAAAGCGAAATGTGATTCGAATTA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref GTGAACATAATATTGAGGAGACAGAACATACAGAGCAAAAGCGAAATGTGATTCGAATTA
               10        20        30        40        50        60

               70        80        90       100       110       120
S10Ex8 TTCCTCACCACAACTACAATGCAGCTATTAATAAGTACAACCATGACATTGCCCTTCTGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref TTCCTCACCACAACTACAATGCAGCTATTAATAAGTACAACCATGACATTGCCCTTCTGG
               70        80        90       100       110       120

              130       140       150       160       170       180
S10Ex8 AACTGGACGAACCCTTAGTGCTAAACAGCTACGTTACACCTATTTGCATTGCTGACAAGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref AACTGGACGAACCCTTAGTGCTAAACAGCTACGTTACACCTATTTGCATTGCTGACAAGG
              130       140       150       160       170       180

              190       200       210       220       230       240
S10Ex8 AATACACGAACATCTTCCTCAAATTTGGATCTGGCTATGTAAGTGGCTGGGGAAGAGTCT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref AATACACGAACATCTTCCTCAAATTTGGATCTGGCTATGTAAGTGGCTGGGGAAGAGTCT
              190       200       210       220       230       240

              250       260       270       280       290       300
S10Ex8 TCCACAAAGGGAGATCAGCTTTAGTTCTTCAGTACCTTAGAGTTCCACTTGTTGACCGAG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref TCCACAAAGGGAGATCAGCTTTAGTTCTTCAGTACCTTAGAGTTCCACTTGTTGACCGAG
              250       260       270       280       290       300

              310       320       330       340       350       360
S10Ex8 CCACATGTCTTCGATCTACAAAGTTCACCATCTATAACAACATGTTCTGTGCTGGCTTCC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref CCACATGTCTTCGATCTACAAAGTTCACCATCTATAACAACATGTTCTGTGCTGGCTTCC
              310       320       330       340       350       360

              370       380       390       400       410       420
S10Ex8 ATGAAGGAGGTAGAGATTCATGTCAAGGAGATAGTGGGGGACCCCATGTTACTGAAGTGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref ATGAAGGAGGTAGAGATTCATGTCAAGGAGATAGTGGGGGACCCCATGTTACTGAAGTGG
              370       380       390       400       410       420

              430       440       450       460       470       480
S10Ex8 AAGGGACCAGTTTCTTAACTGGAATTATTAGCTGGGGTGAAGAGTGTGCAATGAAAGGCA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref AAGGGACCAGTTTCTTAACTGGAATTATTAGCTGGGGTGAAGAGTGTGCAATGAAAGGCA
              430       440       450       460       470       480

              490       500       510       520       530       540
S10Ex8 AATATGGAATATATACCAAGGTATCCCGGTATGTCAACTGGATTAAGGAAAAAACAAAGC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref AATATGGAATATATACCAAGGTATCCCGGTATGTCAACTGGATTAAGGAAAAAACAAAGC
              490       500       510       520       530       540


S10Ex8 TCACTTAA
       ::::::::
Ex8Ref TCACTTAA
```

Participant 11 (S11)

S11: *F9*_Exon 1

```
               10        20        30        40        50        60
S11Ex1 ATGCAGCGCGTGAACATGATCATGGCAGAATCACCAGGCCTCATCACCATCTGCCTTTTA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex1Ref ATGCAGCGCGTGAACATGATCATGGCAGAATCACCAGGCCTCATCACCATCTGCCTTTTA
               10        20        30        40        50        60

               70        80
S11Ex1 GGATATCTACTCAGTGCTGAATGTACAG
       ::::::::::::::::::::::::::::
Ex1Ref GGATATCTACTCAGTGCTGAATGTACAG
               70        80
```

S11: *F9*_Exon 2,3

```
                 10        20        30        40        50        60
S11Ex2,3 TTTTTCTTGATCATGAAAACGCCAACAAAATTCTGAATCGGCCAAAGAGGTATAATTCAG
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref TTTTTCTTGATCATGAAAACGCCAACAAAATTCTGAATCGGCCAAAGAGGTATAATTCAG
                 10        20        30        40        50        60

                 70        80        90       100       110       120
S11Ex2,3 GTAAATTGGAAGAGTTTGTTCAAGGGAACCTTGAGAGAGAATGTATGGAAGAAAAGTGTA
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref GTAAATTGGAAGAGTTTGTTCAAGGGAACCTTGAGAGAGAATGTATGGAAGAAAAGTGTA
                 70        80        90       100       110       120

                130       140       150       160       170       180
S11Ex2,3 GTTTTGAAGAAGCACGAGAAGTTTTTGAAAACACTGAAAGAACAGTGAGTATTTCCACAT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref GTTTTGAAGAAGCACGAGAAGTTTTTGAAAACACTGAAAGAACAGTGAGTATTTCCACAT
                130       140       150       160       170       180

                190       200       210       220       230       240
S11Ex2,3 AATACCCTTCAGATGCAGAGCATAGAATAGAAAATCTTTAAAAAGACACTTCTCTTTAAA
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref AATACCCTTCAGATGCAGAGCATAGAATAGAAAATCTTTAAAAAGACACTTCTCTTTAAA
                190       200       210       220       230       240

                250       260       270       280       290       300
S11Ex2,3 ATTTTAAAGCATCCATATATATTTATGTATGTTAAATGTTATAAAAGATAGGAAATCAAT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref ATTTTAAAGCATCCATATATATTTATGTATGTTAAATGTTATAAAAGATAGGAAATCAAT
                250       260       270       280       290       300

                310       320       330       340       350       360
S11Ex2,3 ACCAAAACACTTTAGATATTACCGTTAATTTGTCTTCTTTTATTCTTTATAGACTGAATT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref ACCAAAACACTTTAGATATTACCGTTAATTTGTCTTCTTTTATTCTTTATAGACTGAATT
                310       320       330       340       350       360

                370
S11Ex2,3 TTGGAAGCAGTATGTTG
         :::::::::::::::::
Ex2,3Ref TTGGAAGCAGTATGTTG
                370
```

S11: *F9*_Exon 4

```
               10        20        30        40        50        60
S11Ex4 ATGGAGATCAGTGTGAGTCCAATCCATGTTTAAATGGCGGCAGTTGCAAGGATGACATTA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex4Ref ATGGAGATCAGTGTGAGTCCAATCCATGTTTAAATGGCGGCAGTTGCAAGGATGACATTA
               10        20        30        40        50        60

               70        80        90       100       110
S11Ex4 ATTCCTATGAATGTTGGTGTCCCTTTGGATTTGAAGGAAAGAACTGTGAATTAG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex4Ref ATTCCTATGAATGTTGGTGTCCCTTTGGATTTGAAGGAAAGAACTGTGAATTAG
               70        80        90       100       110
```

S11: *F9*_Exon 5

```
               10        20        30        40        50        60
S11Ex5 ATGTAACATGTAACATTAAGAATGGCAGATGCGAGCAGTTTTGTAAAAATAGTGCTGATA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex5Ref ATGTAACATGTAACATTAAGAATGGCAGATGCGAGCAGTTTTGTAAAAATAGTGCTGATA
               10        20        30        40        50        60

               70        80        90       100       110       120
S11Ex5 ACAAGGTGGTTTGCTCCTGTACTGAGGGATATCGACTTGCAGAAAACCAGAAGTCCTGTG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex5Ref ACAAGGTGGTTTGCTCCTGTACTGAGGGATATCGACTTGCAGAAAACCAGAAGTCCTGTG
               70        80        90       100       110       120


S11Ex5 AACCAGCAG
       :::::::::
Ex5Ref AACCAGCAG
```

S11: *F9*_Exon 6

```
             10        20        30        40        50        60
S11Ex6  TGCCATTTCCATGTGGAAGAGTTTCTGTTTCACAAACTTCTAAGCTCACCCGTGCTGAGA
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex6Ref  TGCCATTTCCATGTGGAAGAGTTTCTGTTTCACAAACTTCTAAGCTCACCCGTGCTGAGA
               10        20        30        40        50        60

               70        80        90       100       110       120
S11Ex6  CTGTTTTTCCTGATGTGGACTATGTAAATTCTACTGAAGCTGAAACCATTTTGGATAACA
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex6Ref  CTGTTTTTCCTGATGTGGACTATGTAAATTCTACTGAAGCTGAAACCATTTTGGATAACA
               70        80        90       100       110       120

              130       140       150       160       170       180
S11Ex6  TCACTCAAAGCACCCAATCATTTAATGACTTCACTCGGGTTGTTGGTGGAGAAGATGCCA
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex6Ref  TCACTCAAAGCACCCAATCATTTAATGACTTCACTCGGGTTGTTGGTGGAGAAGATGCCA
              130       140       150       160       170       180

              190       200
S11Ex6  AACCAGGTCAATTCCCTTGGCAG
        :::::::::::::::::::::::
Ex6Ref  AACCAGGTCAATTCCCTTGGCAG
              190       200
```

S11: *F9*_Exon 7

```
               10        20        30        40        50        60
S11Ex7  GTTGTTTTGAATGGTAAAGTTGATGCATTCTGTGGAGGCTCTATCGTTAATGAAAAATGG
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex7Ref  GTTGTTTTGAATGGTAAAGTTGATGCATTCTGTGGAGGCTCTATCGTTAATGAAAAATGG
               10        20        30        40        50        60

               70        80        90       100       110
S11Ex7  ATTGTAACTGCTGCCCACTGTGTTGAAACTGGTGTTAAAATTACAGTTGTCGCAG
        :::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex7Ref  ATTGTAACTGCTGCCCACTGTGTTGAAACTGGTGTTAAAATTACAGTTGTCGCAG
               70        80        90       100       110
```

S11: *F9*_Exon 8

```
               10        20        30        40        50        60
S11Ex8  GTGAACATAATATTGAGGAGACAGAACATACAGAGCAAAAGCGAAATGTGATTCGAATTA
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref  GTGAACATAATATTGAGGAGACAGAACATACAGAGCAAAAGCGAAATGTGATTCGAATTA
               10        20        30        40        50        60

               70        80        90       100       110       120
S11Ex8  TTCCTCACCACAACTACAATGCAGCTATTAATAAGTACAACCATGACATTGCCCTTCTGG
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref  TTCCTCACCACAACTACAATGCAGCTATTAATAAGTACAACCATGACATTGCCCTTCTGG
               70        80        90       100       110       120

              130       140       150       160       170       180
S11Ex8  AACTGGACGAACCCTTAGTGCTAAACAGCTACGTTACACCTATTTGCATTGCTGACAAGG
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref  AACTGGACGAACCCTTAGTGCTAAACAGCTACGTTACACCTATTTGCATTGCTGACAAGG
              130       140       150       160       170       180

              190       200       210       220       230       240
S11Ex8  AATACACGAACATCTTCCTCAAATTTGGATCTGGCTATGTAAGTGGCTGGGGAAGAGTCT
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref  AATACACGAACATCTTCCTCAAATTTGGATCTGGCTATGTAAGTGGCTGGGGAAGAGTCT
              190       200       210       220       230       240

              250       260       270       280       290       300
S11Ex8  TCCACAAAGGGAGATCAGCTTTAGTTCTTCAGTACCTTAGAGTTCCACTTGTTGACCGAG
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref  TCCACAAAGGGAGATCAGCTTTAGTTCTTCAGTACCTTAGAGTTCCACTTGTTGACCGAG
              250       260       270       280       290       300

              310       320       330          340       350
S11Ex8  CCACATGTCTTCGATCTACAAAGTTCACCATCTATAACA---TGTTCTGTGCTGGCTTCC
        ::::::::::::::::::::::::::::::::::::::::   ::::::::::::::::::
Ex8Ref  CCACATGTCTTCGATCTACAAAGTTCACCATCTATAACAACATGTTCTGTGCTGGCTTCC
              310       320       330       340       350       360

       360       370       380       390       400       410
S11Ex8  ATGAAGGAGGTAGAGATTCATGTCAAGGAGATAGTGGGGGACCCCATGTTACTGAAGTGG
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref  ATGAAGGAGGTAGAGATTCATGTCAAGGAGATAGTGGGGGACCCCATGTTACTGAAGTGG
              370       380       390       400       410       420

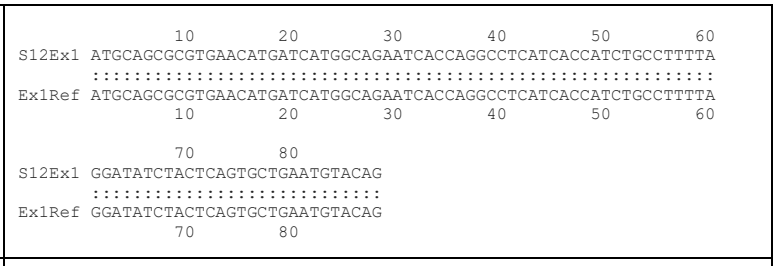
       420       430       440       450       460       470
S11Ex8  AAGGGACCAGTTTCTTAACTGGAATTATTAGCTGGGGTGAAGAGTGTGCAATGAAAGGCA
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref  AAGGGACCAGTTTCTTAACTGGAATTATTAGCTGGGGTGAAGAGTGTGCAATGAAAGGCA
              430       440       450       460       470       480

       480       490       500       510       520       530
S11Ex8  AATATGGAATATATACCAAGGTATCCCGGTATGTCAACTGGATTAAGGAAAAAACAAAGC
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref  AATATGGAATATATACCAAGGTATCCCGGTATGTCAACTGGATTAAGGAAAAAACAAAGC
              490       500       510       520       530       540

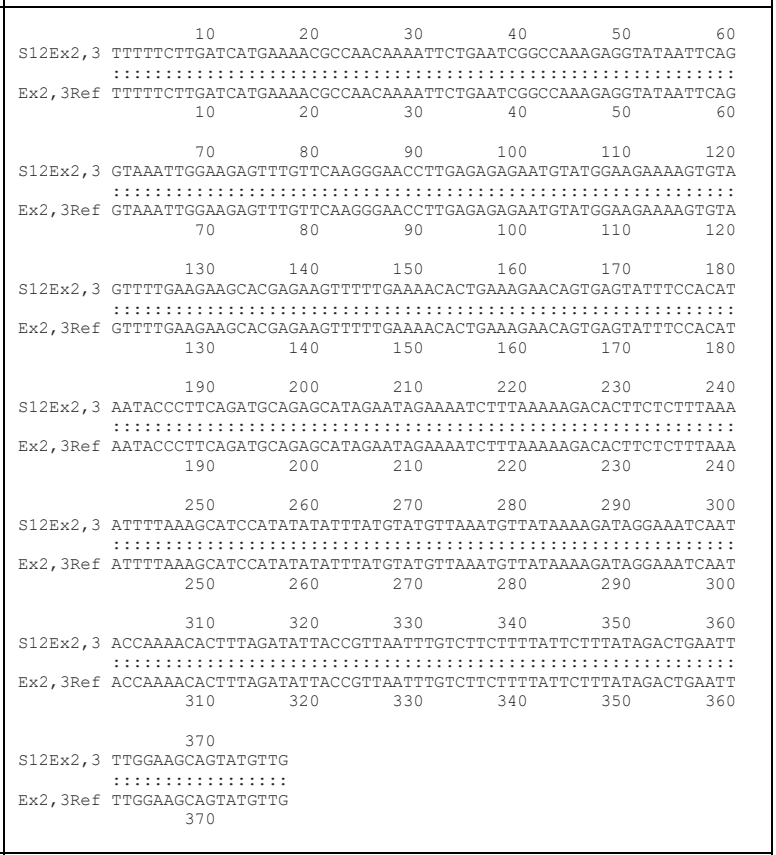
       540
S11Ex8  TCACTTAA
        ::::::::
Ex8Ref  TCACTTAA
```

Participant 12 (S12)

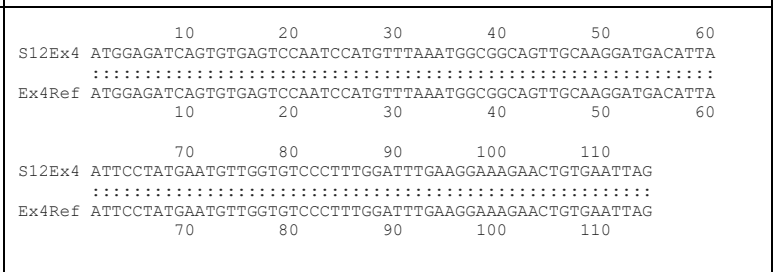
S12: *F9*_Exon 1



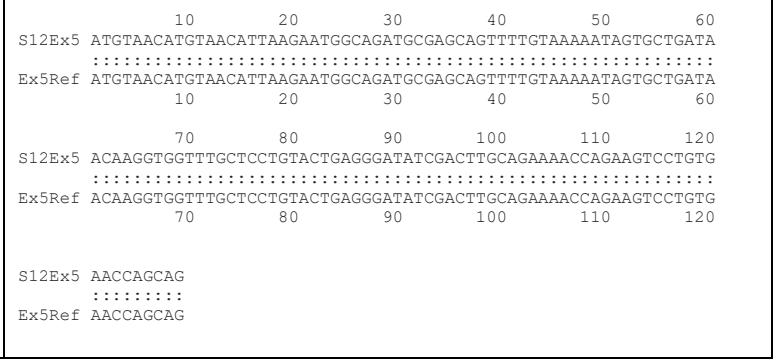
S12: *F9*_Exon 2,3



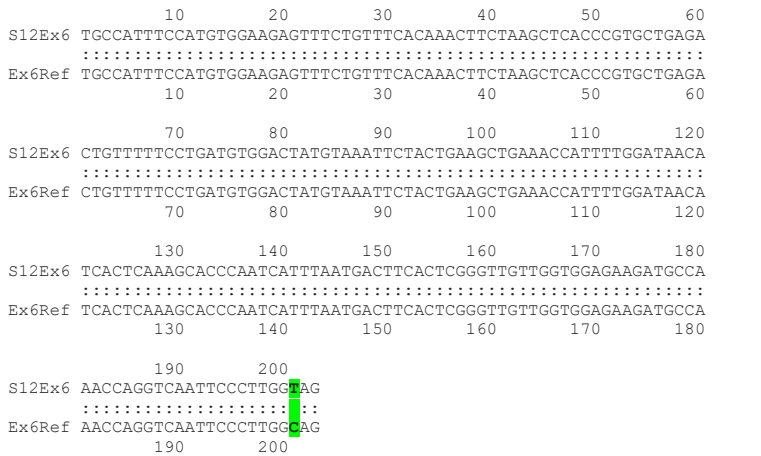
S12: *F9*_Exon 4



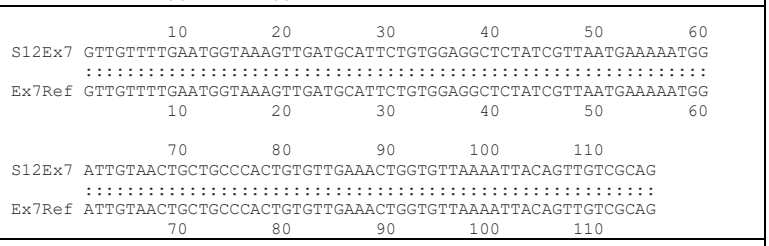
S12: *F9*_Exon 5



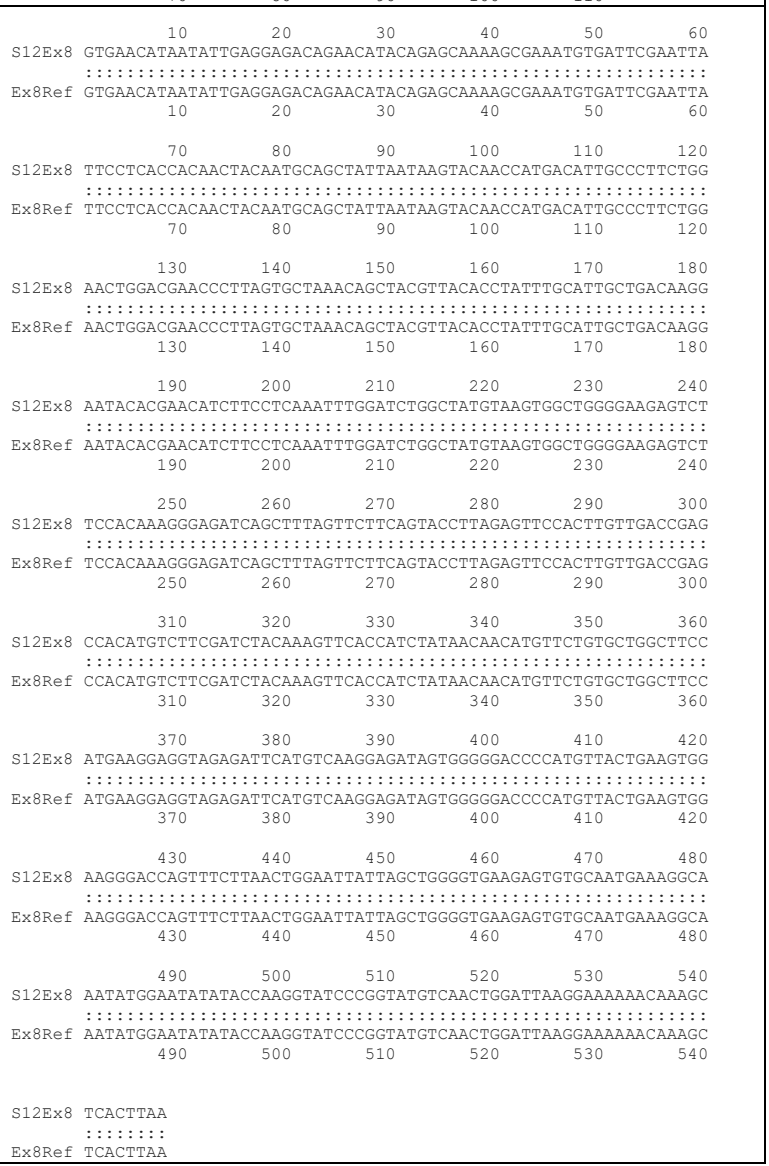
S12: *F9*_Exon 6



S12: *F9*_Exon 7



S12: *F9*_Exon 8



Participant 13 (S13)

S13: *F9*_Exon 1

```
               10        20        30        40        50        60
S13Ex1 ATGCAGCGCGTGAACATGATCATGGCAGAATCACCAGGCCTCATCACCATCTGCCTTTTA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex1Ref ATGCAGCGCGTGAACATGATCATGGCAGAATCACCAGGCCTCATCACCATCTGCCTTTTA
               10        20        30        40        50        60

               70        80
S13Ex1 GGATATCTACTCAGTGCTGAATGTACAG
       ::::::::::::::::::::::::::::
Ex1Ref GGATATCTACTCAGTGCTGAATGTACAG
               70        80
```

S13: *F9*_Exon 2,3

```
                 10        20        30        40        50        60
S13Ex2,3 TTTTTCTTGATCATGAAAACGCCAACAAAATTCTGAATCGGCCAAAGAGGTATAATTCAG
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref TTTTTCTTGATCATGAAAACGCCAACAAAATTCTGAATCGGCCAAAGAGGTATAATTCAG
                 10        20        30        40        50        60

                 70        80        90       100       110       120
S13Ex2,3 GTAAATTGGAAGAGTTTGTTCAAGGGAACCTTGAGAGAGAATGTATGGAAGAAAAGTGTA
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref GTAAATTGGAAGAGTTTGTTCAAGGGAACCTTGAGAGAGAATGTATGGAAGAAAAGTGTA
                 70        80        90       100       110       120

                130       140       150       160       170       180
S13Ex2,3 GTTTTGAAGAAGCACGAGAAGTTTTTGAAAACACTGAAAGAACAGTGAGTATTTCCACAT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref GTTTTGAAGAAGCACGAGAAGTTTTTGAAAACACTGAAAGAACAGTGAGTATTTCCACAT
                130       140       150       160       170       180

                190       200       210       220       230       240
S13Ex2,3 AATACCCTTCAGATGCAGAGCATAGAATAGAAAATCTTTAAAAAGACACTTCTCTTTAAA
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref AATACCCTTCAGATGCAGAGCATAGAATAGAAAATCTTTAAAAAGACACTTCTCTTTAAA
                190       200       210       220       230       240

                250       260       270       280       290       300
S13Ex2,3 ATTTTAAAGCATCCATATATATTTATGTATGTTAAATGTTATAAAAGATAGGAAATCAAT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref ATTTTAAAGCATCCATATATATTTATGTATGTTAAATGTTATAAAAGATAGGAAATCAAT
                250       260       270       280       290       300

                310       320       330       340       350       360
S13Ex2,3 ACCAAAACACTTTAGATATTACCGTTAATTTGTCTTCTTTTATTCTTTATAGACTGAATT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref ACCAAAACACTTTAGATATTACCGTTAATTTGTCTTCTTTTATTCTTTATAGACTGAATT
                310       320       330       340       350       360

                370
S13Ex2,3 TTGGAAGCAGTATGTTG
         :::::::::::::::::
Ex2,3Ref TTGGAAGCAGTATGTTG
                370
```

S13: *F9*_Exon 4

```
               10        20        30        40        50        60
S13Ex4 ATGGAGATCAGTGTGAGTCCAATCCATGTTTAAATGGCGGCAGTTGCAAGGATGACATTA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex4Ref ATGGAGATCAGTGTGAGTCCAATCCATGTTTAAATGGCGGCAGTTGCAAGGATGACATTA
               10        20        30        40        50        60

               70        80        90       100       110
S13Ex4 ATTCCTATGAATGTTGGTGTCCCTTTGGATTTGAAGGAAAGAACTGTGAATTAG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex4Ref ATTCCTATGAATGTTGGTGTCCCTTTGGATTTGAAGGAAAGAACTGTGAATTAG
               70        80        90       100       110
```

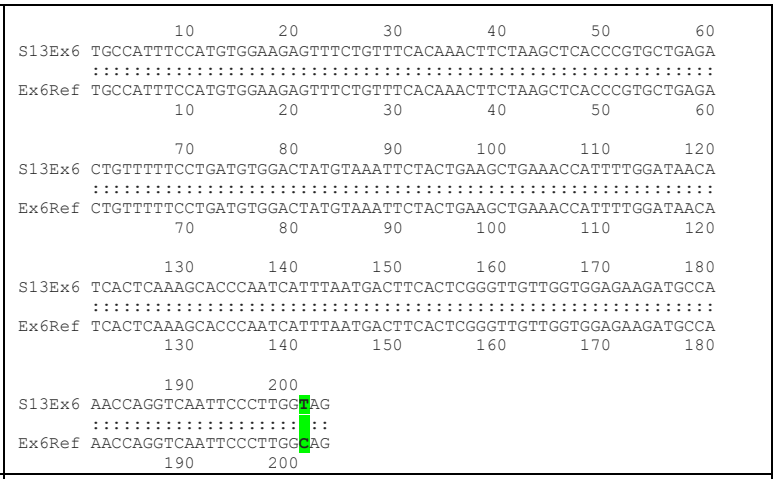
S13: *F9*_Exon 5

```
               10        20        30        40        50        60
S13Ex5 ATGTAACATGTAACATTAAGAATGGCAGATGCGAGCAGTTTTGTAAAAATAGTGCTGATA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex5Ref ATGTAACATGTAACATTAAGAATGGCAGATGCGAGCAGTTTTGTAAAAATAGTGCTGATA
               10        20        30        40        50        60

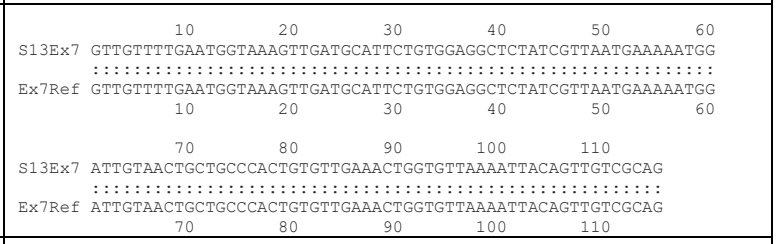
               70        80        90       100       110       120
S13Ex5 ACAAGGTGGTTTGCTCCTGTACTGAGGGATATCGACTTGCAGAAAACCAGAAGTCCTGTG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex5Ref ACAAGGTGGTTTGCTCCTGTACTGAGGGATATCGACTTGCAGAAAACCAGAAGTCCTGTG
               70        80        90       100       110       120


S13Ex5 AACCAGCAG
       :::::::::
Ex5Ref AACCAGCAG
```

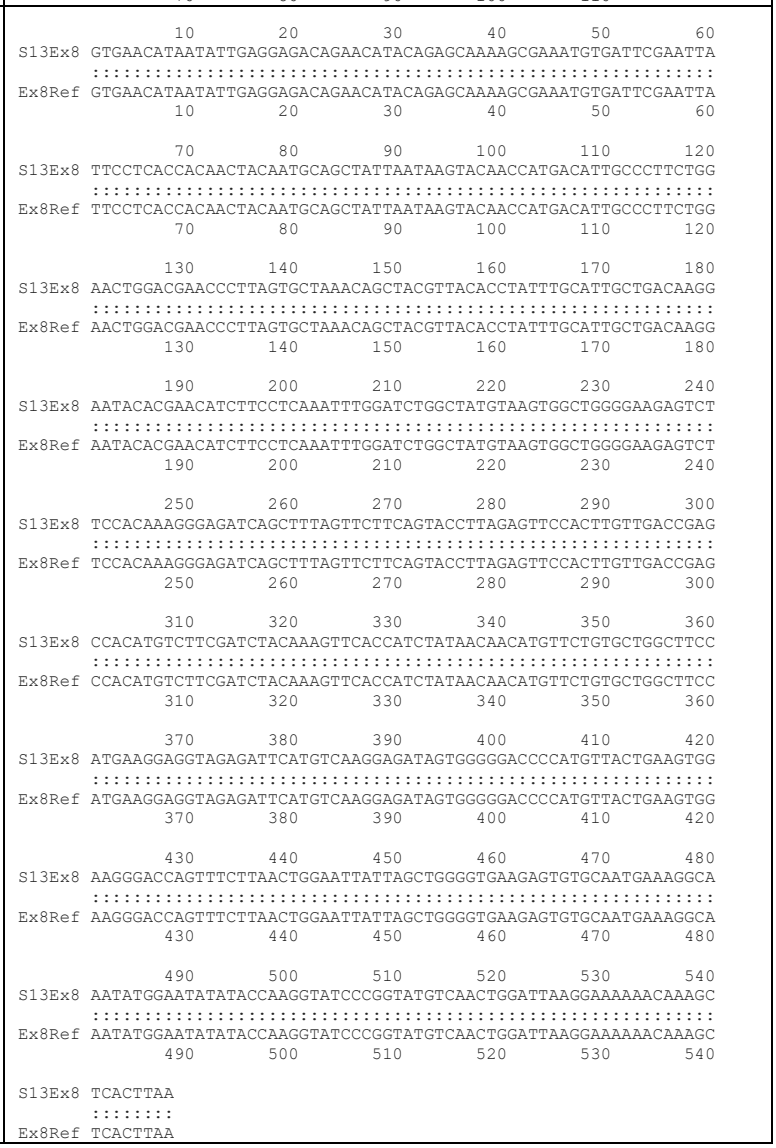
S13: *F9*_Exon 6



S13: *F9*_Exon 7



S13: *F9*_Exon 8



Participant 14 (S14)

S14: *F9*_Exon 1

```
              10        20        30        40        50        60
S14Ex1 ATGCAGCGCGTGAACATGATCATGGCAGAATCACCAGGCCTCATCACCATCTGCCTTTTA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex1Ref ATGCAGCGCGTGAACATGATCATGGCAGAATCACCAGGCCTCATCACCATCTGCCTTTTA
              10        20        30        40        50        60

              70        80
S14Ex1 GGATATCTACTCAGTGCTGAATGTACAG
       ::::::::::::::::::::::::::::
Ex1Ref GGATATCTACTCAGTGCTGAATGTACAG
              70        80
```

S14: *F9*_Exon 2,3

```
                10        20        30        40        50        60
S14Ex2,3 TTTTTCTTGATCATGAAAACGCCAACAAAATTCTGAATCGGCCAAAGAGGTATAATTCAG
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref TTTTTCTTGATCATGAAAACGCCAACAAAATTCTGAATCGGCCAAAGAGGTATAATTCAG
                10        20        30        40        50        60

                70        80        90       100       110       120
S14Ex2,3 GTAAATTGGAAGAGTTTGTTCAAGGGAACCTTGAGAGAGAATGTATGGAAGAAAAGTGTA
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref GTAAATTGGAAGAGTTTGTTCAAGGGAACCTTGAGAGAGAATGTATGGAAGAAAAGTGTA
                70        80        90       100       110       120

               130       140       150       160       170       180
S14Ex2,3 GTTTTGAAGAAGCACGAGAAGTTTTTGAAAACACTGAAAGAACAGTGAGTATTTCCACAT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref GTTTTGAAGAAGCACGAGAAGTTTTTGAAAACACTGAAAGAACAGTGAGTATTTCCACAT
               130       140       150       160       170       180

               190       200       210       220       230       240
S14Ex2,3 AATACCCTTCAGATGCAGAGCATAGAATAGAAAATCTTTAAAAAGACACTTCTCTTTAAA
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref AATACCCTTCAGATGCAGAGCATAGAATAGAAAATCTTTAAAAAGACACTTCTCTTTAAA
               190       200       210       220       230       240

               250       260       270       280       290       300
S14Ex2,3 ATTTTAAAGCATCCATATATATTTATGTATGTTAAATGTTATAAAAGATAGGAAATCAAT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref ATTTTAAAGCATCCATATATATTTATGTATGTTAAATGTTATAAAAGATAGGAAATCAAT
               250       260       270       280       290       300

               310       320       330       340       350       360
S14Ex2,3 ACCAAAACACTTTAGATATTACCGTTAATTTGTCTTCTTTTATTCTTTATAGACTGAATT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref ACCAAAACACTTTAGATATTACCGTTAATTTGTCTTCTTTTATTCTTTATAGACTGAATT
               310       320       330       340       350       360

               370
S14Ex2,3 TTGGAAGCAGTATGTTG
         :::::::::::::::::
Ex2,3Ref TTGGAAGCAGTATGTTG
               370
```

S14: *F9*_Exon 4

```
              10        20        30        40        50        60
S14Ex4 ATGGAGATCAGTGTGAGTCCAATCCATGTTTAAATGGCGGCAGTTGCAAGGATGACATTA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex4Ref ATGGAGATCAGTGTGAGTCCAATCCATGTTTAAATGGCGGCAGTTGCAAGGATGACATTA
              10        20        30        40        50        60

              70        80        90       100       110
S14Ex4 ATTCCTATGAATGTTGGTGTCCCTTTGGATTTGAAGGAAAGAACTGTGAATTAG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex4Ref ATTCCTATGAATGTTGGTGTCCCTTTGGATTTGAAGGAAAGAACTGTGAATTAG
              70        80        90       100       110
```

S14: *F9*_Exon 5

```
              10        20        30        40        50        60
S14Ex5 ATGTAACATGTAACATTAAGAATGGCAGATGCGAGCAGTTTTGTAAAAATAGTGCTGATA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex5Ref ATGTAACATGTAACATTAAGAATGGCAGATGCGAGCAGTTTTGTAAAAATAGTGCTGATA
              10        20        30        40        50        60

              70        80        90       100       110       120
S14Ex5 ACAAGGTGGTTTGCTCCTGTACTGAGGGATATCGACTTGCAGAAAACCAGAAGTCCTGTG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex5Ref ACAAGGTGGTTTGCTCCTGTACTGAGGGATATCGACTTGCAGAAAACCAGAAGTCCTGTG
              70        80        90       100       110       120


S14Ex5 AACCAGCAG
       :::::::::
Ex5Ref AACCAGCAG
```

S14: *F9*_Exon 6

```
             10        20        30        40        50        60
S14Ex6 TGCCATTTCCATGTGGAAGAGTTTCTGTTTCACAAACTTCTAAGCTCACCCGTGCTGAGA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex6Ref TGCCATTTCCATGTGGAAGAGTTTCTGTTTCACAAACTTCTAAGCTCACCCGTGCTGAGA
               10        20        30        40        50        60

               70        80        90       100       110       120
S14Ex6 CTGTTTTTCCTGATGTGGACTATGTAAATTCTACTGAAGCTGAAACCATTTTGGATAACA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex6Ref CTGTTTTTCCTGATGTGGACTATGTAAATTCTACTGAAGCTGAAACCATTTTGGATAACA
               70        80        90       100       110       120

              130       140       150       160       170       180
S14Ex6 TCACTCAAAGCACCCAATCATTTAATGACTTCACTCGGGTTGTTGGTGGAGAAGATGCCA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex6Ref TCACTCAAAGCACCCAATCATTTAATGACTTCACTCGGGTTGTTGGTGGAGAAGATGCCA
              130       140       150       160       170       180

              190       200
S14Ex6 AACCAGGTCAATTCCCTTGGCAG
       :::::::::::::::::::::::
Ex6Ref AACCAGGTCAATTCCCTTGGCAG
              190       200
```

S14: *F9*_Exon 7

```
               10        20        30        40        50        60
S14Ex7 GTTGTTTTGAATGGTAAAGTTGATGCATTCTGTGGAGGCTCTATCGTTAATGAAAAATGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex7Ref GTTGTTTTGAATGGTAAAGTTGATGCATTCTGTGGAGGCTCTATCGTTAATGAAAAATGG
               10        20        30        40        50        60

               70        80        90       100       110
S14Ex7 ATTGTAACTGCTGCCCACTGTGTTGAAACTGGTGTTAAAATTACAGTTGTCGCAG
       :::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex7Ref ATTGTAACTGCTGCCCACTGTGTTGAAACTGGTGTTAAAATTACAGTTGTCGCAG
               70        80        90       100       110
```

S14: *F9*_Exon 8

```
               10        20        30        40        50        60
S14Ex8 GTGAACATAATATTGAGGAGACAGAACATACAGAGCAAAAGCGAAATGTGATTCGAATTA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref GTGAACATAATATTGAGGAGACAGAACATACAGAGCAAAAGCGAAATGTGATTCGAATTA
               10        20        30        40        50        60

               70        80        90       100       110       120
S14Ex8 TTCCTCACCACAACTACAATGCAGCTATTAATAAGTACAACCATGACATTGCCCTTCTGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref TTCCTCACCACAACTACAATGCAGCTATTAATAAGTACAACCATGACATTGCCCTTCTGG
               70        80        90       100       110       120

              130       140       150       160       170       180
S14Ex8 AACTGGACGAACCCTTAGTGCTAAACAGCTACGTTACACCTATTTGCATTGCTGACAAGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref AACTGGACGAACCCTTAGTGCTAAACAGCTACGTTACACCTATTTGCATTGCTGACAAGG
              130       140       150       160       170       180

              190       200       210       220       230       240
S14Ex8 AATACACGAACATCTTCCTCAAATTTGGATCTGGCTATGTAAGTGGCTGGGGAAGAGTCT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref AATACACGAACATCTTCCTCAAATTTGGATCTGGCTATGTAAGTGGCTGGGGAAGAGTCT
              190       200       210       220       230       240

              250       260       270       280       290       300
S14Ex8 TCCACAAAGGGAGATCAGCTTTAGTTCTTCAGTACCTTAGAGTTCCACTTGTTGACCGAG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref TCCACAAAGGGAGATCAGCTTTAGTTCTTCAGTACCTTAGAGTTCCACTTGTTGACCGAG
              250       260       270       280       290       300

              310       320       330       340       350       360
S14Ex8 CCACATGTCTTCGATCTACAAAGTTCACCATCTATAACAACATGTTCTGTGCTGGCTTCC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref CCACATGTCTTCGATCTACAAAGTTCACCATCTATAACAACATGTTCTGTGCTGGCTTCC
              310       320       330       340       350       360

              370       380       390       400       410       420
S14Ex8 ATGAAGGAGGTAGAGATTCATGTCAAGGAGATAGTGGGGGACCCCATGTTACTGAAGTGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref ATGAAGGAGGTAGAGATTCATGTCAAGGAGATAGTGGGGGACCCCATGTTACTGAAGTGG
              370       380       390       400       410       420

              430       440       450       460       470       480
S14Ex8 AAGGGACCAGTTTCTTAACTGGAATTATTAGCTGGGGTGAAGAGTGTGCAATGAAAGGCA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref AAGGGACCAGTTTCTTAACTGGAATTATTAGCTGGGGTGAAGAGTGTGCAATGAAAGGCA
              430       440       450       460       470       480

              490       500       510       520       530       540
S14Ex8 AATATGGAATATATACCAAGGTATCCCGGTATGTCAACTGGATTAAGGAAAAAACAAAGC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref AATATGGAATATATACCAAGGTATCCCGGTATGTCAACTGGATTAAGGAAAAAACAAAGC
              490       500       510       520       530       540


S14Ex8 TCACTTAA
       ::::::::
Ex8Ref TCACTTAA
```

Participant 15 (S15)

S15: *F9*_Exon 1

```
               10        20        30        40        50        60
S15Ex1 ATGCAGCGCGTGAACATGATCATGGCAGAATCACCAGGCCTCATCACCATCTGCCTTTTA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex1Ref ATGCAGCGCGTGAACATGATCATGGCAGAATCACCAGGCCTCATCACCATCTGCCTTTTA
               10        20        30        40        50        60

               70        80
S15Ex1 GGATATCTACTCAGTGCTGAATGTACAG
       ::::::::::::::::::::::::::::
Ex1Ref GGATATCTACTCAGTGCTGAATGTACAG
               70        80
```

S15: *F9*_Exon 2,3

```
                10        20        30        40        50        60
S10Ex2,3 TTTTTCTTGATCATGAAAACGCCAACAAAATTCTGAATCGGCCAAAGAGGTATAATTCAG
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref TTTTTCTTGATCATGAAAACGCCAACAAAATTCTGAATCGGCCAAAGAGGTATAATTCAG
                10        20        30        40        50        60

                70        80        90       100       110       120
S15Ex2,3 GTAAATTGGAAGAGTTTGTTCAAGGGAACCTTGAGAGAGAATGTATGGAAGAAAAGTGTA
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref GTAAATTGGAAGAGTTTGTTCAAGGGAACCTTGAGAGAGAATGTATGGAAGAAAAGTGTA
                70        80        90       100       110       120

               130       140       150       160       170       180
S15Ex2,3 GTTTTGAAGAAGCACGAGAAGTTTTTGAAAACACTGAAAGAACAGTGAGTATTTCCACAT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref GTTTTGAAGAAGCACGAGAAGTTTTTGAAAACACTGAAAGAACAGTGAGTATTTCCACAT
               130       140       150       160       170       180

               190       200       210       220       230       240
S15Ex2,3 AATACCCTTCAGATGCAGAGCATAGAATAGAAAATCTTTAAAAAGACACTTCTCTTTAAA
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref AATACCCTTCAGATGCAGAGCATAGAATAGAAAATCTTTAAAAAGACACTTCTCTTTAAA
               190       200       210       220       230       240

               250       260       270       280       290       300
S15Ex2,3 ATTTTAAAGCATCCATATATATTTATGTATGTTAAATGTTATAAAAGATAGGAAATCAAT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref ATTTTAAAGCATCCATATATATTTATGTATGTTAAATGTTATAAAAGATAGGAAATCAAT
               250       260       270       280       290       300

               310       320       330       340       350       360
S15Ex2,3 ACCAAAACACTTTAGATATTACCGTTAATTTGTCTTCTTTTATTCTTTATAGACTGAATT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref ACCAAAACACTTTAGATATTACCGTTAATTTGTCTTCTTTTATTCTTTATAGACTGAATT
               310       320       330       340       350       360

               370
S15Ex2,3 TTGGAAGCAGTATGTTG
         :::::::::::::::::
Ex2,3Ref TTGGAAGCAGTATGTTG
               370
```

S15: *F9*_Exon 4

```
               10        20        30        40        50        60
S15Ex4 ATGGAGATCAGTGTGAGTCCAATCCATGTTTAAATGGCGGCAGTTGCAAGGATGACATTA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex4Ref ATGGAGATCAGTGTGAGTCCAATCCATGTTTAAATGGCGGCAGTTGCAAGGATGACATTA
               10        20        30        40        50        60

               70        80        90       100       110
S15Ex4 ATTCCTATGAATGTTGGTGTCCCTTTGGATTTGAAGGAAAGAACTGTGAATTAG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex4Ref ATTCCTATGAATGTTGGTGTCCCTTTGGATTTGAAGGAAAGAACTGTGAATTAG
               70        80        90       100       110
```

S15: *F9*_Exon 5

```
               10        20        30        40        50        60
S15Ex5 ATGTAACATGTAACATTAAGAATGGCAGATGCGAGCAGTTTTGTAAAAATAGTGCTGATA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex5Ref ATGTAACATGTAACATTAAGAATGGCAGATGCGAGCAGTTTTGTAAAAATAGTGCTGATA
               10        20        30        40        50        60

               70        80        90       100       110       120
S15Ex5 ACAAGGTGGTTTGCTCCTGTACTGAGGGATATCGACTTGCAGAAAACCAGAAGTCCTGTG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex5Ref ACAAGGTGGTTTGCTCCTGTACTGAGGGATATCGACTTGCAGAAAACCAGAAGTCCTGTG
               70        80        90       100       110       120


S15Ex5 AACCAGCAG
       :::::::::
Ex5Ref AACCAGCAG
```

S15: *F9*_Exon 6

```
             10        20        30        40        50        60
S15Ex6 TGCCATTTCCATGTGGAAGAGTTTCTGTTTCACAAACTTCTAAGCTCACCCGTGCTGAGA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex6Ref TGCCATTTCCATGTGGAAGAGTTTCTGTTTCACAAACTTCTAAGCTCACCCGTGCTGAGA
               10        20        30        40        50        60

               70        80        90       100       110       120
S15Ex6 CTGTTTTTCCTGATGTGGACTATGTAAATTCTACTGAAGCTGAAACCATTTTGGATAACA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex6Ref CTGTTTTTCCTGATGTGGACTATGTAAATTCTACTGAAGCTGAAACCATTTTGGATAACA
               70        80        90       100       110       120

              130       140       150       160       170       180
S15Ex6 TCACTCAAAGCACCCAATCATTTAATGACTTCACTCGGGTTGTTGGTGGAGAAGATGCCA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex6Ref TCACTCAAAGCACCCAATCATTTAATGACTTCACTCGGGTTGTTGGTGGAGAAGATGCCA
              130       140       150       160       170       180

              190       200
S15Ex6 AACCAGGTCAATTCCCTTGGCAG
       :::::::::::::::::::::::
Ex6Ref AACCAGGTCAATTCCCTTGGCAG
              190       200
```

S15: *F9*_Exon 7

```
               10        20        30        40        50        60
S15Ex7 GTTGTTTTGAATGGTAAAGTTGATGCATTCTGTGGAGGCTCTATCGTTAATGAAAAATGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex7Ref GTTGTTTTGAATGGTAAAGTTGATGCATTCTGTGGAGGCTCTATCGTTAATGAAAAATGG
               10        20        30        40        50        60

               70        80        90       100       110
S15Ex7 ATTGTAACTGCTGCCCACTGTGTTGAAACTGGTGTTAAAATTACAGTTGTCGCAG
       :::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex7Ref ATTGTAACTGCTGCCCACTGTGTTGAAACTGGTGTTAAAATTACAGTTGTCGCAG
               70        80        90       100       110
```

S15: *F9*_Exon 8

```
               10        20        30        40        50        60
S15Ex8 GTGAACATAATATTGAGGAGACAGAACATACAGAGCAAAAGCGAAATGTGATTCGAATTA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref GTGAACATAATATTGAGGAGACAGAACATACAGAGCAAAAGCGAAATGTGATTCGAATTA
               10        20        30        40        50        60

               70        80        90       100       110       120
S15Ex8 TTCCTCACCACAACTACAATGCAGCTATTAATAAGTACAACCATGACATTGCCCTTCTGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref TTCCTCACCACAACTACAATGCAGCTATTAATAAGTACAACCATGACATTGCCCTTCTGG
               70        80        90       100       110       120

              130       140       150       160       170       180
S15Ex8 AACTGGACGAACCCTTAGTGCTAAACAGCTACGTTACACCTATTTGCATTGCTGACAAGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref AACTGGACGAACCCTTAGTGCTAAACAGCTACGTTACACCTATTTGCATTGCTGACAAGG
              130       140       150       160       170       180

              190       200       210       220       230       240
S15Ex8 AATACACGAACATCTTCCTCAAATTTGGATCTGGCTATGTAAGTGGCTGGGGAAGAGTCT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref AATACACGAACATCTTCCTCAAATTTGGATCTGGCTATGTAAGTGGCTGGGGAAGAGTCT
              190       200       210       220       230       240

              250       260       270       280       290       300
S15Ex8 TCCACAAAGGGAGATCAGCTTTAGTTCTTCAGTACCTTAGAGTTCCACTTGTTGACCGAG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref TCCACAAAGGGAGATCAGCTTTAGTTCTTCAGTACCTTAGAGTTCCACTTGTTGACCGAG
              250       260       270       280       290       300

              310       320       330       340       350       360
S15Ex8 CCACATGTCTTCGATCTACAAAGTTCACCATCTATAACAACATGTTCTGTGCTGGCTTCC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref CCACATGTCTTCGATCTACAAAGTTCACCATCTATAACAACATGTTCTGTGCTGGCTTCC
              310       320       330       340       350       360

              370       380       390       400       410       420
S15Ex8 ATGAAGGAGGTAGAGATTCATGTCAAGGAGATAGTGGGGGACCCCATGTTACTGAAGTGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref ATGAAGGAGGTAGAGATTCATGTCAAGGAGATAGTGGGGGACCCCATGTTACTGAAGTGG
              370       380       390       400       410       420

              430       440       450       460       470       480
S15Ex8 AAGGGACCAGTTTCTTAACTGGAATTATTAGCTGGGGTGAAGAGTGTGCAATGAAAGGCA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref AAGGGACCAGTTTCTTAACTGGAATTATTAGCTGGGGTGAAGAGTGTGCAATGAAAGGCA
              430       440       450       460       470       480

              490       500       510       520       530
S15Ex8 AATATGGAATATATACCAAGGTATCCCGGTATGTCAACTGGATTAAGGAAAAAACAA-GC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::: ::
Ex8Ref AATATGGAATATATACCAAGGTATCCCGGTATGTCAACTGGATTAAGGAAAAAACAAAGC
              490       500       510       520       530       540

     540
S15Ex8 TCACTTAA
       ::::::::
Ex8Ref TCACTTAA
```

Participant 16 (S16)

S16: *F9*_Exon 1

```
               10        20        30        40        50        60
S16Ex1 ATGCAGCGCGTGAACATGATCATGGCAGAATCACCAGGCCTCATCACCATCTGCCTTTTA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex1Ref ATGCAGCGCGTGAACATGATCATGGCAGAATCACCAGGCCTCATCACCATCTGCCTTTTA
               10        20        30        40        50        60

               70        80
S16Ex1 GGATATCTACTCAGTGCTGAATGTACAG
       ::::::::::::::::::::::::::::
Ex1Ref GGATATCTACTCAGTGCTGAATGTACAG
               70        80
```

S16: *F9*_Exon 2,3

```
                10        20        30        40        50        60
S16Ex2,3 TTTTTCTTGATCATGAAAACGCCAACAAAATTCTGAATCGGCCAAAGAGGTATAATTCAG
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref TTTTTCTTGATCATGAAAACGCCAACAAAATTCTGAATCGGCCAAAGAGGTATAATTCAG
                10        20        30        40        50        60

                70        80        90       100       110       120
S16Ex2,3 GTAAATTGGAAGAGTTTGTTCAAGGGAACCTTGAGAGAGAATGTATGGAAGAAAAGTGTA
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref GTAAATTGGAAGAGTTTGTTCAAGGGAACCTTGAGAGAGAATGTATGGAAGAAAAGTGTA
                70        80        90       100       110       120

               130       140       150       160       170       180
S16Ex2,3 GTTTTGAAGAAGCACGAGAAGTTTTTGAAAACACTGAAAGAACAGTGAGTATTTCCACAT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref GTTTTGAAGAAGCACGAGAAGTTTTTGAAAACACTGAAAGAACAGTGAGTATTTCCACAT
               130       140       150       160       170       180

               190       200       210       220       230       240
S16Ex2,3 AATACCCTTCAGATGCAGAGCATAGAATAGAAAATCTTTAAAAAGACACTTCTCTTTAAA
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref AATACCCTTCAGATGCAGAGCATAGAATAGAAAATCTTTAAAAAGACACTTCTCTTTAAA
               190       200       210       220       230       240

               250       260       270       280       290       300
S16Ex2,3 ATTTTAAAGCATCCATATATATTTATGTATGTTAAATGTTATAAAAGATAGGAAATCAAT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref ATTTTAAAGCATCCATATATATTTATGTATGTTAAATGTTATAAAAGATAGGAAATCAAT
               250       260       270       280       290       300

               310       320       330       340       350       360
S16Ex2,3 ACCAAAACACTTTAGATATTACCGTTAATTTGTCTTCTTTTATTCTTTATAGACTGAATT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref ACCAAAACACTTTAGATATTACCGTTAATTTGTCTTCTTTTATTCTTTATAGACTGAATT
               310       320       330       340       350       360

               370
S16Ex2,3 TTGGAAGCAGTATGTTG
         :::::::::::::::::
Ex2,3Ref TTGGAAGCAGTATGTTG
               370
```

S16: *F9*_Exon 4

```
               10        20        30        40        50        60
S16Ex4 ATGGAGATCAGTGTGAGTCCAATCCATGTTTAAATGGCGGCAGTTGCAAGGATGACATTA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex4Ref ATGGAGATCAGTGTGAGTCCAATCCATGTTTAAATGGCGGCAGTTGCAAGGATGACATTA
               10        20        30        40        50        60

               70        80        90       100       110
S16Ex4 ATTCCTATGAATGTTGGTGTCCCTTTGGATTTGAAGGAAAGAACTGTGAATTAG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex4Ref ATTCCTATGAATGTTGGTGTCCCTTTGGATTTGAAGGAAAGAACTGTGAATTAG
               70        80        90       100       110
```

S16: *F9*_Exon 5

```
               10        20        30        40        50        60
S16Ex5 ATGTAACATGTAACATTAAGAATGGCAGATGCGAGCAGTTTTGTAAAAATAGTGCTGATA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex5Ref ATGTAACATGTAACATTAAGAATGGCAGATGCGAGCAGTTTTGTAAAAATAGTGCTGATA
               10        20        30        40        50        60

               70        80        90       100       110       120
S16Ex5 ACAAGGTGGTTTGCTCCTGTACTGAGGGATATCGACTTGCAGAAAACCAGAAGTCCTGTG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex5Ref ACAAGGTGGTTTGCTCCTGTACTGAGGGATATCGACTTGCAGAAAACCAGAAGTCCTGTG
               70        80        90       100       110       120


S16Ex5 AACCAGCAG
       :::::::::
Ex5Ref AACCAGCAG
```

S16: *F9*_Exon 6

```
             10        20        30        40        50        60
S16Ex6 TGCCATTTCCATGTGGAAGAGTTTCTGTTTCACAAACTTCTAAGCTCACCCGTGCTGAGA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex6Ref TGCCATTTCCATGTGGAAGAGTTTCTGTTTCACAAACTTCTAAGCTCACCCGTGCTGAGA
               10        20        30        40        50        60

               70        80        90       100       110       120
S16Ex6 CTGTTTTTCCTGATGTGGACTATGTAAATTCTACTGAAGCTGAAACCATTTTGGATAACA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex6Ref CTGTTTTTCCTGATGTGGACTATGTAAATTCTACTGAAGCTGAAACCATTTTGGATAACA
               70        80        90       100       110       120

              130       140       150       160       170       180
S16Ex6 TCACTCAAAGCACCCAATCATTTAATGACTTCACTCGGGTTGTTGGTGGAGAAGATGCCA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex6Ref TCACTCAAAGCACCCAATCATTTAATGACTTCACTCGGGTTGTTGGTGGAGAAGATGCCA
              130       140       150       160       170       180

              190       200
S16Ex6 AACCAGGTCAATTCCCTTGGCAG
       :::::::::::::::::::::::
Ex6Ref AACCAGGTCAATTCCCTTGGCAG
              190       200
```

S16: *F9*_Exon 7

```
               10        20        30        40        50        60
S16Ex7 GTTGTTTTGAATGGTAAAGTTGATGCATTCTGTGGAGGCTCTATCGTTAATGAAAAATGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex7Ref GTTGTTTTGAATGGTAAAGTTGATGCATTCTGTGGAGGCTCTATCGTTAATGAAAAATGG
               10        20        30        40        50        60

               70        80        90       100       110
S16Ex7 ATTGTAACTGCTGCCCACTGTGTTGAAACTGGTGTTAAAATTACAGTTGTCGCAG
       :::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex7Ref ATTGTAACTGCTGCCCACTGTGTTGAAACTGGTGTTAAAATTACAGTTGTCGCAG
               70        80        90       100       110
```

S16: *F9*_Exon 8

```
               10        20        30        40        50        60
S16Ex8 GTGAACATAATATTGAGGAGACAGAACATACAGAGCAAAAGCGAAATGTGATTCGAATTA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref GTGAACATAATATTGAGGAGACAGAACATACAGAGCAAAAGCGAAATGTGATTCGAATTA
               10        20        30        40        50        60

               70        80        90       100       110       120
S16Ex8 TTCCTCACCACAACTACAATGCAGCTATTAATAAGTACAACCATGACATTGCCCTTCTGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref TTCCTCACCACAACTACAATGCAGCTATTAATAAGTACAACCATGACATTGCCCTTCTGG
               70        80        90       100       110       120

              130       140       150       160       170       180
S16Ex8 AACTGGACGAACCCTTAGTGCTAAACAGCTACGTTACACCTATTTGCATTGCTGACAAGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref AACTGGACGAACCCTTAGTGCTAAACAGCTACGTTACACCTATTTGCATTGCTGACAAGG
              130       140       150       160       170       180

              190       200       210       220       230       240
S16Ex8 AATACACGAACATCTTCCTCAAATTTGGATCTGGCTATGTAAGTGGCTGGGGAAGAGTCT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref AATACACGAACATCTTCCTCAAATTTGGATCTGGCTATGTAAGTGGCTGGGGAAGAGTCT
              190       200       210       220       230       240

              250       260       270       280       290       300
S16Ex8 TCCACAAAGGGAGATCAGCTTTAGTTCTTCAGTACCTTAGAGTTCCACTTGTTGACCGAG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref TCCACAAAGGGAGATCAGCTTTAGTTCTTCAGTACCTTAGAGTTCCACTTGTTGACCGAG
              250       260       270       280       290       300

              310       320       330       340       350       360
S16Ex8 CCACATGTCTTCGATCTACAAAGTTCACCATCTATAACAACATGTTCTGTGCTGGCTTCC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref CCACATGTCTTCGATCTACAAAGTTCACCATCTATAACAACATGTTCTGTGCTGGCTTCC
              310       320       330       340       350       360

              370       380       390       400       410       420
S16Ex8 ATGAAGGAGGTAGAGATTCATGTCAAGGAGATAGTGGGGGACCCCATGTTACTGAAGTGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref ATGAAGGAGGTAGAGATTCATGTCAAGGAGATAGTGGGGGACCCCATGTTACTGAAGTGG
              370       380       390       400       410       420

              430       440       450       460       470       480
S16Ex8 AAGGGACCAGTTTCTTAACTGGAATTATTAGCTGGGGTGAAGAGTGTGCAATGAAAGGCA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref AAGGGACCAGTTTCTTAACTGGAATTATTAGCTGGGGTGAAGAGTGTGCAATGAAAGGCA
              430       440       450       460       470       480

              490       500       510       520       530       540
S16Ex8 AATATGGAATATATACCAAGGTATCCCGGTATGTCAACTGGATTAAGGAAAAAACAAAGC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref AATATGGAATATATACCAAGGTATCCCGGTATGTCAACTGGATTAAGGAAAAAACAAAGC
              490       500       510       520       530       540


S16Ex8 TCACTTAA
       ::::::::
Ex8Ref TCACTTAA
```

Participant 17 (S17)

S17: *F9*_Exon 1

```
               10        20        30        40        50        60
S17Ex1 ATGCAGCGCGTGAACATGATCATGGCAGAATCACCAGGCCTCATCACCATCTGCCTTTTA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex1Ref ATGCAGCGCGTGAACATGATCATGGCAGAATCACCAGGCCTCATCACCATCTGCCTTTTA
               10        20        30        40        50        60

               70        80
S17Ex1 GGATATCTACTCAGTGCTGAATGTACAG
       ::::::::::::::::::::::::::::
Ex1Ref GGATATCTACTCAGTGCTGAATGTACAG
               70        80
```

S17: *F9*_Exon 2,3

```
                 10        20        30        40        50        60
S17Ex2,3 TTTTTCTTGATCATGAAAACGCCAACAAAATTCTGAATCGGCCAAAGAGGTATAATTCAG
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref TTTTTCTTGATCATGAAAACGCCAACAAAATTCTGAATCGGCCAAAGAGGTATAATTCAG
                 10        20        30        40        50        60

                 70        80        90       100       110       120
S17Ex2,3 GTAAATTGGAAGAGTTTGTTCAAGGGAACCTTGAGAGAGAATGTATGGAAGAAAAGTGTA
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref GTAAATTGGAAGAGTTTGTTCAAGGGAACCTTGAGAGAGAATGTATGGAAGAAAAGTGTA
                 70        80        90       100       110       120

                130       140       150       160       170       180
S17Ex2,3 GTTTTGAAGAAGCACGAGAAGTTTTTGAAAACACTGAAAGAACAGTGAGTATTTCCACAT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref GTTTTGAAGAAGCACGAGAAGTTTTTGAAAACACTGAAAGAACAGTGAGTATTTCCACAT
                130       140       150       160       170       180

                190       200       210       220       230       240
S17Ex2,3 AATACCCTTCAGATGCAGAGCATAGAATAGAAAATCTTTAAAAAGACACTTCTCTTTAAA
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref AATACCCTTCAGATGCAGAGCATAGAATAGAAAATCTTTAAAAAGACACTTCTCTTTAAA
                190       200       210       220       230       240

                250       260       270       280       290       300
S17Ex2,3 ATTTTAAAGCATCCATATATATTTATGTATGTTAAATGTTATAAAAGATAGGAAATCAAT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref ATTTTAAAGCATCCATATATATTTATGTATGTTAAATGTTATAAAAGATAGGAAATCAAT
                250       260       270       280       290       300

                310       320       330       340       350       360
S17Ex2,3 ACCAAAACACTTTAGATATTACCGTTAATTTGTCTTCTTTTATTCTTTATAGACTGAATT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref ACCAAAACACTTTAGATATTACCGTTAATTTGTCTTCTTTTATTCTTTATAGACTGAATT
                310       320       330       340       350       360

                370
S17Ex2,3 TTGGAAGCAGTATGTTG
         :::::::::::::::::
Ex2,3Ref TTGGAAGCAGTATGTTG
                370
```

S17: *F9*_Exon 4

```
               10        20        30        40        50        60
S17Ex4 ATGGAGATCAGTGTGAGTCCAATCCATGTTTAAATGGCGGCAGTTGCAAGGATGACATTA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex4Ref ATGGAGATCAGTGTGAGTCCAATCCATGTTTAAATGGCGGCAGTTGCAAGGATGACATTA
               10        20        30        40        50        60

               70        80        90       100       110
S17Ex4 ATTCCTATGAATGTTGGTGTCCCTTTGGATTTGAAGGAAAGAACTGTGAATTAG
       :::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex4Ref ATTCCTATGAATGTTGGTGTCCCTTTGGATTTGAAGGAAAGAACTGTGAATTAG
               70        80        90       100       110
```

S17: *F9*_Exon 5

```
               10        20        30        40        50        60
S17Ex5 ATGTAACATGTAACATTAAGAATGGCAGATGCGAGCAGTTTTGTAAAAATAGTGCTGATA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex5Ref ATGTAACATGTAACATTAAGAATGGCAGATGCGAGCAGTTTTGTAAAAATAGTGCTGATA
               10        20        30        40        50        60

               70        80        90       100       110       120
S17Ex5 ACAAGGTGGTTTGCTCCTGTACTGAGGGATATCGACTTGCAGAAAACCAGAAGTCCTGTG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex5Ref ACAAGGTGGTTTGCTCCTGTACTGAGGGATATCGACTTGCAGAAAACCAGAAGTCCTGTG
               70        80        90       100       110       120


S17Ex5 AACCAGCAG
       :::::::::
Ex5Ref AACCAGCAG
```

S17: *F9*_Exon 6

```
             10        20        30        40        50        60
S17Ex6 TGCCATTTCCATGTGGAAGAGTTTCTGTTTCACAAACTTCTAAGCTCACCCGTGCTGAGA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex6Ref TGCCATTTCCATGTGGAAGAGTTTCTGTTTCACAAACTTCTAAGCTCACCCGTGCTGAGA
               10        20        30        40        50        60

               70        80        90       100       110       120
S17Ex6 CTGTTTTTCCTGATGTGGACTATGTAAATTCTACTGAAGCTGAAACCATTTTGGATAACA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex6Ref CTGTTTTTCCTGATGTGGACTATGTAAATTCTACTGAAGCTGAAACCATTTTGGATAACA
               70        80        90       100       110       120

              130       140       150       160       170       180
S17Ex6 TCACTCAAAGCACCCAATCATTTAATGACTTCACTCGGGTTGTTGGTGGAGAAGATGCCA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex6Ref TCACTCAAAGCACCCAATCATTTAATGACTTCACTCGGGTTGTTGGTGGAGAAGATGCCA
              130       140       150       160       170       180

              190       200
S17Ex6 AACCAGGTCAATTCCCTTGGCAG
       :::::::::::::::::::::::
Ex6Ref AACCAGGTCAATTCCCTTGGCAG
              190       200
```

S17: *F9*_Exon 7

```
               10        20        30        40        50        60
S17Ex7 GTTGTTTTGAATGGTAAAGTTGATGCATTCTGTGGAGGCTCTATCGTTAATGAAAAATGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex7Ref GTTGTTTTGAATGGTAAAGTTGATGCATTCTGTGGAGGCTCTATCGTTAATGAAAAATGG
               10        20        30        40        50        60

               70        80        90       100       110
S17Ex7 ATTGTAACTGCTGCCCACTGTGTTGAAACTGGTGTTAAAATTACAGTTGTCGCAG
       :::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex7Ref ATTGTAACTGCTGCCCACTGTGTTGAAACTGGTGTTAAAATTACAGTTGTCGCAG
               70        80        90       100       110
```

S17: *F9*_Exon 8

```
               10        20        30        40        50        60
S17Ex8 GTGAACATAATATTGAGGAGACAGAACATACAGAGCAAAAGCGAAATGTGATTCGAATTA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref GTGAACATAATATTGAGGAGACAGAACATACAGAGCAAAAGCGAAATGTGATTCGAATTA
               10        20        30        40        50        60

               70        80        90       100       110       120
S17Ex8 TTCCTCACCACAACTACAATGCAGCTATTAATAAGTACAACCATGACATTGCCCTTCTGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref TTCCTCACCACAACTACAATGCAGCTATTAATAAGTACAACCATGACATTGCCCTTCTGG
               70        80        90       100       110       120

              130       140       150       160       170       180
S17Ex8 AACTGGACGAACCCTTAGTGCTAAACAGCTACGTTACACCTATTTGCATTGCTGACAAGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref AACTGGACGAACCCTTAGTGCTAAACAGCTACGTTACACCTATTTGCATTGCTGACAAGG
              130       140       150       160       170       180

              190       200       210       220       230       240
S17Ex8 AATACACGAACATCTTCCTCAAATTTGGATCTGGCTATGTAAGTGGCTGGGGAAGAGTCT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref AATACACGAACATCTTCCTCAAATTTGGATCTGGCTATGTAAGTGGCTGGGGAAGAGTCT
              190       200       210       220       230       240

              250       260       270       280       290       300
S17Ex8 TCCACAAAGGGAGATCAGCTTTAGTTCTTCAGTACCTTAGAGTTCCACTTGTTGACCGAG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref TCCACAAAGGGAGATCAGCTTTAGTTCTTCAGTACCTTAGAGTTCCACTTGTTGACCGAG
              250       260       270       280       290       300

              310       320       330       340       350       360
S17Ex8 CCACATGTCTTCGATCTACAAAGTTCACCATCTATAACAACATGTTCTGTGCTGGCTTCC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref CCACATGTCTTCGATCTACAAAGTTCACCATCTATAACAACATGTTCTGTGCTGGCTTCC
              310       320       330       340       350       360

              370       380       390       400       410       420
S17Ex8 ATGAAGGAGGTAGAGATTCATGTCAAGGAGATAGTGGGGGACCCCATGTTACTGAAGTGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref ATGAAGGAGGTAGAGATTCATGTCAAGGAGATAGTGGGGGACCCCATGTTACTGAAGTGG
              370       380       390       400       410       420

              430       440       450       460       470       480
S17Ex8 AAGGGACCAGTTTCTTAACTGGAATTATTAGCTGGGGTGAAGAGTGTGCAATGAAAGGCA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref AAGGGACCAGTTTCTTAACTGGAATTATTAGCTGGGGTGAAGAGTGTGCAATGAAAGGCA
              430       440       450       460       470       480

              490       500       510       520       530       540
S17Ex8 AATATGGAATATATACCAAGGTATCCCGGTATGTCAACTGGATTAAGGAAAAAACAAAGC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref AATATGGAATATATACCAAGGTATCCCGGTATGTCAACTGGATTAAGGAAAAAACAAAGC
              490       500       510       520       530       540


S17Ex8 TCACTTAA
       ::::::::
Ex8Ref TCACTTAA
```

Participant 18 (S18)

S18: *F9*_Exon 1

```
                10        20        30        40        50        60
S18Ex1 ATGCAGCGCGTGAACATGATCATGGCAGAATCACCAGGCCTCATCACCATCTGCCTTTTA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex1Ref ATGCAGCGCGTGAACATGATCATGGCAGAATCACCAGGCCTCATCACCATCTGCCTTTTA
                10        20        30        40        50        60

                70        80
S18Ex1 GGATATCTACTCAGTGCTGAATGTACAG
       ::::::::::::::::::::::::::::
Ex1Ref GGATATCTACTCAGTGCTGAATGTACAG
                70        80
```

S18: *F9*_Exon 2,3

```
                 10        20        30        40        50        60
S18Ex2,3 TTTTTCTTGATCATGAAAACGCCAACAAAATTCTGAATCGGCCAAAGAGGTATAATTCAG
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref TTTTTCTTGATCATGAAAACGCCAACAAAATTCTGAATCGGCCAAAGAGGTATAATTCAG
                 10        20        30        40        50        60

                 70        80        90       100       110       120
S18Ex2,3 GTAAATTGGAAGAGTTTGTTCAAGGGAACCTTGAGAGAGAATGTATGGAAGAAAAGTGTA
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref GTAAATTGGAAGAGTTTGTTCAAGGGAACCTTGAGAGAGAATGTATGGAAGAAAAGTGTA
                 70        80        90       100       110       120

                130       140       150       160       170       180
S18Ex2,3 GTTTTGAAGAAGCACGAGAAGTTTTTGAAAACACTGAAAGAACAGTGAGTATTTCCACAT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref GTTTTGAAGAAGCACGAGAAGTTTTTGAAAACACTGAAAGAACAGTGAGTATTTCCACAT
                130       140       150       160       170       180

                190       200       210       220       230       240
S18Ex2,3 AATACCCTTCAGATGCAGAGCATAGAATAGAAAATCTTTAAAAAGACACTTCTCTTTAAA
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref AATACCCTTCAGATGCAGAGCATAGAATAGAAAATCTTTAAAAAGACACTTCTCTTTAAA
                190       200       210       220       230       240

                250       260       270       280       290       300
S18Ex2,3 ATTTTAAAGCATCCATATATATTTATGTATGTTAAATGTTATAAAAGATAGGAAATCAAT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref ATTTTAAAGCATCCATATATATTTATGTATGTTAAATGTTATAAAAGATAGGAAATCAAT
                250       260       270       280       290       300

                310       320       330       340       350       360
S18Ex2,3 ACCAAAACACTTTAGATATTACCGTTAATTTGTCTTCTTTTATTCTTTATAGACTGAATT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref ACCAAAACACTTTAGATATTACCGTTAATTTGTCTTCTTTTATTCTTTATAGACTGAATT
                310       320       330       340       350       360

                370
S18Ex2,3 TTGGAAGCAGTATGTTG
         :::::::::::::::::
Ex2,3Ref TTGGAAGCAGTATGTTG
                370
```

S18: *F9*_Exon 4

```
                10        20        30         40        50
S18Ex4 ATGGAGATCAGTGTGAGTCCAATCCATGTTTAA-TGGCGGCAGTTGCAAGGATGACATTA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex4Ref ATGGAGATCAGTGTGAGTCCAATCCATGTTTAAATGGCGGCAGTTGCAAGGATGACATTA
                10        20        30        40        50        60

      60        70        80        90       100       110
S18Ex4 ATTCCTATGAATGTTGGTGTCCCTTTGGATTTGAAGGAAAGAACTGTGAATTAG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex4Ref ATTCCTATGAATGTTGGTGTCCCTTTGGATTTGAAGGAAAGAACTGTGAATTAG
                70        80        90       100       110
```

S18: *F9*_Exon 5

```
                10        20        30        40        50        60
S18Ex5 ATGTAACATGTAACATTAAGAATGGCAGATGCGAGCAGTTTTGTAAAAATAGTGCTGATA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex5Ref ATGTAACATGTAACATTAAGAATGGCAGATGCGAGCAGTTTTGTAAAAATAGTGCTGATA
                10        20        30        40        50        60

                70        80        90       100       110       120
S18Ex5 ACAAGGTGGTTTGCTCCTGTACTGAGGGATATCGACTTGCAGAAAACCAGAAGTCCTGTG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex5Ref ACAAGGTGGTTTGCTCCTGTACTGAGGGATATCGACTTGCAGAAAACCAGAAGTCCTGTG
                70        80        90       100       110       120


S18Ex5 AACCAGCAG
       :::::::::
Ex5Ref AACCAGCAG
```

S18: *F9*_Exon 6

```
            10        20        30        40        50        60
S18Ex6 TGCCATTTCCATGTGGAAGAGTTTCTGTTTCACAAACTTCTAAGCTCACCCGTGCTGAGA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex6Ref TGCCATTTCCATGTGGAAGAGTTTCTGTTTCACAAACTTCTAAGCTCACCCGTGCTGAGA
               10        20        30        40        50        60

               70        80        90       100       110       120
S18Ex6 CTGTTTTTCCTGATGTGGACTATGTAAATTCTACTGAAGCTGAAACCATTTTGGATAACA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex6Ref CTGTTTTTCCTGATGTGGACTATGTAAATTCTACTGAAGCTGAAACCATTTTGGATAACA
               70        80        90       100       110       120

              130       140       150       160       170       180
S18Ex6 TCACTCAAAGCACCCAATCATTTAATGACTTCACTCGGGTTGTTGGTGGAGAAGATGCCA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex6Ref TCACTCAAAGCACCCAATCATTTAATGACTTCACTCGGGTTGTTGGTGGAGAAGATGCCA
              130       140       150       160       170       180

              190       200
S18Ex6 AACCAGGTCAATTCCCTTGGCAG
       :::::::::::::::::::::::
Ex6Ref AACCAGGTCAATTCCCTTGGCAG
              190       200
```

S18: *F9*_Exon 7

```
               10        20        30        40        50        60
S18Ex7 GTTGTTTTGAATGGTAAAGTTGATGCATTCTGTGGAGGCTCTATCGTTAATGAAAAATGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex7Ref GTTGTTTTGAATGGTAAAGTTGATGCATTCTGTGGAGGCTCTATCGTTAATGAAAAATGG
               10        20        30        40        50        60

               70        80        90       100       110
S18Ex7 ATTGTAACTGCTGCCCACTGTGTTGAAACTGGTGTTAAAATTACAGTTGTCGCAG
       :::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex7Ref ATTGTAACTGCTGCCCACTGTGTTGAAACTGGTGTTAAAATTACAGTTGTCGCAG
               70        80        90       100       110
```

S18: *F9*_Exon 8

```
               10        20        30        40        50        60
S18Ex8 GTGAACATAATATTGAGGAGACAGAACATACAGAGCAAAAGCGAAATGTGATTCGAATTA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref GTGAACATAATATTGAGGAGACAGAACATACAGAGCAAAAGCGAAATGTGATTCGAATTA
               10        20        30        40        50        60

               70        80        90       100       110       120
S18Ex8 TTCCTCACCACAACTACAATGCAGCTATTAATAAGTACAACCATGACATTGCCCTTCTGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref TTCCTCACCACAACTACAATGCAGCTATTAATAAGTACAACCATGACATTGCCCTTCTGG
               70        80        90       100       110       120

              130       140       150       160       170       180
S18Ex8 AACTGGACGAACCCTTAGTGCTAAACAGCTACGTTACACCTATTTGCATTGCTGACAAGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref AACTGGACGAACCCTTAGTGCTAAACAGCTACGTTACACCTATTTGCATTGCTGACAAGG
              130       140       150       160       170       180

              190       200       210       220       230       240
S18Ex8 AATACACGAACATCTTCCTCAAATTTGGATCTGGCTATGTAAGTGGCTGGGGAAGAGTCT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref AATACACGAACATCTTCCTCAAATTTGGATCTGGCTATGTAAGTGGCTGGGGAAGAGTCT
              190       200       210       220       230       240

              250       260       270       280       290       300
S18Ex8 TCCACAAAGGGAGATCAGCTTTAGTTCTTCAGTACCTTAGAGTTCCACTTGTTGACCGAG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref TCCACAAAGGGAGATCAGCTTTAGTTCTTCAGTACCTTAGAGTTCCACTTGTTGACCGAG
              250       260       270       280       290       300

              310       320       330       340       350       360
S18Ex8 CCACATGTCTTCGATCTACAAAGTTCACCATCTATAACAACATGTTCTGTGCTGGCTTCC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref CCACATGTCTTCGATCTACAAAGTTCACCATCTATAACAACATGTTCTGTGCTGGCTTCC
              310       320       330       340       350       360

              370       380       390       400       410       420
S18Ex8 ATGAAGGAGGTAGAGATTCATGTCAAGGAGATAGTGGGGGACCCCATGTTACTGAAGTGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref ATGAAGGAGGTAGAGATTCATGTCAAGGAGATAGTGGGGGACCCCATGTTACTGAAGTGG
              370       380       390       400       410       420

              430       440       450       460       470       480
S18Ex8 AAGGGACCAGTTTCTTAACTGGAATTATTAGCTGGGGTGAAGAGTGTGCAATGAAAGGCA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref AAGGGACCAGTTTCTTAACTGGAATTATTAGCTGGGGTGAAGAGTGTGCAATGAAAGGCA
              430       440       450       460       470       480

              490       500       510       520       530       540
S18Ex8 AATATGGAATATATACCAAGGTATCCCGGTATGTCAACTGGATTAAGGAAAAAACAAAGC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref AATATGGAATATATACCAAGGTATCCCGGTATGTCAACTGGATTAAGGAAAAAACAAAGC
              490       500       510       520       530       540


S18Ex8 TCACTTAA
       ::::::::
Ex8Ref TCACTTAA
```

Participant 19 (S19)

S19: *F9*_Exon 1

```
              10        20        30        40        50        60
S19Ex1 ATGCAGCGCGTGAACATGATCATGGCAGAATCACCAGGCCTCATCACCATCTGCCTTTTA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex1Ref ATGCAGCGCGTGAACATGATCATGGCAGAATCACCAGGCCTCATCACCATCTGCCTTTTA
               10        20        30        40        50        60

               70        80
S19Ex1 GGATATCTACTCAGTGCTGAATGTACAG
       ::::::::::::::::::::::::::::
Ex1Ref GGATATCTACTCAGTGCTGAATGTACAG
               70        80
```

S19: *F9*_Exon 2,3

```
                10        20        30        40        50        60
S19Ex2,3 TTTTTCTTGATCATGAAAACGCCAACAAAATTCTGAATCGGCCAAAGAGGTATAATTCAG
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref TTTTTCTTGATCATGAAAACGCCAACAAAATTCTGAATCGGCCAAAGAGGTATAATTCAG
                10        20        30        40        50        60

                70        80        90       100       110       120
S19Ex2,3 GTAAATTGGAAGAGTTTGTTCAAGGGAACCTTGAGAGAGAATGTATGGAAGAAAAGTGTA
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref GTAAATTGGAAGAGTTTGTTCAAGGGAACCTTGAGAGAGAATGTATGGAAGAAAAGTGTA
                70        80        90       100       110       120

               130       140       150       160       170       180
S19Ex2,3 GTTTTGAAGAAGCACGAGAAGTTTTTGAAAACACTGAAAGAACAGTGAGTATTTCCACAT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref GTTTTGAAGAAGCACGAGAAGTTTTTGAAAACACTGAAAGAACAGTGAGTATTTCCACAT
               130       140       150       160       170       180

               190       200       210       220       230       240
S19Ex2,3 AATACCCTTCAGATGCAGAGCATAGAATAGAAAATCTTTAAAAAGACACTTCTCTTTAAA
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref AATACCCTTCAGATGCAGAGCATAGAATAGAAAATCTTTAAAAAGACACTTCTCTTTAAA
               190       200       210       220       230       240

               250       260       270       280       290       300
S19Ex2,3 ATTTTAAAGCATCCATATATATTTATGTATGTTAAATGTTATAAAAGATAGGAAATCAAT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref ATTTTAAAGCATCCATATATATTTATGTATGTTAAATGTTATAAAAGATAGGAAATCAAT
               250       260       270       280       290       300

               310       320       330       340       350       360
S19Ex2,3 ACCAAAACACTTTAGATATTACCGTTAATTTGTCTTCTTTTATTCTTTATAGACTGAATT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref ACCAAAACACTTTAGATATTACCGTTAATTTGTCTTCTTTTATTCTTTATAGACTGAATT
               310       320       330       340       350       360

               370
S19Ex2,3 TTGGAAGCAGTATGTTG
         :::::::::::::::::
Ex2,3Ref TTGGAAGCAGTATGTTG
               370
```

S19: *F9*_Exon 4

```
             10        20        30        40        50
S19Ex4 ATGGAGATCAGTGTGAGTCCAATCCATGTTTAA-TGGCGGCAGTTGCAAGGATGACATTA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex4Ref ATGGAGATCAGTGTGAGTCCAATCCATGTTTAAATGGCGGCAGTTGCAAGGATGACATTA
              10        20        30        40        50        60

     60        70        80        90       100       110
S19Ex4 ATTCCTATGAATGTTGGTGTCCCTTTGGATTTGAAGGAAAGAACTGTGAATTAG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex4Ref ATTCCTATGAATGTTGGTGTCCCTTTGGATTTGAAGGAAAGAACTGTGAATTAG
               70        80        90       100       110
```

S19: *F9*_Exon 5

```
               10        20        30        40        50        60
S19Ex5 ATGTAACATGTAACATTAAGAATGGCAGATGCGAGCAGTTTTGTAAAAATAGTGCTGATA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex5Ref ATGTAACATGTAACATTAAGAATGGCAGATGCGAGCAGTTTTGTAAAAATAGTGCTGATA
               10        20        30        40        50        60

               70        80        90       100       110       120
S19Ex5 ACAAGGTGGTTTGCTCCTGTACTGAGGGATATCGACTTGCAGAAAACCAGAAGTCCTGTG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex5Ref ACAAGGTGGTTTGCTCCTGTACTGAGGGATATCGACTTGCAGAAAACCAGAAGTCCTGTG
               70        80        90       100       110       120


S19Ex5 AACCAGCAG
       :::::::::
Ex5Ref AACCAGCAG
```

S19: *F9*_Exon 6

```
             10        20        30        40        50        60
S19Ex6 TGCCATTTCCATGTGGAAGAGTTTCTGTTTCACAAACTTCTAAGCTCACCCGTGCTGAGA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex6Ref TGCCATTTCCATGTGGAAGAGTTTCTGTTTCACAAACTTCTAAGCTCACCCGTGCTGAGA
               10        20        30        40        50        60

               70        80        90       100       110       120
S19Ex6 CTGTTTTTCCTGATGTGGACTATGTAAATTCTACTGAAGCTGAAACCATTTTGGATAACA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex6Ref CTGTTTTTCCTGATGTGGACTATGTAAATTCTACTGAAGCTGAAACCATTTTGGATAACA
               70        80        90       100       110       120

              130       140       150       160       170       180
S19Ex6 TCACTCAAAGCACCCAATCATTTAATGACTTCACTCGGGTTGTTGGTGGAGAAGATGCCA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex6Ref TCACTCAAAGCACCCAATCATTTAATGACTTCACTCGGGTTGTTGGTGGAGAAGATGCCA
              130       140       150       160       170       180

              190       200
S19Ex6 AACCAGGTCAATTCCCTTGGCAG
       :::::::::::::::::::::::
Ex6Ref AACCAGGTCAATTCCCTTGGCAG
              190       200
```

S19: *F9*_Exon 7

```
               10        20        30        40        50        60
S19Ex7 GTTGTTTTGAATGGTAAAGTTGATGCATTCTGTGGAGGCTCTATCGTTAATGAAAAATGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex7Ref GTTGTTTTGAATGGTAAAGTTGATGCATTCTGTGGAGGCTCTATCGTTAATGAAAAATGG
               10        20        30        40        50        60

               70        80        90       100       110
S19Ex7 ATTGTAACTGCTGCCCACTGTGTTGAAACTGGTGTTAAAATTACAGTTGTCGCAG
       :::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex7Ref ATTGTAACTGCTGCCCACTGTGTTGAAACTGGTGTTAAAATTACAGTTGTCGCAG
               70        80        90       100       110
```

S19: *F9*_Exon 8

```
               10        20        30        40        50        60
S19Ex8 GTGAACATAATATTGAGGAGACAGAACATACAGAGCAAAAGCGAAATGTGATTCGAATTA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref GTGAACATAATATTGAGGAGACAGAACATACAGAGCAAAAGCGAAATGTGATTCGAATTA
               10        20        30        40        50        60

               70        80        90       100       110       120
S19Ex8 TTCCTCACCACAACTACAATGCAGCTATTAATAAGTACAACCATGACATTGCCCTTCTGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref TTCCTCACCACAACTACAATGCAGCTATTAATAAGTACAACCATGACATTGCCCTTCTGG
               70        80        90       100       110       120

              130       140       150       160       170       180
S19Ex8 AACTGGACGAACCCTTAGTGCTAAACAGCTACGTTACACCTATTTGCATTGCTGACAAGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref AACTGGACGAACCCTTAGTGCTAAACAGCTACGTTACACCTATTTGCATTGCTGACAAGG
              130       140       150       160       170       180

              190       200       210       220       230       240
S19Ex8 AATACACGAACATCTTCCTCAAATTTGGATCTGGCTATGTAAGTGGCTGGGGAAGAGTCT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref AATACACGAACATCTTCCTCAAATTTGGATCTGGCTATGTAAGTGGCTGGGGAAGAGTCT
              190       200       210       220       230       240

              250       260       270       280       290       300
S19Ex8 TCCACAAAGGGAGATCAGCTTTAGTTCTTCAGTACCTTAGAGTTCCACTTGTTGACCGAG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref TCCACAAAGGGAGATCAGCTTTAGTTCTTCAGTACCTTAGAGTTCCACTTGTTGACCGAG
              250       260       270       280       290       300

              310       320       330       340       350       360
S19Ex8 CCACATGTCTTCGATCTACAAAGTTCACCATCTATAACAACATGTTCTGTGCTGGCTTCC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref CCACATGTCTTCGATCTACAAAGTTCACCATCTATAACAACATGTTCTGTGCTGGCTTCC
              310       320       330       340       350       360

              370       380       390       400       410       420
S19Ex8 ATGAAGGAGGTAGAGATTCATGTCAAGGAGATAGTGGGGGACCCCATGTTACTGAAGTGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref ATGAAGGAGGTAGAGATTCATGTCAAGGAGATAGTGGGGGACCCCATGTTACTGAAGTGG
              370       380       390       400       410       420

              430       440       450       460       470       480
S19Ex8 AAGGGACCAGTTTCTTAACTGGAATTATTAGCTGGGGTGAAGAGTGTGCAATGAAAGGCA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref AAGGGACCAGTTTCTTAACTGGAATTATTAGCTGGGGTGAAGAGTGTGCAATGAAAGGCA
              430       440       450       460       470       480

              490       500       510       520       530       540
S19Ex8 AATATGGAATATATACCAAGGTATCCCGGTATGTCAACTGGATTAAGGAAAAAACAAAGC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref AATATGGAATATATACCAAGGTATCCCGGTATGTCAACTGGATTAAGGAAAAAACAAAGC
              490       500       510       520       530       540


S19Ex8 TCACTTAA
       ::::::::
Ex8Ref TCACTTAA
```

Participant 20 (S20)

S20: *F9*_Exon 1

```
               10        20        30        40        50        60
S20Ex1 ATGCAGCGCGTGAACATGATCATGGCAGAATCACCAGGCCTCATCACCATCTGCCTTTTA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex1Ref ATGCAGCGCGTGAACATGATCATGGCAGAATCACCAGGCCTCATCACCATCTGCCTTTTA
               10        20        30        40        50        60

               70        80
S20Ex1 GGATATCTACTCAGTGCTGAATGTACAG
       ::::::::::::::::::::::::::::
Ex1Ref GGATATCTACTCAGTGCTGAATGTACAG
               70        80
```

S20: *F9*_Exon 2,3

```
                 10        20        30        40        50        60
S20Ex2,3 TTTTTCTTGATCATGAAAACGCCAACAAAATTCTGAATCGGCCAAAGAGGTATAATTCAG
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref TTTTTCTTGATCATGAAAACGCCAACAAAATTCTGAATCGGCCAAAGAGGTATAATTCAG
                 10        20        30        40        50        60

                 70        80        90       100       110       120
S20Ex2,3 GTAAATTGGAAGAGTTTGTTCAAGGGAACCTTGAGAGAGAATGTATGGAAGAAAAGTGTA
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref GTAAATTGGAAGAGTTTGTTCAAGGGAACCTTGAGAGAGAATGTATGGAAGAAAAGTGTA
                 70        80        90       100       110       120

                130       140       150       160       170       180
S20Ex2,3 GTTTTGAAGAAGCACGAGAAGTTTTTGAAAACACTGAAAGAACAGTGAGTATTTCCACAT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref GTTTTGAAGAAGCACGAGAAGTTTTTGAAAACACTGAAAGAACAGTGAGTATTTCCACAT
                130       140       150       160       170       180

                190       200       210       220       230       240
S20Ex2,3 AATACCCTTCAGATGCAGAGCATAGAATAGAAAATCTTTAAAAAGACACTTCTCTTTAAA
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref AATACCCTTCAGATGCAGAGCATAGAATAGAAAATCTTTAAAAAGACACTTCTCTTTAAA
                190       200       210       220       230       240

                250       260       270       280       290       300
S20Ex2,3 ATTTTAAAGCATCCATATATATTTATGTATGTTAAATGTTATAAAAGATAGGAAATCAAT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref ATTTTAAAGCATCCATATATATTTATGTATGTTAAATGTTATAAAAGATAGGAAATCAAT
                250       260       270       280       290       300

                310       320       330       340       350       360
S20Ex2,3 ACCAAAACACTTTAGATATTACCGTTAATTTGTCTTCTTTTATTCTTTATAGACTGAATT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref ACCAAAACACTTTAGATATTACCGTTAATTTGTCTTCTTTTATTCTTTATAGACTGAATT
                310       320       330       340       350       360

                370
S20Ex2,3 TTGGAAGCAGTATGTTG
         :::::::::::::::::
Ex2,3Ref TTGGAAGCAGTATGTTG
                370
```

S20: *F9*_Exon 4

```
               10        20        30        40        50        60
S20Ex4 ATGGAGATCAGTGTGAGTCCAATCCATGTTTAAATGGCGGCAGTTGCAAGGATGACATTA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex4Ref ATGGAGATCAGTGTGAGTCCAATCCATGTTTAAATGGCGGCAGTTGCAAGGATGACATTA
               10        20        30        40        50        60

               70        80        90       100       110
S20Ex4 ATTCCTATGAATGTTGGTGTCCCTTTGGATTTGAAGGAAAGAACTGTGAATTAG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex4Ref ATTCCTATGAATGTTGGTGTCCCTTTGGATTTGAAGGAAAGAACTGTGAATTAG
               70        80        90       100       110
```

S20: *F9*_Exon 5

```
               10        20        30        40        50        60
S20Ex5 ATGTAACATGTAACATTAAGAATGGCAGATGCGAGCAGTTTTGTAAAAATAGTGCTGATA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex5Ref ATGTAACATGTAACATTAAGAATGGCAGATGCGAGCAGTTTTGTAAAAATAGTGCTGATA
               10        20        30        40        50        60

               70        80        90       100       110       120
S20Ex5 ACAAGGTGGTTTGCTCCTGTACTGAGGGATATCGACTTGCAGAAAACCAGAAGTCCTGTG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex5Ref ACAAGGTGGTTTGCTCCTGTACTGAGGGATATCGACTTGCAGAAAACCAGAAGTCCTGTG
               70        80        90       100       110       120


S20Ex5 AACCAGCAG
       :::::::::
Ex5Ref AACCAGCAG
```

S20: *F9*_Exon 6

```
             10        20        30        40        50        60
S20Ex6 TGCCATTTCCATGTGGAAGAGTTTCTGTTTCACAAACTTCTAAGCTCACCCGTGCTGAGA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex6Ref TGCCATTTCCATGTGGAAGAGTTTCTGTTTCACAAACTTCTAAGCTCACCCGTGCTGAGA
               10        20        30        40        50        60

               70        80        90       100       110       120
S20Ex6 CTGTTTTTCCTGATGTGGACTATGTAAATTCTACTGAAGCTGAAACCATTTTGGATAACA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex6Ref CTGTTTTTCCTGATGTGGACTATGTAAATTCTACTGAAGCTGAAACCATTTTGGATAACA
               70        80        90       100       110       120

              130       140       150       160       170       180
S20Ex6 TCACTCAAAGCACCCAATCATTTAATGACTTCACTCGGGTTGTTGGTGGAGAAGATGCCA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex6Ref TCACTCAAAGCACCCAATCATTTAATGACTTCACTCGGGTTGTTGGTGGAGAAGATGCCA
              130       140       150       160       170       180

              190       200
S20Ex6 AACCAGGTCAATTCCCTTGGCAG
       :::::::::::::::::::::::
Ex6Ref AACCAGGTCAATTCCCTTGGCAG
              190       200
```

S20: *F9*_Exon 7

```
               10        20        30        40        50        60
S20Ex7 GTTGTTTTGAATGGTAAAGTTGATGCATTCTGTGGAGGCTCTATCGTTAATGAAAAATGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex7Ref GTTGTTTTGAATGGTAAAGTTGATGCATTCTGTGGAGGCTCTATCGTTAATGAAAAATGG
               10        20        30        40        50        60

               70        80        90       100       110
S20Ex7 ATTGTAACTGCTGCCCACTGTGTTGAAACTGGTGTTAAAATTACAGTTGTCGCAG
       :::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex7Ref ATTGTAACTGCTGCCCACTGTGTTGAAACTGGTGTTAAAATTACAGTTGTCGCAG
               70        80        90       100       110
```

S20: *F9*_Exon 8

```
               10        20        30        40        50        60
S20Ex8 GTGAACATAATATTGAGGAGACAGAACATACAGAGCAAAAGCGAAATGTGATTCGAATTA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref GTGAACATAATATTGAGGAGACAGAACATACAGAGCAAAAGCGAAATGTGATTCGAATTA
               10        20        30        40        50        60

               70        80        90       100       110       120
S20Ex8 TTCCTCACCACAACTACAATGCAGCTATTAATAAGTACAACCATGACATTGCCCTTCTGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref TTCCTCACCACAACTACAATGCAGCTATTAATAAGTACAACCATGACATTGCCCTTCTGG
               70        80        90       100       110       120

              130       140       150       160       170       180
S20Ex8 AACTGGACGAACCCTTAGTGCTAAACAGCTACGTTACACCTATTTGCATTGCTGACAAGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref AACTGGACGAACCCTTAGTGCTAAACAGCTACGTTACACCTATTTGCATTGCTGACAAGG
              130       140       150       160       170       180

              190       200       210       220       230       240
S20Ex8 AATACACGAACATCTTCCTCAAATTTGGATCTGGCTATGTAAGTGGCTGGGGAAGAGTCT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref AATACACGAACATCTTCCTCAAATTTGGATCTGGCTATGTAAGTGGCTGGGGAAGAGTCT
              190       200       210       220       230       240

              250       260       270       280       290       300
S20Ex8 TCCACAAAGGGAGATCAGCTTTAGTTCTTCAGTACCTTAGAGTTCCACTTGTTGACCGAG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref TCCACAAAGGGAGATCAGCTTTAGTTCTTCAGTACCTTAGAGTTCCACTTGTTGACCGAG
              250       260       270       280       290       300

              310       320       330       340       350       360
S20Ex8 CCACATGTCTTCGATCTACAAAGTTCACCATCTATAACAACATGTTCTGTGCTGGCTTCC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref CCACATGTCTTCGATCTACAAAGTTCACCATCTATAACAACATGTTCTGTGCTGGCTTCC
              310       320       330       340       350       360

              370       380       390       400       410       420
S20Ex8 ATGAAGGAGGTAGAGATTCATGTCAAGGAGATAGTGGGGGACCCCATGTTACTGAAGTGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref ATGAAGGAGGTAGAGATTCATGTCAAGGAGATAGTGGGGGACCCCATGTTACTGAAGTGG
              370       380       390       400       410       420

              430       440       450       460       470       480
S20Ex8 AAGGGACCAGTTTCTTAACTGGAATTATTAGCTGGGGTGAAGAGTGTGCAATGAAAGGCA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref AAGGGACCAGTTTCTTAACTGGAATTATTAGCTGGGGTGAAGAGTGTGCAATGAAAGGCA
              430       440       450       460       470       480

              490       500       510       520       530
S20Ex8 AATATGGAATATATACCAAGGTATCCCGGTATGTCAACTGGATTAAGGAAAAAACAA-GC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::: ::
Ex8Ref AATATGGAATATATACCAAGGTATCCCGGTATGTCAACTGGATTAAGGAAAAAACAAAGC
              490       500       510       520       530       540

     540
S20Ex8 TCACTTAA
       ::::::::
Ex8Ref TCACTTAA
```

Participant 21 (S21)

S21: *F9*_Exon 1

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              10        20        30        40        50        60
S21Ex1 ATGCAGCGCGTGAACATGATCATGGCAGAATCACCAGGCCTCATCACCATCTGCCTTTTA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex1Ref ATGCAGCGCGTGAACATGATCATGGCAGAATCACCAGGCCTCATCACCATCTGCCTTTTA
               10        20        30        40        50        60

               70        80
S21Ex1 GGATATCTACTCAGTGCTGAATGTACAG
       ::::::::::::::::::::::::::::
Ex1Ref GGATATCTACTCAGTGCTGAATGTACAG
               70        80
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S21: *F9*_Exon 2,3

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                10        20        30        40        50        60
S21Ex2,3 TTTTTCTTGATCATGAAAACGCCAACAAAATTCTGAATCGGCCAAAGAGGTATAATTCAG
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref TTTTTCTTGATCATGAAAACGCCAACAAAATTCTGAATCGGCCAAAGAGGTATAATTCAG
                10        20        30        40        50        60

                70        80        90       100       110       120
S21Ex2,3 GTAAATTGGAAGAGTTTGTTCAAGGGAACCTTGAGAGAGAATGTATGGAAGAAAAGTGTA
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref GTAAATTGGAAGAGTTTGTTCAAGGGAACCTTGAGAGAGAATGTATGGAAGAAAAGTGTA
                70        80        90       100       110       120

               130       140       150       160       170       180
S21Ex2,3 GTTTTGAAGAAGCACGAGAAGTTTTTGAAAACACTGAAAGAACAGTGAGTATTTCCACAT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref GTTTTGAAGAAGCACGAGAAGTTTTTGAAAACACTGAAAGAACAGTGAGTATTTCCACAT
               130       140       150       160       170       180

               190       200       210       220       230       240
S21Ex2,3 AATACCCTTCAGATGCAGAGCATAGAATAGAAAATCTTTAAAAAGACACTTCTCTTTAAA
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref AATACCCTTCAGATGCAGAGCATAGAATAGAAAATCTTTAAAAAGACACTTCTCTTTAAA
               190       200       210       220       230       240

               250       260       270       280       290       300
S21Ex2,3 ATTTTAAAGCATCCATATATATTTATGTATGTTAAATGTTATAAAAGATAGGAAATCAAT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref ATTTTAAAGCATCCATATATATTTATGTATGTTAAATGTTATAAAAGATAGGAAATCAAT
               250       260       270       280       290       300

               310       320       330       340       350       360
S21Ex2,3 ACCAAAACACTTTAGATATTACCGTTAATTTGTCTTCTTTTATTCTTTATAGACTGAATT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex2,3Ref ACCAAAACACTTTAGATATTACCGTTAATTTGTCTTCTTTTATTCTTTATAGACTGAATT
               310       320       330       340       350       360

               370
S21Ex2,3 TTGGAAGCAGTATGTTG
         :::::::::::::::::
Ex2,3Ref TTGGAAGCAGTATGTTG
               370
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S21: *F9*_Exon 4

```
               10        20        30        40        50        60
S21Ex4 ATGGAGATCAGTGTGAGTCCAATCCATGTTTAAATGGCGGCAGTTGCAAGGATGACATTA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex4Ref ATGGAGATCAGTGTGAGTCCAATCCATGTTTAAATGGCGGCAGTTGCAAGGATGACATTA
               10        20        30        40        50        60

               70        80        90       100       110
S21Ex4 ATTCCTATGAATGTTGGTGTCCCTTTGGATTTGAAGGAAAGAACTGTGAATTAG
       :::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex4Ref ATTCCTATGAATGTTGGTGTCCCTTTGGATTTGAAGGAAAGAACTGTGAATTAG
               70        80        90       100       110
```

S21: *F9*_Exon 5

```
               10        20        30        40        50        60
S21Ex5 ATGTAACATGTAACATTAAGAATGGCAGATGCGAGCAGTTTTGTAAAAATAGTGCTGATA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex5Ref ATGTAACATGTAACATTAAGAATGGCAGATGCGAGCAGTTTTGTAAAAATAGTGCTGATA
               10        20        30        40        50        60

               70        80        90       100       110       120
S21Ex5 ACAAGGTGGTTTGCTCCTGTACTGAGGGATATCGACTTGCAGAAAACCAGAAGTCCTGTG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex5Ref ACAAGGTGGTTTGCTCCTGTACTGAGGGATATCGACTTGCAGAAAACCAGAAGTCCTGTG
               70        80        90       100       110       120


S21Ex5 AACCAGCAG
       :::::::::
Ex5Ref AACCAGCAG
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S21: *F9*_Exon 6

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            10        20        30        40        50        60
S21Ex6 TGCCATTTCCATGTGGAAGAGTTTCTGTTTCACAAACTTCTAAGCTCACCCGTGCTGAGA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex6Ref TGCCATTTCCATGTGGAAGAGTTTCTGTTTCACAAACTTCTAAGCTCACCCGTGCTGAGA
               10        20        30        40        50        60

               70        80        90       100       110       120
S21Ex6 CTGTTTTTCCTGATGTGGACTATGTAAATTCTACTGAAGCTGAAACCATTTTGGATAACA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex6Ref CTGTTTTTCCTGATGTGGACTATGTAAATTCTACTGAAGCTGAAACCATTTTGGATAACA
               70        80        90       100       110       120

              130       140       150       160       170       180
S21Ex6 TCACTCAAAGCACCCAATCATTTAATGACTTCACTCGGGTTGTTGGTGGAGAAGATGCCA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex6Ref TCACTCAAAGCACCCAATCATTTAATGACTTCACTCGGGTTGTTGGTGGAGAAGATGCCA
              130       140       150       160       170       180

              190       200
S21Ex6 AACCAGGTCAATTCCCTTGGCAG
       :::::::::::::::::::::::
Ex6Ref AACCAGGTCAATTCCCTTGGCAG
              190       200
```

S21: *F9*_Exon 7

```
               10        20        30        40        50        60
S21Ex7 GTTGTTTTGAATGGTAAAGTTGATGCATTCTGTGGAGGCTCTATCGTTAATGAAAAATGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex7Ref GTTGTTTTGAATGGTAAAGTTGATGCATTCTGTGGAGGCTCTATCGTTAATGAAAAATGG
               10        20        30        40        50        60

               70        80        90       100       110
S21Ex7 ATTGTAACTGCTGCCCACTGTGTTGAAACTGGTGTTAAAATTACAGTTGTCGCAG
       :::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex7Ref ATTGTAACTGCTGCCCACTGTGTTGAAACTGGTGTTAAAATTACAGTTGTCGCAG
               70        80        90       100       110
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S21: *F9*_Exon 8

```
               10        20        30        40        50        60
S21Ex8 GTGAACATAATATTGAGGAGACAGAACATACAGAGCAAAAGCGAAATGTGATTCGAATTA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref GTGAACATAATATTGAGGAGACAGAACATACAGAGCAAAAGCGAAATGTGATTCGAATTA
               10        20        30        40        50        60

               70        80        90       100       110       120
S21Ex8 TTCCTCACCACAACTACAATGCAGCTATTAATAAGTACAACCATGACATTGCCCTTCTGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref TTCCTCACCACAACTACAATGCAGCTATTAATAAGTACAACCATGACATTGCCCTTCTGG
               70        80        90       100       110       120

              130       140       150       160       170       180
S21Ex8 AACTGGACGAACCCTTAGTGCTAAACAGCTACGTTACACCTATTTGCATTGCTGACAAGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref AACTGGACGAACCCTTAGTGCTAAACAGCTACGTTACACCTATTTGCATTGCTGACAAGG
              130       140       150       160       170       180

              190       200       210       220       230       240
S21Ex8 AATACACGAACATCTTCCTCAAATTTGGATCTGGCTATGTAAGTGGCTGGGGAAGAGTCT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref AATACACGAACATCTTCCTCAAATTTGGATCTGGCTATGTAAGTGGCTGGGGAAGAGTCT
              190       200       210       220       230       240

              250       260       270       280       290       300
S21Ex8 TCCACAAAGGGAGATCAGCTTTAGTTCTTCAGTACCTTAGAGTTCCACTTGTTGACCGAG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref TCCACAAAGGGAGATCAGCTTTAGTTCTTCAGTACCTTAGAGTTCCACTTGTTGACCGAG
              250       260       270       280       290       300

              310       320       330       340       350       360
S21Ex8 CCACATGTCTTCGATCTACAAAGTTCACCATCTATAACAACATGTTCTGTGCTGGCTTCC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref CCACATGTCTTCGATCTACAAAGTTCACCATCTATAACAACATGTTCTGTGCTGGCTTCC
              310       320       330       340       350       360

              370       380       390       400       410       420
S21Ex8 ATGAAGGAGGTAGAGATTCATGTCAAGGAGATAGTGGGGGACCCCATGTTACTGAAGTGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref ATGAAGGAGGTAGAGATTCATGTCAAGGAGATAGTGGGGGACCCCATGTTACTGAAGTGG
              370       380       390       400       410       420

              430       440       450       460       470       480
S21Ex8 AAGGGACCAGTTTCTTAACTGGAATTATTAGCTGGGGTGAAGAGTGTGCAATGAAAGGCA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref AAGGGACCAGTTTCTTAACTGGAATTATTAGCTGGGGTGAAGAGTGTGCAATGAAAGGCA
              430       440       450       460       470       480

              490       500       510       520       530
S21Ex8 AATATGGAATATATACCAAGGTATCCCGGTATGTCAACTGGATTAAGGAAAAAACAA-GC
       :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Ex8Ref AATATGGAATATATACCAAGGTATCCCGGTATGTCAACTGGATTAAGGAAAAAACAAAGC
              490       500       510       520       530       540

     540
S21Ex8 TCACTTAA
       ::::::::
Ex8Ref TCACTTAA
```

Masters of Medical Science (MMedSc)

Human Molecular Biology

Mrs C Bester