

The occurrence of mitochondrial DNA polymerase gamma gene mutations in mitochondrial deficiencies, in a selection of South African paediatric patients

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Psalm 8

For the director of music. A psalm of David.

*O Lord, our Lord,
how majestic is Your Name in all the earth!*

You have set Your glory above the heavens.

From the lips of children and infants

You have ordained praise

because of Your enemies,

to silence the foe and the avenger.

When I consider your heavens, the work of Your fingers,

the moon and the stars which you have set in place,

what is man that You are mindful of him,

the son of man that You care for him?

You made him a little lower than the heavenly beings

and crowned him with glory and honor.

You made him ruler over the works of your hands;

you put everything under his feet:

all flocks and herds, and the beasts of the field,

the birds of the air, and the fish of the sea,

all that swim the paths of the seas.

O Lord, our Lord, how majestic is Your name in all the earth!

(NIV)

This dissertation is dedicated to my husband, Karl, and to my family.

All the glory and honour to God, the Father of our Lord Jesus Christ, who gives us the Spirit of wisdom and revelation in the knowledge of Him (Ephesians 1:17)

Amen!

The lives of a cell: (Thomas, 1974; Singh, 2000)

"I am intimately involved, and obliged to do a great deal of essential work for my mitochondria. My nuclei code out the outer membranes of each, and a good many of the enzymes attached to the cristae must be synthesised by me. Each of them, by all accounts, makes only enough of its own materials to get along and the rest must come from me. And I am the one who has to do the worrying. Now that I know the situation, I can find all kinds of things to worry about..."

"... Then there's the question of my estate. Do my mitochondria all die with me, or did my children get some of mine along with their mother's. This sort of thing should not worry me, I know, but it does."

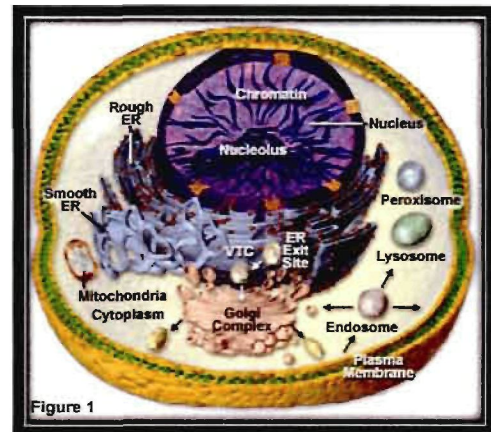


Figure 1.1 Graphical presentation of a human cell.

This image illustrates the location of a mitochondrion within the cell. Image courtesy: Mr. M.W. Davidson. (Molecular Expressions, Cell Biology and Microscopy)

Opsomming:

Die voorkoms van mitochondriale DNA polymerase gamma geenmutasies in mitochondriale defekte in 'n seleksie van Suid-Afrikaanse pediatriese pasiënte.

Mitochondrieë is die sel se belangrikste energievoorskaffer en voorsien die nodige energie aan elke sel in die vorm van adenosientrifosfaat (ATP). ATP is die finale produk wat gevorm word tydens sellulêre oksidatiewe fosforilering (Eng: OXPHOS). Gedurende hierdie proses word verskeie reduserende bronne gebruik vir die uiteindelijke produksie van ATP, deur die laaste ensiem van OXPHOS. Suurstof tree as finale elektronontvanger op in die elektrontransportketting (ETK), wat saamgestel is uit die eerste vier ensieme van die OXPHOS sisteem.

Die OXPHOS sisteem is 'n elektrochemiese pomp wat geleë is in die mitochondriale binnemembraan. Dit bestaan uit kern- en mitochondriaal-gekodeerde subeenhede. Mitochondriale DNA (mtDNA) kodeer vir 13 peptiede van die OXPHOS sisteem, 22 transport RNA's en 2 ribosomale RNA's. Defekte van hierdie sisteem, wat kan ontstaan as gevolg van kern- en mitochondriale DNA mutasies, kan lei tot sogenaamde mitochondriale defekte wat die algemeenste aangebore metaboliese defek in kinders is. 'n Verskeidenheid toestande met verskillende grade van erns word toegeskryf aan mitochondriale defekte wat die sel se energieproduksie beïnvloed. Die integriteit van mtDNA is daarom uiters belangrik om voldoende ATP produksie in die sel te verseker.

Kerngekodeerde mitochondriale DNA polimerase gamma (mtDNA POLG of POLG), is die enigste DNA polimerase verantwoordelik vir mtDNA replisering. Dit is onlangs bevind dat daar 'n hoë voorkoms (tot 25%) van *POLG1* mutasies by mitochondriale defekte bestaan.

Daar is min inligting bekend rakende die etiologie van mitochondriale defekte in die Suid-Afrikaanse populasie. Hierdie studie vorm deel van 'n studie met verskeie medewerkers om die etiologie van mitochondriale toestande in Suid-Afrikaanse pediatriese pasiënte te ondersoek. Pasiënte was voorheen gediagnoseer op grond van die kliniese beeld en/of biochemiese analises, sonder die ondersteuning van genetiese toetsing. Hierdie studie was onderneem om die rol van moontlike mutasies in die mtDNA *POLG1* geen in 'n klinies-geselekteerde teikengroep te ondersoek. Die keuse van agt pasiënte uit 'n groep van 38, was gemaak op grond van abnormale oogfunksie wat voorheen al toegeskryf is aan moontlike abnormale funksionering van POLG. Die doel van hierdie studie was om die genetiese volgorde van die *POLG1* geen in die groep geselekteerde pasiënte, sowel as die relatiewe mitochondriale DNA kopiegetal van die volledige groep van 38 pasiënte, te bepaal.

In die lig van die resultate verkry uit die mitochondriale kopiegetal van die groep van 38 pasiënte, blyk dit in hierdie studie dat pasiënt seleksie op grond van uitsluitlik kliniese fenotiepe 'n onbetroubare metode is om

POLG1 mutasies op te spoor. In hierdie studie is geen patogeniese mutasies, invoegings of delesies in die geselekteerde groep gevind nie, maar agt enkel nukleotied polimorfismes – insluitende twee “invoeging-delesies”, was gevind.

Geen mutasies was in die *POLG* geen van die pasiënte, wat op grond van hul kliniese beeld moontlike *POLG* wanfunksionering kon toon, gevind nie. Aangesien die pasiënt getal baie klein was, is hierdie bevindinge egter nie verteenwoordigend van al die Suid-Afrikaanse pasiënte nie. Dit word daarom aanbeveel dat die *POLG* geen se volgorde in alle Suid-Afrikaanse pasiënte met bevestigde mitochondriale toestande bepaal moet word om moontlike mutasies te vind, alvorens enige finale afleiding gemaak kan word.

Abstract

Mitochondria are the "power houses" of each cell, sustaining the cell's energy demands by providing energy in the form of adenosine triphosphate (ATP). ATP is produced inside the mitochondria during cellular oxidative phosphorylation (OXPHOS). During this process, numerous reducing agents work together to finally produce ATP by the last enzyme of the OXPHOS system. Oxygen acts as the final electron acceptor in the electron transport chain (ETC), which is composed by the first four enzymes of the OXPHOS system.

The OXPHOS system is an electrochemical pump situated in the mitochondrial inner membrane. It contains subunits that are encoded by nuclear and mitochondrial DNA respectively. Mitochondrial DNA (mtDNA) encodes 13 peptides (part of the OXPHOS system), 22 transfer RNAs and 2 ribosomal RNAs. Disorders of this system can arise from mutations in either mitochondrial or nuclear DNA and are responsible for the most prevalent inborn (inherited) errors of metabolism in children. Various disorders with ranging severity have been linked to mitochondrial disorders that affect the energy production of the cell. This is especially evident when mutations in mtDNA occur. Therefore, the integrity of mtDNA is of utmost importance to primarily ensure the sufficient production of ATP within each cell.

Mitochondrial DNA polymerase gamma (mtDNA POLG or POLG) is the only nuclear encoded DNA polymerase involved in the replication of mtDNA. It has recently been shown that there is a high probability of *POLG1* gene mutations (~25%) in mitochondrial disorders.

Very little is known about the aetiology of mitochondrial disorders in the South African population. This study is part of a collaborative study initiated in order to investigate the aetiology of mitochondrial disorders in South African paediatric patients. Patients were previously diagnosed on clinical presentation and/or biochemical enzyme analysis only, without the support of genetic testing. This study was undertaken to elucidate the role of possible mutations in the mtDNA *POLG1* gene in a clinically selected paediatric target patient group (TPG). The clinical selection of eight patients from a group of 38 paediatric patients was mainly based on the occurrences of impaired eye function which has previously been associated with possible POLG malfunctioning. The aim of this study was to determine the *POLG1* genetic sequence in the selected target patient group and to determine the relative mitochondrial copy number (RMCN) of the entire group of 38 patients.

Results obtained from RMCN analysis of the larger paediatric patient group, suggests that the clinical selection of patients for possible POLG mutations is inadequate. No pathogenic mutations, insertions or deletions were found in the selected TPG, but eight known intronic single nucleotide polymorphisms (SNPs), which include two insertion-deletions, were detected.

No mutations were found in the *POLG* gene of patients that, based on their clinical profiles, were suspected to have *POLG* malfunctioning. However, the number of patients investigated in this study was small and therefore these results are not representative of all South African patients. It is therefore suggested that all the South African patients with confirmed mitochondrial disorders should be sequenced for possible *POLG* gene mutations, before any final conclusions can be drawn.

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I am greatly indebted to my supervisor and co-supervisor, Prof. Francois van der Westhuizen and Prof. Izelle Smuts, for making special arrangements to accommodate my study during the year. It has been an honour and privilege to work with them during this study and great thanks go to them for their input, patience and excellent guidance during this study. Not only did I enjoy learning new techniques, but also found this study inspiring and motivating. I particularly take joy in the possibility that my research could contribute to the understanding of the disease and to aid people in the diagnosis of the specific mitochondrial disorders.

A word of thanks goes to Inqaba Biotec industries for their accommodation and assistance with my practical.

Furthermore, I would like to thank my family for their continual support and especially my husband for his motivation and encouragement.

All thanks to the Lord our God for blessing me with the abilities and privilege to be able to complete this work.

Declaration

I hereby declare that the work presented in this dissertation is that of the author.

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List of abbreviations and symbols

ADP	Adenosine diphosphate
ANS	Ataxia-neuropathy syndrome,
AS	Alpers syndrome
ATP	Adenosine triphosphate
bp	Base pair
CNS	Central Nervous System
ddNTP	Dideoxynucleotide-triphosphate
D-loop	Displacement loop
dNTP	Deoxynucleotide-triphosphate
dsDNA	Double stranded DNA
H ₂ O	Water
HL	Mitochondrial light strand origin of replication
HO	Mitochondrial heavy strand origin of replication
KSS	Kearns-Sayre syndrome
LHON	Leber's hereditary optic neuropathy
LSP	Light strand promoter
MELAS	Mitochondrial encephalomyopathy, lactic acidosis, and strokelike episodes
MERFF	Myoclonic epilepsy and ragged red fibres
mg	Milligram
min	Minutes
MIRAS,	Mitochondrial recessive ataxia syndrome
ml	Milliliter
MNGIE	Mitochondrial neurogastrointestinal encephalomyopathy
mtDNA	Mitochondrial deoxyribonucleic acid
mtDNA POLG	Mitochondrial DNA polymerase gamma
mtSSB	Mitochondrial single stranded binding protein
NAD ⁺	Nicotinamide adenine dinucleotide (oxidised)
NADH	Nicotinamide adenine dinucleotide (reduced)
NARP	Neuropathy, ataxia and retinitis pigmentosa
ND2	Mitochondrial gene encoding NADH dehydrogenase subunit 2
nDNA	Nuclear DNA (genomic DNA)
ng/μL	Nanogram per microliter
OXPHOS	Oxidative phosphorylation
PCR	Polymerase chain reaction
PEO	Progressive external ophthalmoplegia
POLG	mtDNA polymerase gamma (protein)
<i>POLG</i>	mtDNA polymerase gamma (gene)
POLG A	mtDNA polymerase gamma alpha subunit (protein)
POLG B	mtDNA polymerase gamma accessory beta subunit (protein)
<i>POLG1</i>	mtDNA polymerase gamma alpha subunit gene
<i>POLG2</i>	mtDNA polymerase gamma accessory beta subunit gene
RCMN	Relative mitochondrial copy number
SSBP	Single stranded binding protein

Ta	Estimated optimum primer annealing temperature
Taq	DNA polymerase from <i>Thermus aquaticus</i>
TBE	Tris, Boric acid and EDTA.
TCA	Tricarboxylic acid
TFAM	Mitochondrial transcription factor A
TFB1 M	Mitochondrial transcription factor B1
TFB2M	Mitochondrial transcription factor B2
Tm	Melting temperature
[DNA]	DNA concentration
µg	Microgram
µL	Microliter
µM	Micromolar
A260/280	DNA optical density absorbance ratio measured at 260 nm and 280 nm

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1 Introduction

The individual cell is the basic structural and functional unit of the human body. It contains cytoplasmic organelles as well as all the genetic material (chromosomal and extra-chromosomal) that encodes all structural and functional elements in the human body. The mitochondrion (plural: mitochondria) is the organelle that, amongst other important functions, generates most of the energy for the cell.

The majority of mitochondrial proteins and structures are encoded by nuclear DNA. However, mitochondria contain their own DNA, i.e. extra-chromosomal DNA, that also encodes 13 proteins, tRNAs and rRNAs involved in mitochondrial structure and function. Mitochondrial biogenesis is thus reliant on the integrity of both nuclear and mitochondrial DNA and the interaction of the two genomes. Not surprisingly, research areas concerning mitochondrial disorders involve the investigation of nuclear and mtDNA fidelity and cell signalling as means of inter – and intracellular communication (Biswas *et al.*, 2005; Brookes *et al.*, 2004; Ermak and Davies, 2002; Reinecke *et al.*, 2009).

To sustain life, all cells need energy in the form of a molecule called adenosine tri-phosphate (ATP), which is produced during cellular respiration by mitochondria. Mitochondria are therefore of primary importance to provide and sustain necessary energy levels within cells. This is emphasised by the severity of mitochondrial disorders which are described in more detail in Chapter 2, Section 2.4 (Scheffler, 2000).

Cells in different organs or tissues require different amounts of energy. Correspondingly, the cellular content of mitochondria containing varying numbers of mtDNA, vary from cell to cell according to cellular energy demand and the cellular regulation (Davis *et al.*, 1996; Hudson and Chinnery, 2006) Therefore, an abnormal decline or increase in the number of mtDNA copies in patients is often indicative of mitochondrial disorders (Bai and Wong, 2005).

The relative number of mitochondria can be investigated by determining the relative mitochondrial DNA copy number (RMCN). This technique was utilized during this study to give an indication of the up- or downwards regulation of mtDNA within the specific patients' tissue samples and is described in more detail in Chapter 2, Section 2.4.5.

Mitochondrial DNA polymerase gamma (mtDNA POLG) is the only nuclear encoded DNA polymerase responsible for the replication of mitochondrial DNA (Nguyen *et al.*, 2006). Therefore when POLG malfunctions, it severely alters the mitochondrial copy number and subsequently mtDNA fidelity, which results in severe mitochondrial deficiency.

Mitochondrial disorders due to mtDNA mutations in humans can result in several disease phenotypes. Although the prevalence of mitochondrial disorders is estimated to be 1 in every 2000 to 5000 people (Naviaux and Nguyen, 2004, Skladal et al., 2003), it was recently reported that as many as 1 in 200 people carry a pathogenic mutation in their mtDNA (Cree et al., 2009). The terms mitochondrial disorders and OXPHOS disorders are often used interchangeably since mitochondrial disorders due to mtDNA mutations have been found to account for at least 20-25% of mitochondrial oxidative phosphorylation (OXPHOS) disorders. Therefore, mitochondrial disorders are the group of inborn errors of metabolism with the highest prevalence (Thorburn, 2004).

Furthermore, recent reports have indicated that mutations in the *POLG1* gene are relatively frequent among patients with mitochondrial disorders (Chinnery and Zeviani, 2008). Therefore, this study primarily focused on the investigation of the *POLG1* gene sequence and the relative mtDNA copy number estimation of a selected group of South African paediatric patients. This approach was followed because of POLG's direct and important involvement in mtDNA replication and because *POLG1* sequence investigation has proven to be fruitful in elucidating mitochondrial disorders in similar studies (Chinnery and Zeviani, 2008). Therefore, the aims of this study were to investigate the occurrence of *POLG1* mutations in clinically selected group of patients diagnosed with mitochondrial disorders. Furthermore, these patients were suspected to have possible POLG malfunctioning as contributing factor to their mitochondrial disorders.

The investigation presented here forms part of a broader investigation of the aetiology of mitochondrial disorders in South African paediatric patients.

A literature overview on aspects relevant to this study is presented in Chapter 2, which includes a detailed problem statement and aim. The methodology of the study is given in Chapter 3, results and discussion in Chapter 4, followed by conclusions in Chapter 5.

2 Literature overview

2.1 Introduction

The discovery and identification of mitochondria, as well as their function, occurred more than a century ago and has progressed over great a number of years. The organelle became officially known as “mitochondria” (mitochondrion singular) in 1901 (Scheffler I.E., 2000). The name “mitochondria” was given by the microbiologist Carl Benda (1857–1933) in 1898, who derived the word from the Greek words: *mitos*, meaning “thread” and *khondrion*, “little granule” (Harper, 2008).

We inherit our mitochondria from our mothers and they are present within the ovum even before fertilization. Each oocyte receives a number of mitochondria at random during gametogenesis. Although there might initially be sperm mitochondria present at fertilization (it is estimated that a sperm can contribute ~ 100 mitochondria during fertilization (Sutovsky *et al.*, 1999)), they are tagged by the oocyte’s (egg cell’s) cytoplasmic recycling marker protein, ubiquitin, destining them for destruction (Sutovsky *et al.*, 1999). As the cells in the ovum start to develop and replicate after fertilization, so also do mitochondria.

The process of mitochondrial replication and maintenance requires the functions of both nuclear and mitochondrial encoded elements. Mitochondrial biogenesis therefore entails nuclear and mitochondrial gene involvement in the synthesis and import of proteins and enzymes into the mitochondrion, as well as regulation of mitochondrial replication and turnover (or mitochondrial replacement) (Díaz and Moraes, 2008). Malfunctioning in any of these genes - or proteins encoded by them - could lead to mitochondrial deficiencies. Mitochondrial dysfunction, and therefore energy metabolism, is regarded as the underlying cause of many of the major debilitating diseases in humans today. These include neurodegenerative disorders, cardiovascular disorders and diabetes mellitus (Dröge, 2002; Thorburn, 2004). Mitochondrial disorders have also been found to be the group of inherited metabolic disorders with the highest prevalence (Skladal *et al.*, 2003; Bourgeron *et al.*, 1995). These topics will be discussed in more detail later in this chapter.

Since underlying causes of mitochondrial disorders are numerous, this study focused on a specific aspect of the aetiology of mitochondrial dysfunction. The presence of genetic alterations (mutations or deletions) in the mitochondrial DNA polymerase gamma gene was investigated in this study. This study consequently aimed to detect *POLG1* mutations, which could influence mtDNA replication and fidelity, in a cohort of patients with clinical phenotypes suggestive of POLG-related mitochondrial disorders. This included the determination of the relative mitochondrial copy number in order to establish whether an association exists between POLG mutations and a change in mitochondrial copy number.

2.2 Mitochondrial structure and function

2.2.1 Introduction

Inside the cytoplasm of each eukaryotic cell we find ranging numbers of mitochondria – the ‘powerhouses’ of a cell. Mitochondrial distribution and abundance vary in different tissue types, with the highest abundance of mitochondria in brain, skeletal and cardiac muscle tissue – tissues with high energy requirements (Chabi *et al.*, 2003). It is estimated that there are roughly 10 to 10000 mitochondria per cell, depending on the tissue type (Davis *et al.*, 1996; Hudson and Chinnery, 2006; Gaspari *et al.*, 2004), although this is subject to extreme inter and intra-individual variability, making it difficult to precisely determine the mitochondrial copy number (Gaspari *et al.*, 2004).

The double mitochondrial membranes (inner and outer membranes) compartmentalize the mitochondrion into the “inter membrane space” (area between the inner and outer membrane) and the “matrix” (the area inside the inner membrane). The inner membrane is folded into small folds called “cristae”, where oxidative phosphorylation (OXPHOS) takes place. OXPHOS is the process in which oxygen serves as a final electron acceptor at the end of the electron transport chain. Therefore, the major objective of cellular respiration is adenosine triphosphate (ATP) synthesis (Davis *et al.*, 1996; Zeviani and Di Donato, 2004). This makes mitochondrial function in ATP production essential for normal life and sustainability within the body.

The most important functions of mitochondria are the biochemical pathways of oxidative phosphorylation, amino acid metabolism, fatty acid oxidation and ion homeostasis. In these we can see that mitochondria are the centre of energy metabolism, together with intermediary metabolism and biosynthetic processes (Elstner *et al.*, 2008). The important activities involved in ATP synthesis take place between the inter membrane space and the inner membrane. The electron transport chain (ETC) is part of the mitochondrial inner membrane as illustrated in Figure 2.1. It consists of four multi-subunit enzyme complexes, trivially called complex I-IV. ATP synthase, or complex V, uses the electrochemical potential generated by complex I-IV as energy for ATP synthesis. The subunits of complex II of the ETC are encoded by the nuclear genome only, whereas the subunits of the other complexes are encoded by both nuclear and mtDNA. These complexes function in creating a reduction-oxidation (redox) potential by pumping protons (H⁺), via the action of complexes I, III and IV, over the inner membrane into the inter membrane space. The redox carriers, ubiquinone (coenzyme Q) and cytochrome c, aid in the electron transfer between these complexes. Furthermore, as illustrated in Figure 2.1, oxygen (O₂) functions as final electron acceptor to form water (H₂O) at complex IV - a process often called “respiration”. The redox potential formed by the ETC is used by complex V to release the H⁺ gradient back across the inner membrane into the matrix. This action by complex V drives the phosphorylation of ADP to ATP, thus completing the OXPHOS process.

The body utilizes different energy sources in order to drive the functions mentioned. Every function ultimately aids in generating the required energy demanded by each cell. Carbohydrates, amino acids and fatty acids are examples of the utilized energy sources in OXPHOS. These compounds may all undergo oxidation, providing reducing equivalents (NADH from the Krebs cycle or FADH produced via fatty acid oxidation), to drive the ETC and subsequently ATP synthesis.

Carbohydrates undergo glycolysis during anaerobic metabolism in the cytoplasm of the cell, creating pyruvate and four ATP molecules. To sustain normal cellular function, it is imperative to synthesise higher quantities of ATP molecules. Therefore, further utilization of pyruvate takes place in the mitochondria through the Krebs (or TCA) cycle. Fatty acids can be utilized in mitochondria through the processes of beta oxidation and the by-product, acetyl-CoA, also enters the Krebs cycle (Childs, 1996a).

However, other important metabolic processes also occur in the mitochondrion. These organelles play a vital role in reactive oxygen species (ROS) generation. Although other sources exist, ROS is a by product of electron leakage during cellular respiration. When ROS levels surpass levels tolerated by the antioxidant control system, they can cause oxidative damage. However, they are also key regulators of the cellular redox state and modulators of expression of several genes (Zhang and Gutterman, 2007; Genestra, 2007). ROS, along with other modulators such as Ca^{2+} , modulates the nuclear expression of defensive genes such as superoxide dismutase (SOD), metallothioneins and other antioxidant enzymes. It also modulates the expression of mitochondrial DNA replicating and transcription elements (Reinecke *et al.*, 2009). Furthermore, mitochondria are essential in controlling programmed cell-death (apoptosis) and calcium homeostasis within cells (Diaz and Moraes, 2008).

It is important to note that in order to retain normal cellular homeostasis, mitochondrial turnover must take place. Mitochondrial turnover commences during a period of 9 to 24 days after mitochondrial synthesis (depending on the tissue type) (Menzies and Gold, 1971) or when defective mitochondria are present, they are targeted and destroyed (Diaz and Moraes, 2008). Precise mechanisms involved in targeting mitochondria for destruction are not known. However, the process of turnover proceeds in a similar fashion to autophagy and is termed "mitophagy" (Mijaljica *et al.*, 2007). The process of mitophagy generates a double membranous phagosome which forms an autolysosome through fusion with a lysosome (Kim *et al.*, 2007). This process is also governed by cell signalling mechanisms, and involves both the nuclear and mitochondrial genomes.

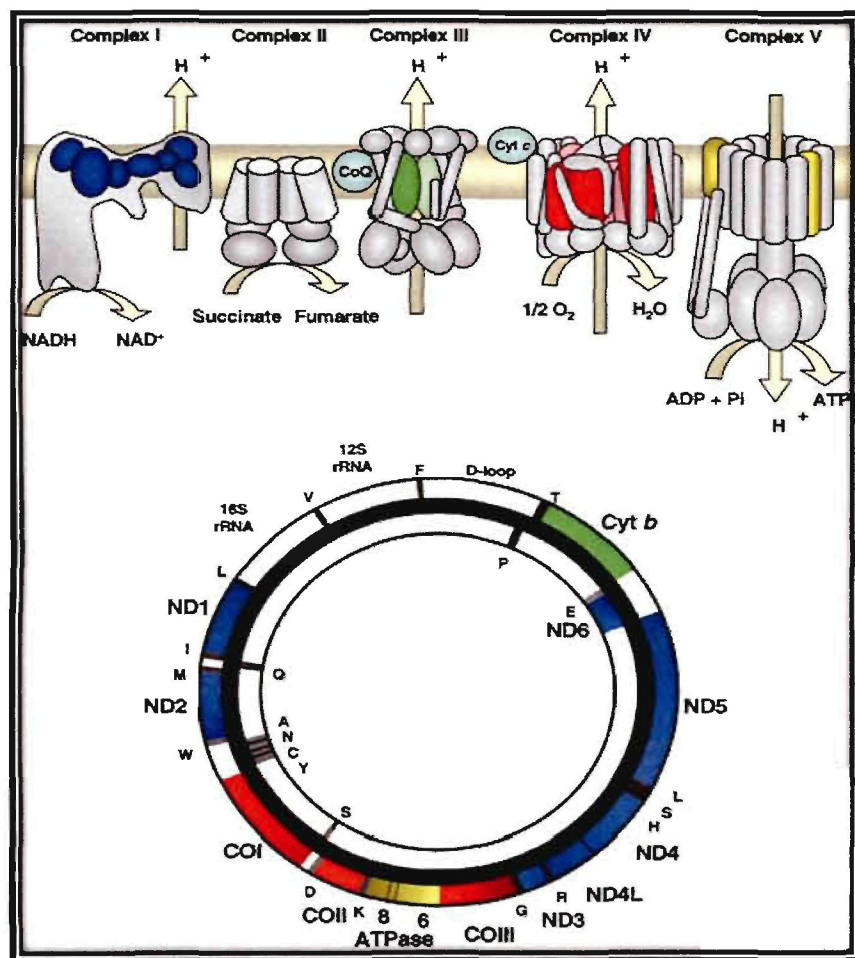


Figure 2.1 Human mitochondrial DNA and the OXPHOS subunits encoded by it.

A creative drawing of the mitochondrial OXPHOS system (top) and human mitochondrial DNA (bottom). Mitochondrial encoded subunits, which are embedded in the midst of nuclear-encoded subunits, are shown in different colors: complex I subunits = blue; complex III subunit = green; complex IV subunits = red; complex V subunits = yellow. Pi = inorganic phosphate; Cyt c = cytochrome c; CoQ = coenzyme Q. Bottom: mtDNA encoded genes: complex I genes = blue; complex III (Cytb) gene = green; complex IV genes = red; complex V genes = yellow. tRNA genes = grey; rRNA genes = purple. Cyt b = cytochrome b. (From Zeviani and Di Donato, 2004. Copy right permission no. 2067590637987)

Apart from nuclear DNA present in each cell, only mitochondria contain extra chromosomal DNA in human cells (Rabinowitz *et al.*, 1965; Mounolou and Lacroute, 2005; Chabi *et al.*, 2003). Encoding elements of mitochondrial biogenesis, replication, transcription and translation are found on both mitochondrial and nuclear DNA (nDNA). Keeping the mitochondrial genome intact is therefore of utmost importance (Hudson and Chinnery, 2006).

It is estimated that the mitochondrial structure constitutes more than 1500 proteins, of which approximately 900 have, with certainty, been assigned to the mitochondrion (Mootha *et al.*, 2003; Pagliarini *et al.*, 2008). Only 37 of these proteins are encoded by mtDNA (Anderson *et al.*, 1981; Elstner *et al.*, 2008).

More recently, mitochondria's importance and involvement in the processes of inter- and intracellular communication, by means of cell signalling, cellular differentiation and apoptosis has been studied (Biswas *et al.*, 2005; Brookes *et al.*, 2004; Ermak and Davies, 2002; Reinecke *et al.*, 2009). This is an area of continuous research, and will hopefully result in a better understanding of the diverse role of mitochondria in different types of eukaryotic cells.

2.2.2 The mitochondrial genome

Mitochondrial DNA (mtDNA) is located in the mitochondrial matrix in structures known as "nucleoids" which can each contain up to five copies of the mtDNA (Childs, 1996b; Diaz and Moraes, 2008; Copeland *et al.*, 2003). The complete mitochondrial genome was first sequenced and published by Anderson *et al.*, in 1981. It consists of a 16 569bp circular, double stranded genome. The structure and elements of this genome can be seen in Figure 2.1. No introns are found in this mini genome and all coding sequences are therefore contiguous. The displacement loop (D-loop) is the only non-coding area (~1Kb) and is the location of the promoter region for both strands of mitochondrial transcription (Anderson *et al.*, 1981).

Through alkaline gradient centrifugation experiments, using cesium chloride, the double stranded mitochondrial genome was separated due to each strand's different GC-content. The strands were identified as the heavy and light strands respectively (Flavell and Jones, 1970; Gaspari *et al.*, 2004; Diaz and Moraes, 2008). The heavy strand consists of 28 genes and also contains an origin for replication (OH), whereas the light strand consists of only nine genes and also contains an origin for replication (OL). Eight of the nine genes on the light strand encode mitochondrial tRNA's (Anderson *et al.*, 1981). Mitochondrial DNA replication and expression is consequently mostly governed by the nuclear genome. Two origins of replication can be observed, one on each strand of the complementary double stranded genome, respectively. The mitochondrial genome has one regulatory unit, which contains both origins of replication. The complete mitochondrial genome consensus sequence can be viewed at a database called "MITOMAP" (A Human Mitochondrial Genome Database, <http://www.mitomap.org>, 2008).

Replication and repair of mtDNA are achieved by mtDNA polymerase γ (POLG) (Nguyen *et al.*, 2006). This is the only known cellular DNA polymerase present inside the mitochondria (Longley *et al.*, 2005). Due to the low constituency of mtDNA within cells Loeb *et al.*, (1986) estimate that only 1% of cellular DNA is constituted by the mitochondrial genome. Correspondingly, POLG only has 1-5% of the total polymerase

activity within the cell (Davies *et al.*, 1996). It has recently been estimated that POLG deficiencies can account for as much as 25% of mitochondrial disorders (Chinnery and Zeviani, 2008).

2.2.2.1 Mitochondrial transcription, replication and translation

2.2.2.1.1 Mitochondrial transcription:

The process in which mitochondrial DNA is rewritten into messenger RNA (mRNA) is called transcription. This is followed by the process of translation which ultimately generates functional proteins or enzymes from the mRNA intermediates. These mRNA intermediates undergo complicated post-transcriptional changes or modifications before translation can commence (Bowmaker *et al.*, 2003).

Three promoters initiate transcription on the mitochondrial genome:

1. H1 (Heavy strand 1), for transcription of the heavy strand of mtDNA.
2. H2 (Heavy strand 2), for transcription of two mitochondrial rRNA's.
3. L (Light strand promoter), for transcription of the light strand of mtDNA.

Initiation of transcription is governed by three main protein types:

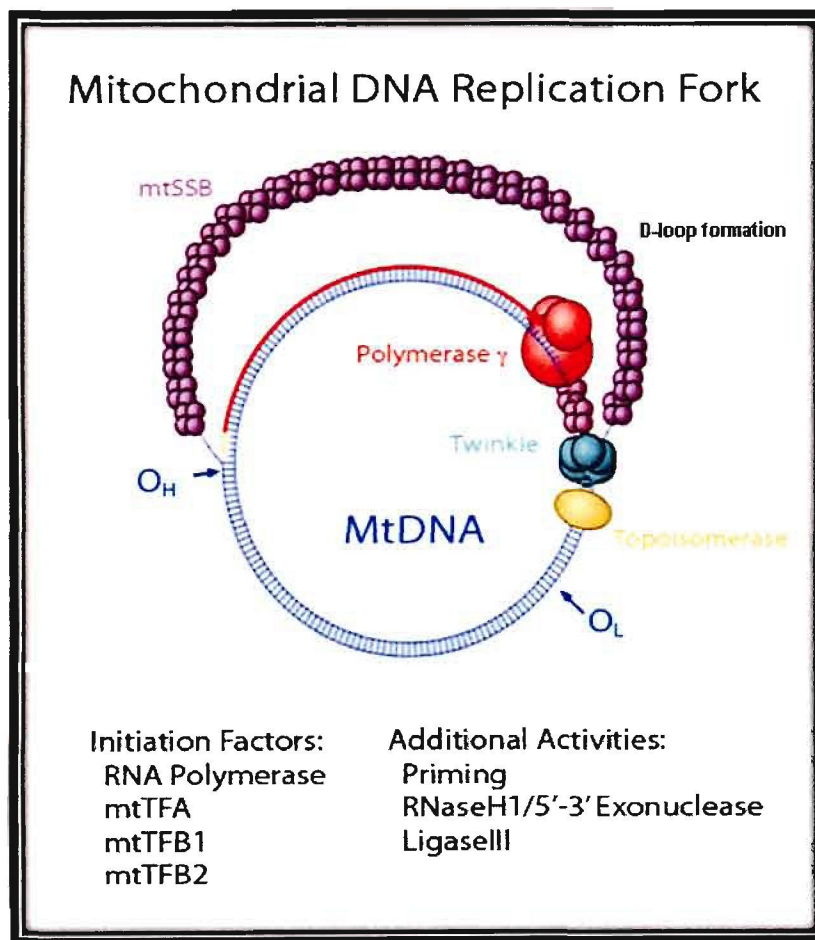
1. The mitochondrial RNA polymerase (POLRMT),
2. Mitochondrial transcription factor A (mtTFA or TFAM), and
3. Mitochondrial transcription factors B1 and B2 (TFB1M, TFB2M) (Gaspari *et al.*, 2004; Bowmaker *et al.*, 2003).

POLRMT, TFAM, and TFB1M or TFB2M are essential for transcription initiation at the mitochondrial promoter sights. Transcription generates polycistronic RNA transcripts (several polypeptides encoded by the same mRNA) which include the total genetic information for the specific strand (Gaspari *et al.*, 2004).

Light strand transcription, which is processed by mitochondrial RNase MRP (mitochondrial RNA processing), produces primers for mtDNA replication (Bowmaker *et al.*, 2003). These primers are essential for mtDNA replication and, therefore, mitochondrial transcription is closely linked to mitochondrial replication (Gaspari *et al.*, 2004). However, the same transcript can also be elongated to produce the light strand mtDNA polycistronic transcript. Endonucleolytic cleavage of the primary transcripts created from both the heavy and light strands produces the separate and functional mitochondrial 22 tRNAs, 12S and 16S rRNAs, and 13 mRNA molecules (Sheffler I.E., 2000).

2.2.2.1.2 Mitochondrial replication:

Some mitochondria may undergo multiple replications, whereas others might not undergo fission at all. For cells to operate and function normally, sufficient energy supply for individual cells is imperative. Energy demands vary between different cells and therefore, mitochondrial biogenesis must take place in accordance with the individual cells' energy demands. The mitochondrial replication process is very similar to that of bacterial replication. Intricate cell signalling mechanisms are involved in the activation and/or deactivation of expression of genes involved in the regulation of mitochondrial replication (Bowmaker *et al.*, 2003). Therefore, mitochondrial replication seems to be a highly variable process and the influences of different role players are still being studied for further elucidation and clarity (Figure 2.2) (Sheffler I.E., 2000).



One of the rate limiting factors of mitochondrial replication is the number of mtDNA copies present within a single mitochondrion. Mitochondria grow in size and also replicate intracellular elements, such as membrane proteins and mtDNA, to create sustainable daughter mitochondria. They only undergo replication/fission when they are large enough, contain the correct amount of mtDNA and all the intracellular elements, to create two functional daughter mitochondria (Childs, 1996:1).

Therefore, replication of the mitochondrial genome is of utmost importance, since the genome contributes greatly to elements involved in the structure and function of the mitochondrion. As mentioned before, human mtDNA replication and repair are accomplished by the nuclear encoded polymerase, POLG (Bowmaker *et al.*, 2003).

This process requires the replication of the total mtDNA to create an identical copy of the original. mtDNA is replicated by nuclear genes in accordance with the energy requirements of the individual cell. mtDNA replication is not necessarily synchronised with nDNA replication, and the mitochondrial DNA in mitochondria closest to the nucleus tend to undergo replication more often than some mitochondria on the outskirts of the cell (Sheffler I.E., 2000).

Elucidation of the process of mtDNA replication has been the topic of much scientific research and debate and no conclusive evidence as to a precise mechanism exists up to date (Luoma, 2007). The principles of mtDNA replication were first elucidated by Clayton (1982) in a study on budding yeast. He proposed the 'strand asymmetric model' of replication - also known as the displacement loop (D-loop) replication model (Figure 2.3A). To date, this is the most commonly accepted model for mtDNA replication. Different mechanisms have since been hypothesised to elucidate the precise mechanism of mtDNA replication. Up to now no conclusive evidence has been obtained that supports any of these hypotheses. Before discussing two hypotheses of replication, a brief description of mitochondrial translation follows.

2.2.2.1.3 Mitochondrial Translation

According to Soleimanpour-Lichaei *et al.*, (2007), the mechanisms of mitochondrial translation are still very uncertain. This is partly attributed to the fact that the mitochondrial genome only contributes to 2 rRNAs and 22tRNAs for translation, while all the other proteins and regulatory elements are nuclear encoded. As is the case with nuclear translation, mitochondria also require cytosolic mitochondrial ribosomes – termed mitoribosomes – acting as areas of mitochondrial translation (O'Brien and Kalf, 1967). Initiation factors are required for mitochondrial translation, however the only known mitochondrial initiation factor up to date is mitochondrial initiation factor 2 (mtIF-2) (Ma and Spernelli, 1995). This protein facilitates binding of the mitochondrial ribosome to fMet-tRNA. Elongation, initiation and release factors, ribosomal proteins and tRNA synthetases are all elements involved in translation. Some elongation and release factors have been

identified (Xin *et al.*, 1995; Soleimanpour-Lichaei *et al.*, 2007) although further investigation and elucidation of their function is still required.

2.2.3 Mitochondrial DNA replication hypotheses:

2.2.3.1 Strand displacement model (Clayton 1982):

This model, as illustrated in Figure 2.3 A and Figure 2.4, is also referred to as the unidirectional model; the asynchronous replication model or the displacement loop (D-Loop) replication model (Diaz and Moraes, 2008).

The hypothesis states that mtDNA replication takes place in an anti-clockwise manner, starting at two distinct origins of replication. As seen in Figure 2.4, replication is initiated and an RNA primer is generated by POLRMT (Scheffler I.E., 2000). To produce the 3' end of the RNA primer, endoribonuclease MRP is necessary (Chang and Clayton, 1987). This initiates heavy strand replication at the H1 promoter in the D-Loop region. Heavy strand replication displaces the original heavy strand and synthesises a new one (Luoma, 2007). Replication continues on the heavy strand until two thirds downstream of O_H , where O_L is revealed by the displaced heavy strand. The generated secondary D-loop structure serves as a signal for mtDNA primase to synthesise a new transcript from the revealed light strand promoter (Shadel and Clayton, 1993).

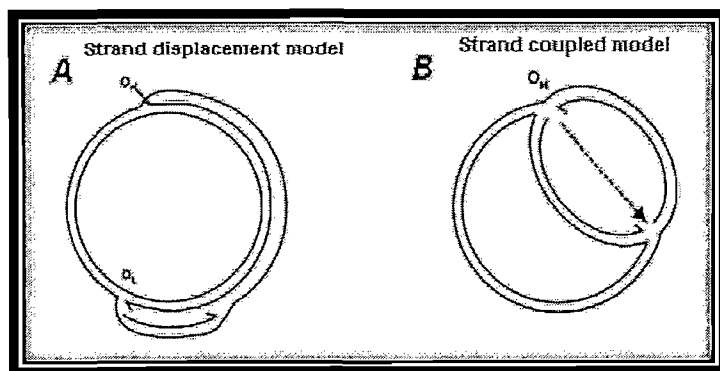


Figure 2.3 Illustration of two models for mtDNA replication: the strand displacement model (A) and the strand coupled model (B) of replication.

A: both strands are synthesized continuously from physically and temporally distinct sites termed O_H and O_L . The diagram depicts a replication intermediate where the leading strand has traversed two-thirds of the genome, exposing O_L and thereby enabling second-strand synthesis to begin in the opposite direction. B: replication from a discrete origin (O_H) which defines both the start site for replication and the terminus; the broken line with an arrowhead indicates the direction of replication away from O_H . (Bowmaker *et al.*, 2003; Permission from the publishers: Journal of Biological Chemistry)

After this, replication commences in both clockwise and anti-clockwise directions, thus continuing on the heavy strand and starting at the light strand promoter transcript (L), at the O_L . The two single strands are

ligated and the end result is the generation of an identical copy of the original mitochondrial genome (Díaz and Moraes, 2008).

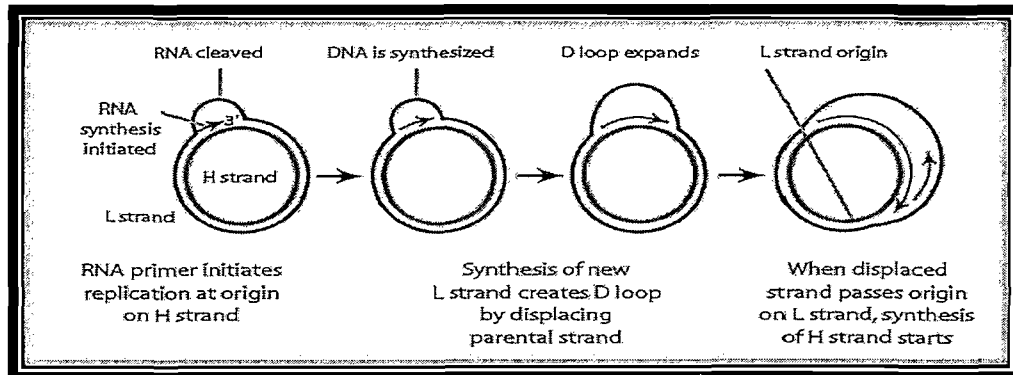


Figure 2.4 The strand displacement replication model. (Yasukawa *et al.*, 2006; with copy right permission)

2.2.3.2 "Strand coupled" or "Unidirectional model"

This model was first proposed by Holt *et al.*, (2000) and Yang *et al.*, (2002) and is illustrated in Figure 2.3 B. According to this hypothesis, the start and finish sites of replication are located within a so called 'zone of replication' downstream of O_H (Bowmaker *et al.*, 2003). Within this area, both strands are synthesised simultaneously from the respective promoter areas, continuing unidirectionally around the mtDNA (Luoma, 2007). Transcription is again initiated by POLRMT and assisted by mtTFA, TFB1M and TFB2M (Falkenberg *et al.*, 2002). Thus, mtDNA transcription initiates simultaneously from the individual HSP and LSP, respectively. Polycistronic RNA transcripts are therefore produced from H1, H2 and L promoter sights (Díaz and Moraes, 2008). Termination of the short transcript on the heavy strand (H1) is regulated by MTERF (Martin *et al.*, 2005).

This model has recently been revised (Luoma, 2007; Yasukawa *et al.*, 2006). It is believed that replication initiation can occur at multiple sites on the mtDNA and that replication continues bidirectionally. This also includes the incorporation of so-called RITOLS (ribonucleotide incorporation throughout the lagging strand) (Yasukawa *et al.*, 2006). These short RNA primers are only found on the light strand and both their origin and function are not clearly understood (Luoma, 2007). This replication suggests fork arrest at O_H creating double stranded replication which extends until the whole mtDNA is replicated (Bowmaker *et al.*, 2003).

2.2.4 Elements involved in replication

During replication, no single molecule or enzyme functions independently. Rather, different elements are combined to work together as a unit or 'mini factory' so to speak. Over recent years the components that participate in the replication machinery have been identified and their functions have been combined into a model called the "minimal mitochondrial replisome". This involves mainly three agents: POLG, Twinkle helicase and single stranded binding protein (SSBP) (Farge *et al.*, 2007).

As stated previously, POLG is the sole DNA polymerase responsible for mtDNA synthesis/replication. However, POLG cannot use double stranded DNA (dsDNA) as a template and therefore the dsDNA must first be unwound to expose the individual strands' origins of replication (Farge *et al.*, 2007). This is done by Twinkle helicase, the DNA helicase that unwinds the double stranded mitochondrial genome in front of the POLG polymerase. Twinkle helicase can however only unwind short stretches of ~ 2kbp of dsDNA at a time, before it dissociates from the DNA strand. In order for Twinkle and POLG to be bound to the DNA strand for longer stretches of DNA, SSBP is needed. With the addition of SSBP to POLG and Twinkle helicase, this processive replication machinery can replicate longer stretches of dsDNA – lengths that compare with the size of the mitochondrial genome (Farge *et al.*, 2007).

2.2.5 Intercellular communication

Cellular communication involving both genomes is imperative when any change in the cells' environment or homeostasis is detected. Therefore, mitochondria must interact with the nucleus and *vice versa*. Changes such as heat shock or reduced protein levels can trigger a mitochondrial stress response which activates a nuclear response.

"Retrograde signalling" is the term used when mitochondria send signals to the nucleus. These signals are usually related to information about the functional state of the mitochondria. Calcium and reactive oxygen species (ROS) signalling have been shown to be key cell signalling effectors in retrograde signalling. Calcium activates calcium-dependent protein kinases, which in turn activates coactivators in order to regulate expression of genes encoding mitochondrial elements (Szabadkai, 2008; Díaz and Moraes, 2008). ROS species such as hydrogen peroxide (H₂O₂) and superoxide were thought to only cause cellular and DNA destruction. However, Zhang and Gutterman (2007) indicate that ROS also regulates vascular tone, cell growth and proliferation and plays a role in apoptosis and inflammatory responses. Similar findings were made by Genestra (2007), with evidence of ROS activated signalling pathways and cascades.

According to Díaz and Moraes (2008), the nucleus sends signals to activate transcription factors responsible for regulating mitochondrial genes, essential for mitochondrial biogenesis (anterograde signalling). It is

therefore important to note that, although retrograde regulation occurs between mitochondria and the nucleus, mtDNA synthesis and maintenance are controlled by the nucleus.

2.3 Mitochondrial DNA polymerase gamma

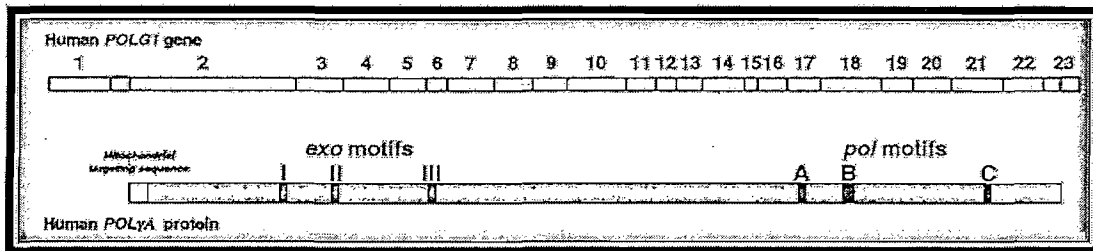
2.3.1 Introduction

A RNA-dependent polymerase was first discovered in 1970 where after it was positively identified to be mitochondrial DNA polymerase gamma (POLG) in 1977 (Bolden *et al.*, 1977). Ropp and Copeland (1996) state that POLG is the only known human DNA polymerase primarily responsible for mtDNA synthesis and repair. POLG, together with other nuclear encoded proteins, is responsible for mtDNA replication, transcription, and maintenance.

2.3.2 mtDNA polymerase gamma structure and function

POLG is a heterotrimer, encoded by nuclear *POLG1* and *POLG2* loci and transported into the mitochondrion (Luoma, 2007). It consists of a 140kD catalytic alpha subunit situated on the *POLG1* locus on chromosome 15q25 (Longley *et al.*, 2005) (see also Appendix A for an illustration of a molecular model for POLG). This subunit functions in 3'-5' exonuclease and 5'dRP lyase activities. The heterotrimer also has two identical 55kD accessory beta subunits, located on the *POLG2* locus, chromosome 17q23. It functions in promoting DNA binding affinity, thereby increasing protein processivity. The alpha subunit (POLG A) as illustrated in Figure 2.5 is encoded by 23 exons (from the *POLG1* gene), while the beta subunit (POLG B) is encoded by eight exons (from the *POLG2* gene) (Nguyen *et al.*, 2006). The beta subunits are referred to as the accessory subunits and are of utmost importance in the functionality of the heterotrimer as it enhances the process of polymerization and increases POLG's nucleotide binding activity (Farge *et al.*, 2007).

The POLG catalytic subunit of POLG A has three domains: The exonuclease, polymerase and linker (or spacer) domain (Nguyen *et al.*, 2006). These domains encompass a C-terminal with DNA polymerizing activity (polymerase domain), an N-terminal with proofreading activity (the exonuclease domain), and a spacer or linker domain which separates these domains and interacts with the POLG B processivity subunit by enhancing its DNA binding properties (Luoma, 2007). The exonuclease activity increases POLG replication fidelity by providing proofreading activity to the enzyme (Lamantea *et al.*, 2002). Mutations can occur in any of these functionally important domains, causing ranging disease severity and clinical presentations (Di Fonzo *et al.*, 2003).



The POLG enzyme functions optimally between pH ranges of 7.5 and 9.5, further requiring coupling to a divalent metal cation (Luoma, 2007). It can, however, perform exonuclease activity under a broad pH optimum, provided that a metal cation and high sodium chloride (NaCl) concentrations are present (Murakami *et al.*, 2003). Exonuclease activity is evident at the C-terminal in particular, illustrating high mismatch repair fidelity with ranging efficacy depending on the specific template involved. Furthermore, POLG demonstrates reverse transcriptase activity, distinguishing it from other DNA polymerases (Longley *et al.*, 1998).

Kunkel and Mosbaugh (1989) found, in a study on *POLG* purified from chicken embryos, that *POLG* replication results in one mutation in every 3.8×10^6 nucleotides. Therefore, it is estimated that mtDNA mutation rates are 10-20 times higher than nuclear DNA. *POLG* has its own proofreading activity and demonstrates well established base selection ability that allows high base substitution fidelity and exonucleolytic proofreading (Longley *et al.*, 2001). However, it functions poorly in detection of frameshifts in areas of homopolymeric sequence repetition (Copeland *et al.*, 2003).

Exposure to ROS, radiation and harmful chemicals contribute to POLG damage by protein oxidation and DNA damage. The latter requires DNA repair to prevent possible malformation and subsequent malfunctioning of the polymerase protein. Repair is done by means of base excision repair (BER). Firstly, DNA errors are detected by the polymerase domain, which subjects the error to exonucleolytic proofreading by transferring it to the exonuclease domain (Copeland *et al.*, 2003).

BER of damaged bases is achieved by DNA glycosylase and AP endonuclease activities. The DNA glycosylase is responsible for removal of the incorrect base with the help of an incision made in the DNA by AP endonuclease. This is referred to as the 2-deoxyribose-5-phosphate (dRP) lyase activity of POLG (Longley *et al.*, 1998). It allows POLG to remove the dRP residue allowing resynthesis of the appropriate nucleotide base and subsequent ligation to fill the resultant gap (Shigenaga *et al.*, 1994). The POLG beta subunit increases

the efficacy of this lyase activity by enhancing POLG's DNA binding affinity for the single stranded DNA (Luoma, 2007). However, should the excision of the mispaired base fail, the polymerase may dissociate from the DNA, possibly affecting POLG's synthetic fidelity and base selection properties (Copeland *et al.*, 2003).

It has been found that POLG is normally expressed and regulated even in the absence of mtDNA – i.e. mitochondria void of mtDNA (Davis *et al.*, 1996). Furthermore, POLG can use a number of different templates as substrates to perform its polymerization activities. These two factors have raised the suspicion that POLG might be responsible for other intracellular functions unrelated to mitochondrial DNA replication. However, no conclusive evidence for these functions currently exists (Luoma, 2007).

The POLG accessory subunit attributes to heterotrimer functionality by acting as a DNA-clamp and forming a high-affinity salt-stable complex with the POLG alpha subunit (Lamantea *et al.*, 2002; Luoma, 2007). It is estimated that this increases holoenzyme processivity 100-fold by restoring salt tolerance, stimulating polymerase and exonuclease activity and binding dsDNA with moderate strength and specificity (Chinnery and Zeviani, 2008; Luoma, 2007).

2.4 Mitochondrial disorders

2.4.1 Introduction

The term 'mitochondrial disorders' collectively refers to heterogeneous clinical phenotypes, occurring due to malfunctioning of nuclear and (or) mitochondrial genes, responsible for maintaining mitochondrial structure and function (Díaz and Moraes, 2008). (Refer to the OMIM catalog for additional information regarding mitochondrial disorders: <http://www.ncbi.nlm.nih.gov/omim>.) As mentioned previously, mitochondrial DNA (mtDNA) is exclusively maternally inherited during fertilization, thus mutated mtDNA is already present in the oocyte mitochondria before fertilization (Figure 2.6). When an oocyte with a high percentage of heteroplasmic mitochondria becomes fertilized, it has a predisposition toward mitochondrial disorders, depending on the heteroplasmic threshold (Rovio *et al.*, 1999) (Figure 2.6).

One study conducted by Schwartz and Vissing, (2002) claimed that they had found a certain case of so called "paternal leakage", where some of the sperm mitochondria weren't degraded and resulted in paternal mitochondrial inheritance. This could be possible since destruction of sperm mitochondria only takes place after fertilization has occurred. Therefore in this particular case, instead of total sperm destruction, resulting in the ovum with only the mother's mitochondrial DNA copies, some sperm mitochondrial DNA copies could have been inherited along with the maternal mtDNA copies. This is not the accepted norm, however, and only disorders of maternally inherited mtDNA will be discussed.

In addition to maternally inherited defective mtDNA, mitochondrial disorders are also inherited through autosomal (somatic and X-linked) inheritance. This consists of nDNA alterations of genes involved in structural or functional aspects of mitochondria. These alterations can be present on the chromosomes of either or both parents, resulting in autosomal dominant or recessive, or X-linked dominant or recessive inheritance.

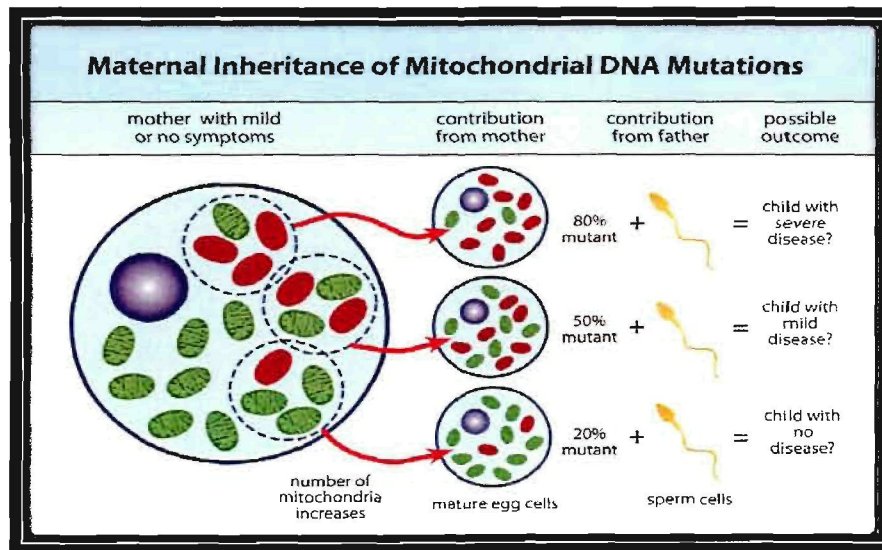


Figure 2.6 The process of heteroplasmy illustrated in maternal mitochondrial inheritance.

(Taken from Hestrelé, Quest, vol 6, No4, Aug, 99; Used with permission of the Muscular Dystrophy Association of the United States)

It is furthermore important to elucidate the concepts called "heteroplasmy" and "homoplasmy" as illustrated in Figure 2.6. The state of a mitochondrion, cell or tissue having combinations of two forms of mtDNA, where one may contain a mutation or deletion, is termed "heteroplasmy" (Copeland *et al.*, 2003). A homogenous mtDNA population, whether containing a mutation or not, is termed "homoplasmy".

Mutated and wild-type mtDNA exist simultaneously in different quantities and ratios in affected areas (Chabi *et al.*, 2003). mtDNA pathogenic mutations or single nucleotide polymorphisms (SNPs) can be homoplasmic, affecting all mtDNA molecules, although they are frequently heteroplasmic where only a percentage of cellular mtDNA is mutated. In addition, a so-called threshold effect (mutation load) is generated in heteroplasmic tissues (Bai and Wong, 2005). This threshold is different for each tissue type and is correlated to a specific ratio of mutated vs. wild type mtDNA. Only when this threshold is reached will the person present with a clinical phenotype. Therefore, a certain percentage of heteroplasmy is often found to be indicative of the disease severity (Figure 2.6) (Bai and Wong, 2005).

Mutations can occur in specific tissues or could even be multi-systemic. It can present as SNPs, single or multiple mtDNA mutations or deletions, mtDNA rearrangements (Table 2.1) and altered mitochondrial copy number (Chabi *et al.*, 2003; Bai and Wong, 2005). In the case of maternal inheritance, it is important to remember that mitochondrial distribution and abundance varies significantly throughout the body, creating different patterns of heteroplasmy (Chabi *et al.*, 2003). Therefore, clinical presentation of the disease could be confined to specific organs or tissues, such as the eye in Leber hereditary optic neuropathy (LHON) (Chinnery *et al.*, 2000), which accounts for various clinical phenotypes (Rovio *et al.*, 1999).

Table 2.1 summarizes the scope of mutations associated with inherited mitochondrial disorders. This indicates the complexity and intricacy of mitochondrial disorders.

Table 2.1 A summary of autosomal and maternally inherited mutations linked to mitochondrial disorders.

mtDNA Polymorphisms (includes mini insertions & deletions)	mtDNA Mutations with Reports of Disease-Associations		Major Rearrangements	Nuclear Genes Involved in Mitochondrial Disease		Mitochondrial Pseudogenes
	Organized by mtDNA location:	Organized by phenotype:		Non-Structural Nuclear Genes in Mitochondrial Disease	Structural Nuclear Genes in Mitochondrial Disease	
Control Region (16024-576)	rRNA/tRNA Mutations	rRNA/tRNA Mutations	mtDNA Deletions	Genes involved in complex assembly		
Coding & RNA (577-16023, MTTF-MTTP)	Coding & Control Region Mutations	Coding & Control Region Mutations	Multiple mtDNA Deletions Within Individuals	mtDNA Stability		
Somatic Mutations		LHON Mutations	MtDNA Inversions	Mitochondrial Import		
Unpublished Polymorphisms			MtDNA Simple Insertions	Mitochondrial Protein Synthesis		
			MtDNA Complex Rearrangements	Iron Homeostasis		
				Chaperone Function		
				Mitochondrial Integrity		
				Mitochondrial Metabolism		

(MITOMAP: A Human Mitochondrial Genome Database. <http://www.mitomap.org>. 2008.)

Tables 2.2 and 2.3 give an indication of primary mitochondrial disorders associated with mutated mtDNA. These are only a few of the possibilities where mtDNA mutations can occur. Table 2.1 indicates sporadic and maternally inherited mitochondrial disorders associated with mtDNA rearrangements which consist of large-scale partial deletions and duplications. Similarly, Table 2.2 summarizes the disorders associated with point mutations in transfer-RNA genes, protein-coding genes and ribosomal-RNA genes.

Table 2.2 Genetic classification of Human Mitochondrial Disorders: Primary mitochondrial disorders originating from mtDNA rearrangements

Rearrangements (large-scale partial deletions and duplications)	
DISEASE TYPE	INHERITANCE
Chronic progressive external ophthalmoplegia (CPEO)	S or M
Kearns-Sayre syndrome	S or M
Diabetes and deafness	S
Pearson marrow-pancreas syndrome	S or M
Sporadic tubulopathy	S

M = Maternal; S = Sporadic. (Adapted from P.F. Chinnery, (2000). [Web]: <http://www.ncbi.nlm.nih.gov/bookshelf/br.fcgi?book=gene&part=mt-overview>. Date of use: 28 Nov 2008)

Table 2.3 Genetic classification of Human Mitochondrial Disorders: Primary mitochondrial disorders originating from mtDNA point mutations

Point mutations					
TRANSFER-RNA GENES	I	PROTEIN-ENCODING GENES	I	RIBOSOMAL-RNA GENES	I
MELAS*	M	LHON*	M	Nonsyndromic sensorineural deafness	M
MERRF*	M	NARP/Leigh syndrome*	M	Aminoglycoside induced nonsyndromic deafness	M
CPEO*	M				
Myopathy	M				
Cardiomyopathy	M				
Diabetes and deafness	M				
Encephalomyopathy	M				

I = Inheritance; M = Maternal;

** = Refer to key words index. (Adapted from P.F. Chinnery, (2000). [Web]: <http://www.ncbi.nlm.nih.gov/bookshelf/br.fcgi?book=gene&part=mt-overview>. Date of use: 28 Nov 2008)*

Table 2.4 illustrates the effect of autosomally inherited mitochondrial disorders, which are the most common cause for mitochondrial disorders. Nuclear mutations can result in primary mitochondrial disorders in areas such as mtDNA maintenance, disorders of the respiratory chain and its assembly, mitochondrial protein import, RNA metabolism and disorders of the mitochondrial lipid membrane. All of these indicate the severity and impact of nuclear DNA alterations.

2.4.2 Clinical phenotypes

Before a patient presents with a clinical phenotype, a heteroplasmic threshold must first be reached in cases where mtDNA mutations or deletions exist (Bai and Wong, 2005). Furthermore, when certain mutations

occur as compound heterozygous mutations, their mutational effect escalates as opposed to their individual occurrence (Longley *et al.*, 2005). This is referred to as “pathogenic synergy between the mutant residues”, illustrating how the presence of the second mutation enhances the severity of the disorder (Longley *et al.*, 2005). This emphasises that clinical phenotypes cannot be wholly elucidated by molecular diagnosis of single point mutations and/or large deletions. Rather, qualitative as well as quantitative analysis of mitochondrial disorders becomes imperative (Bai and Wong, 2005; Chabi *et al.*, 2003). Therefore, ranging clinical phenotypes and severity of disorders are influenced by the extent of genetic heterogeneity, mutation location, mutation pathogenicity and the affected organ or tissues’ energy dependence as illustrated in Tables 2.1 - 2.4 (Zeviani and Di Donato, 2004).

The following systems and organs are most sensitive to depleted energy and when affected, often correlate to more severe phenotypes: visual and auditory systems, the central nervous system, the heart, muscle and endocrine system, pancreas, kidney and liver (Zeviani and Di Donato, 2004). Clinical phenotypes often consist of one, or a combination of, the following presentations: encephalopathy and liver failure, seizures, mental retardation, exercise intolerance, external ophthalmoplegia, ataxia, myopathy, epilepsy and ptosis, to name but a few (Horvath *et al.*, 2006).

Mitochondrial disorders with discrete clinical phenotypes include Alpers syndrome, MELAS (mitochondrial encephalomyopathy; lactic acidosis with stroke-like episodes), MERFF (myoclonic epilepsy; ragged red fibres) and PEO (progressive external opthalmoplegia) (Scheffler I.E., 2000). Table 2.5 summarizes some of the most common clinical phenotypes.

Table 2.4 Selected well known mitochondrial disorders and their clinical presentation.

Disorder	Clinical Presentation
MELAS	Stroke-like episodes, seizures and dementia, lactic acidosis and/or RRFs
MERRF	Myoclonus, epilepsy, cerebellar ataxia, muscle weakness, deafness and dementia
NARP	Peripheral neuropathy, ataxia, pigmentary retinopathy,
Leigh syndrome	Infantile onset, subacute relapsing encephalopathy, basal ganglia lucencies
LHON	Painless sub-acute bilateral visual failure, males more commonly affected
Non syndrom. Deafness	Moderate to profound hearing loss with no other systemic findings
CPEO	External ophthalmoplegia, bilateral ptosis, proximal muscle weakness
KSS	Multisystemic, pigmentary retinopathy, cardiac conduction block, cerebellar ataxia
Pearson syndrome	Usually fatal in infancy, sideroblastic anaemia, exocrine pancreatic failure

Refer to key word index for definitions of acronyms used. (Adapted from Westwood, 2007)

2.4.3 Occurrence and diagnosis

Mitochondrial disorders are far more prevalent than was previously believed (Chinnery, 2006). It can present at any age although patients with nuclear mutations tend to present earlier than patients with mtDNA mutations, which tend to have a later disorder onset (Chinnery and Zeviani, 2008). Arpa *et al.*, (2003) estimate mitochondrial disorder prevalence of Spanish people aged 14 years and over, to be 5.7 in every 100,000 individuals. Chinnery (2006) estimates that ~1 person in every 8500 is affected with a mitochondrial disorder.

Individual diagnosis of patients is extremely difficult and challenging since a single mutation can result in a spectrum of diseases and, conversely, different mutations could result in a single disorder. Autosomal mutations are found to be sporadic whereas mtDNA mutations are more common. Accordingly, point mutations are more attributable to maternal inheritance, as opposed to mtDNA depletions and deletions which seem to be prone to autosomal dominant or recessive inheritance (Rovio *et al.*, 1999).

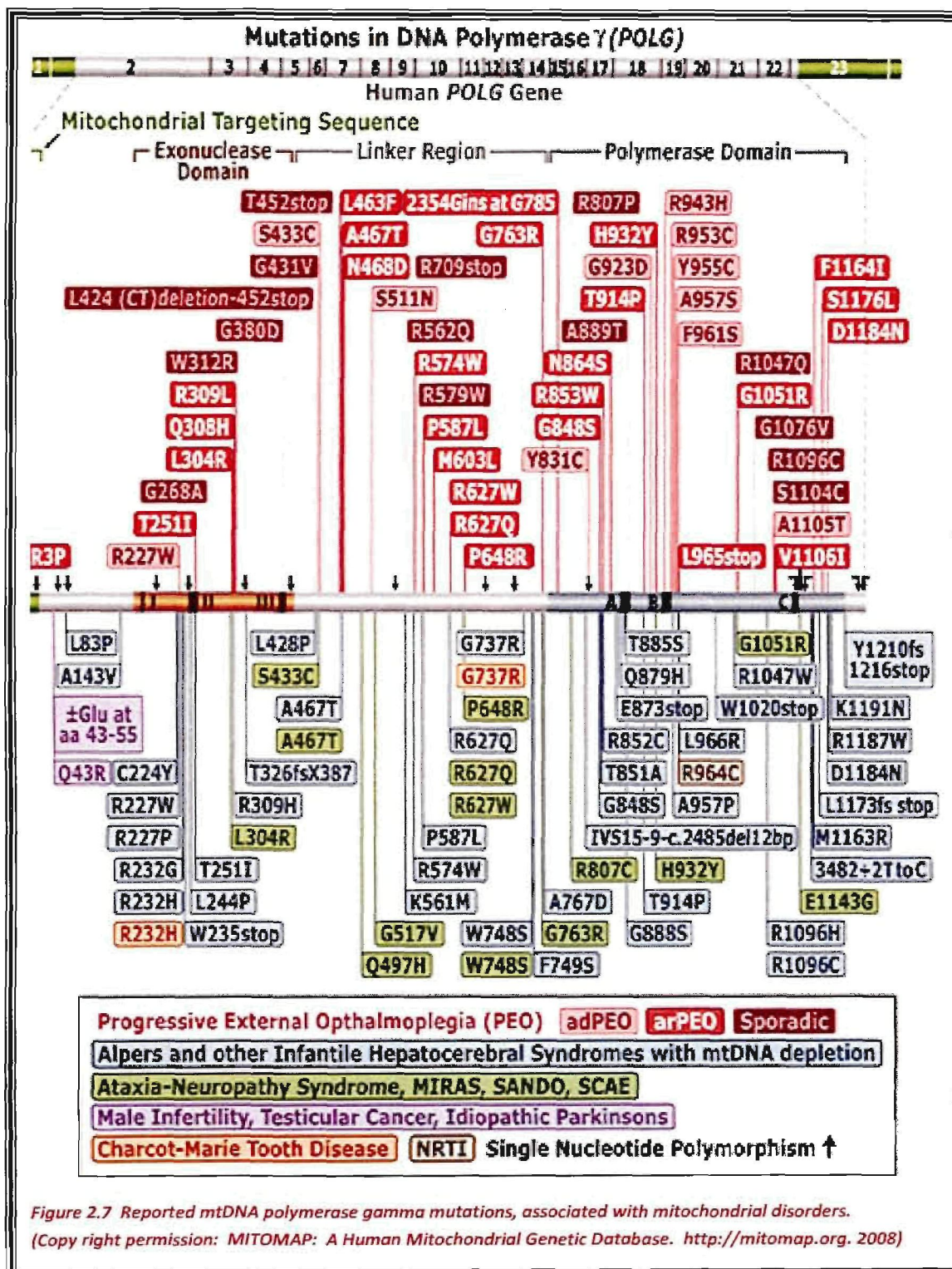
Patients are first diagnosed based on clinical presentation of a disorder. The general rule of thumb used by physicians to evaluate when to suspect a mitochondrial disorder, is when the clinical phenotype involves three or more organ systems (Naviaux and Nguyen, 2004). Thereafter, muscle biopsies are usually collected for enzyme and molecular genetic analysis.

2.4.4 Mitochondrial disease due to *POLG1* mutations

Mutations in *POLG1* were only recently recognized to attribute to mitochondrial disorders (Hudson and Chinnery, 2006) and might be accountable for up to 25% of mitochondrial disorders (Chinnery and Zeviani, 2008). When *POLG1* is mutated, adverse secondary effects such as deletions, point mutations and depletions could be evident in mtDNA (Luoma, 2007), which will affect mitochondrial function (Longley *et al.*, 2005).

Clinical syndromes associated specifically with *POLG* mutations include Alpers-Huttenlocher syndrome (AHS), PEO, MERRF, MELAS, MNGIE, mitochondrial recessive ataxia syndrome (MIRAS) and SANDO (Longley *et al.*, 2005). It is extremely difficult to single out a patient with a *POLG*-related mitochondrial disorder. Chinnery and Zeviani (2008) have therefore established guidelines for diagnosis of patients with a high probability of having a *POLG*-related mitochondrial disorder. They found *POLG* involvement in unexplained neurological features, especially with concurrent liver dysfunction, cerebellar and sensory ataxia and occipital migraine with intractable epilepsy (Chinnery and Zeviani, 2008). PEO, with multiple mtDNA deletions, is also indicated in a skeletal muscle biopsy.

Figure 2.7 summarizes known *POLG* mutations (~150) and associated phenotypes.



2.4.5 Mitochondrial DNA copy number and its variations

As POLG is the key enzyme involved in mtDNA replication, it is important to consider mtDNA copy number. When referring to the number of mitochondrial DNA in a given cell, relative to the amount of nuclear DNA present, the term '*relative mitochondrial copy number*' (RMCN) is used (Davies *et al.*, 1996). Roughly ten (10) mitochondrial DNA copies exist per mitochondrion; however this estimate varies greatly in relation to specific tissues. The number of mitochondrial copies in a given cell is normally kept within constant ranges in varying tissue types and according to the individual cell requirements (Davis *et al.*, 1996; Hudson and Chinnery, 2006).

An interesting condition often associated with mitochondrial disorders, affecting mitochondrial copy number, is known as "mitochondrial over replication" (Diaz and Moraes, 2008). When defective mitochondria cannot supply the necessary energy demanded by the cell, excessive biogenesis of mitochondria (and mtDNA) takes place in order to compensate for depleted cellular energy. Decreased energy levels could arise from severe genetic mutations altering e.g. the respiratory chain complex functionality, resulting in biogenesis to restore necessary energy levels (Chabi *et al.*, 2003).

Conversely, mtDNA depletion could occur, thereby decreasing the relative mitochondrial copy number. This might be attributed partly to defective nuclear genes creating proteins or enzymes whose function in processes concerned with mitochondrial replication is altered. This renders defective processes with too little nucleotides (depleted nucleotide pool) to be incorporated into the mitochondrial genome (Bai and Wong, 2005).

Therefore, determining the relative mitochondrial copy number can give a good indication of possible POLG-related mitochondrial disorders, since POLG is responsible for the variation in mtDNA abundance.

2.5 Problem statement, aims and strategy

Limited information exists on the aetiology of mitochondrial disorders in South African patients (*problem statement*). A better knowledge and understanding of these disorders could greatly enhance the diagnosis and treatment of patients. The acquired information could be used during genetic counselling of these families. Several hundreds of genes may theoretically be involved in the spectrum of mitochondrial disorders. Given this complexity, a complete investigation of the causes of mitochondrial disorders is a daunting task.

An approach to simplify this problem is to target a specific number of genes that, based on existing data, have a greater probability of involvement in mitochondrial disorders. mtDNA is one such a 'gene cluster', which consists of 37 genes, totalling only ~16.6 kbp. These genes are involved in at least 10% of

mitochondrial disorders described in paediatric patients – possibly accounting for as much as 25% if adult patients are included. It has furthermore been found that the investigation of POLG fidelity could possibly account for an additional 25% of mitochondrial disorders (Chinnery and Zeviani, 2008).

A group (38) of mainly paediatric patients have been diagnosed at the Department of Paediatrics, University of Pretoria (UP), over recent years. South African paediatric patients with confirmed mitochondrial disorders have been diagnosed by means of clinical presentation and subsequent biochemical enzyme analyses on muscle biopsies. Molecular genetic tests have not been included so far. Therefore, eight patients were selected from the group of 38 patients for further *POLG1* genetic analysis during this study.

In an attempt to investigate the involvement of mtDNA in the existing patient group (38) mentioned above, a broader project was initiated between the Division of Biochemistry, North-West University (NWU), Potchefstroom and the Department of Paediatrics, UP. This *broader project* has the aims to 1) determine mtDNA point mutations/deletions, 2) mtDNA integrity, 3) mtDNA copy number and 4) *POLG* mutations in the target patient group of eight patients.

For the study presented in this dissertation, the *specific aims* where points 3 and 4 mentioned above. These were thus to evaluate the mtDNA copy number in the whole target patient group and determine if *POLG1* mutations exist in a clinically selected patient group that, based on clinical phenotypes, seemed to be the best candidates for POLG malfunctioning.

To fulfil these aims, the following *strategy*, as illustrated in Figure 2.8, was devised using existing material and knowledge of current methodologies. The detail of the methodologies is described in Chapter 3. Briefly, however, this strategy was directed by the available type of patient- and control material, i.e. *frozen* muscle biopsies stored after surgery. This material was useful for both mtDNA copy number, which should preferably be done in affected tissue, as well as for the sequencing of the *POLG1* gene. An alternative approach for *POLG1* sequencing would have been to sequence cDNA prepared from RNA. This approach would give the same information using less sequencing reaction. However, for RNA preparation it is best to use fresh material which is specifically and immediately prepared for RNA isolation. This was not practically possible in this study.

The strategy shown in Figure 2.8 combines the current sample preparation used for ETC enzyme analyses, which uses the supernatant after tissue homogenization. DNA used for mtDNA copy number can be isolated from the whole homogenate before centrifugation at 600 x g (shown as the right side of the scheme in Figure 2.8). This sample contains the unaffected ratio of nDNA and mtDNA. However, after centrifugation at 600 x g the pellet, which is an enriched nuclear pellet, could be used for nDNA isolation and subsequent mutation

analysis of POLG (shown on the left side of the scheme in Figure 2.8). In the following chapters the methodology (Chapter 3), results (Chapter 4), discussion and conclusions (Chapter 5) will be given.

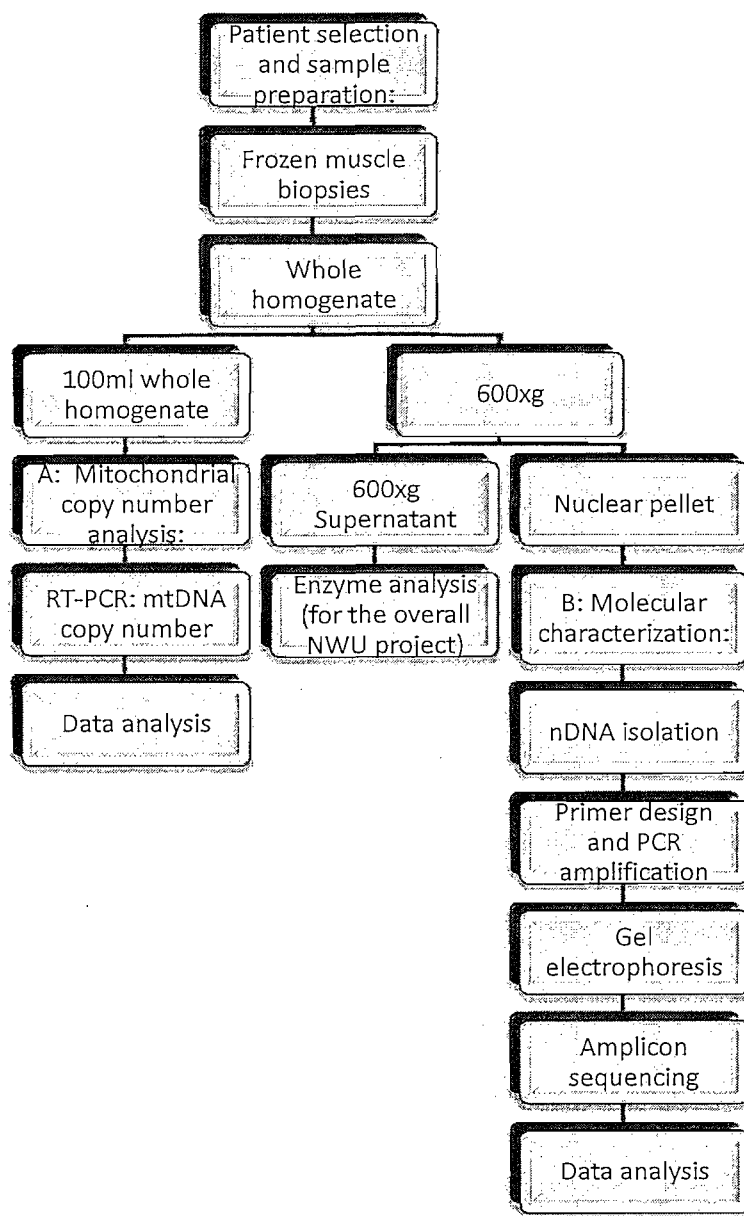


Figure 2.8 Schematic strategy for A) mtDNA copy number analysis and B) POLG1 mutation analysis in muscle samples of selected patients and controls.

3. Materials and Methods

3.1 Introduction

As discussed in Chapter 2, Section 2.4, this study focussed on 38 patients with a diagnosed mitochondrial disorder. For mitochondrial DNA copy number analysis all of these patients were included. However, sequence analysis of the *POLG1* gene was only performed on selected patients (eight). They were clinically selected based on their probability of presenting with a POLG-related mitochondrial disorder. In this chapter the materials and methods and preliminary sample preparation for these two analytical procedures will be described.

3.2 Patients and controls

As part of the broader study described in Section 2.4, most of the patients were selected prior to the onset of this study. Ethical approval had previously been obtained from the University of Pretoria (UP) (approval number 91/98, titled "Mitochondrial disorders in the South African context.", and amendments – see Appendix B for an example of the informed consent papers given to patients). Legal informed consent and assent for collection of muscle biopsies from all participants were obtained by Prof. I. Smuts, Department of Paediatrics, Faculty of Health Sciences, UP. These participants included healthy controls that underwent orthopaedic surgery, where muscle material was made available. Most of the muscle sample biopsies had been collected by the onset of the project, although a few were collected during the study.

Thirty-eight patients were initially diagnosed with a mitochondrial disorder, based on clinical presentation and RC enzyme analysis in muscle biopsies. In addition, 18 control muscle samples had also been collected. Patients presenting with clinical phenotypes were identified by Prof. I. Smuts. Table 3.1 summarizes the clinical and muscle enzyme deficiencies that were identified in these patients. The patients selected for *POLG1* mutation analysis are indicated in this table and were selected based on eye involvement, ptosis, PEO, RP strabismus or visual impairment. Patient nine was included because of an initially suspicious Southern blot analysis, indicating a possible mtDNA structure aberration. These patients will henceforth be referred to as the 'target patient group' (TPG).

Confirmatory enzyme analysis was done on muscle biopsies prior to the onset of this study. During this analysis, a nuclear pellet containing genomic DNA formed as by-product and was subsequently used in this study to isolate genomic DNA for *POLG1* mutation analysis. Furthermore, additional DNA was isolated from a small piece of stored frozen muscle since this would yield more accurate results for comparative mitochondrial DNA copy number analysis.

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Patient Nr	Sex	Clinical involvement								RC enzyme deficiency						
		CNS	Eye	Muscle	Renal	Endocrine	Liver	Skeletal	Rad biology detected changes in	Muscle biopsy changes	CI	CII	CIII	CIII-III	CIV	PDHc
1	M		✓	✓					✓	✓	✓					
2	F		✓	✓					✓	✓		✓				
3	F			✓						✓						
4	F		✓	✓						✓			✓			
5	F			✓					✓	✓	✓					
6	F	✓		✓					✓	✓						✓
7	M		✓	✓						✓		✓	✓	✓		✓
8	M	✓		✓	✓				✓							✓
9	M			✓											✓	
10	M	✓		✓					✓		✓					
11	M	✓	✓	✓					✓		✓		✓			
12	M		✓	✓						✓	✓			✓	✓	
13	F			✓		✓	✓			✓						✓
14	F		✓	✓					✓	✓	✓			✓		✓
15	F		✓	✓	✓				✓	✓			✓			✓
16	M		✓	✓					✓	✓				✓		✓
17	F			✓					✓	✓			✓	✓		✓
18	M	✓							✓	✓						
19	F		✓				✓	✓	✓	✓						
20	M	✓	✓	✓					✓	✓	✓					
21	F		✓	✓		✓			✓	✓	✓	✓	✓	✓	✓	
22	M	✓		✓					✓	✓						
23	M	✓		✓			✓		✓	✓		✓				
24	F			✓						✓	✓	✓	✓	✓	✓	✓
25	M						✓		✓							
26	F	✓		✓					✓					✓	✓	
27	F	✓	✓	✓					✓		✓			✓		-
28	F	✓		✓					✓							-
29	M			✓						✓				✓		-
30	M	✓				✓	✓		✓	✓	✓					-
31	M	✓				✓	✓	✓	✓		✓		✓	✓	✓	-
32	M			✓	✓	✓	✓	✓	✓		✓		✓	✓	✓	-
33	M	✓		✓					✓		✓					-
34	F		✓	✓					✓	✓	✓		✓	✓	✓	-
35	F		✓	✓					✓	✓			✓	✓	✓	-
36	F	✓		✓					✓				✓	✓	✓	-
37	M	✓	✓	✓			✓		✓				✓	✓	✓	-
38	M			✓	✓			✓			✓		✓	✓	✓	-

The following table (Table 3.2) represents the TPG with their clinical phenotypes:

Patient Information		Clinical Phenotype	Enzyme deficiency*					
ID	Sex		CI	CII	CII+III	CIII	CIV	PDHC
P4	F	Myopathic patient with ptosis, short stature, scoliosis, slight dysmorphism and developmental delay				X		
P7	M	Myopathic patient with ptosis, developmental delay, clubfeet and a cleft palate		X	X	X		X
P9	M	Exercise intolerance and myalgia					X	
P12	M	Male with muscle weakness and exercise intolerance, ptosis, PED, RP, lactic acidosis and ragged red fibres	X		X		X	
P14	F	Myopathic patient with developmental regression, nystagmus, epilepsy and head bobbing, abnormal mitochondria seen on EM	X		X			X
P16	M	Young man with strabismus, visual disturbance, extreme tiredness and exercise intolerance			X			
P21	F	Myopathic patient with ptosis, developmental delay, lactic acidosis and TCA metabolites in the urine	X	X	X	X	X	
P27	F	Visual impairment with West syndrome, deafness, white matter loss on MRI, Lactic acidosis	X		X			ND

3.3 DNA isolation

DNA (nuclear and mtDNA) was isolated from previously collected muscle tissues. Upon collection of muscle biopsies, they were transported on ice in a Homogenization Buffer (225 mannitol, 70 mM sucrose, 5 mM HEPES, 0.1 mM EGTA, pH 7.2; all reagents of ultra purity grade purchased from Sigma-Aldrich, St. Louis, Mo., USA). The samples were removed from the buffer, wrapped in tin-foil and frozen at -80°C within two hours after collection.

On the day of sample preparation, ~50 mg of each muscle sample was removed and homogenized in Homogenization Buffer, to produce a 10% weight per volume (w/v) homogenate. This was done using a Weaton glass grinder (Weaton Science, Millville, NJ). Twenty strokes were applied to each sample, while keeping the homogenizer immersed in ice slurry. Of this homogenate, 100 µl was used for DNA isolation (to be used for mtDNA copy number analysis) and the remaining homogenate was centrifuged at 600 x g for 10 minutes at 4°C. The supernatants were used by Prof. F.H. van der Westhuizen (supervisor), Division of Biochemistry, NWU, Potchefstroom, for respiratory chain (RC) enzyme analyses (as reported in Table 2.1). The resultant pellet, containing enriched nuclei, was stored at -80°C for DNA isolation (to be used for *POLG1* mutation analysis in this study).

Macherey-Nagel's NucleoSpin® Tissue kit (Macherey-Nagel, Düren, Germany) was subsequently used for the DNA isolation from the tissue homogenates. The instructions for DNA isolation from human or animal tissue and cultured cells as provided with the kit were followed. The exact composition of the kit reagents is not provided by the manufacturer but the presumed critical reagents are indicated in brackets.

Sample preparation consisted of pre-lysis by re-suspending the homogenate (~100 µL) in a premix of 200 µL Buffer T1 (containing an alkaline- SDS detergent for breaking cellular and nuclear membranes) and 25 µL Proteinase K solution (to digest proteins, including nuclear membrane proteins). This is followed by incubation for 10-15 minutes at 70°C. Further cell lysis was acquired by adding 200 µL of Buffer B3 (containing chaotropic salts for precipitation) to the sample followed by vigorous vortexing and further incubation for 10 minutes at 70°C.

DNA binding conditions were adjusted by adding 210 µL (96-100%) cold ethanol to the sample, before applying the samples to a NucleoSpin® Tissue column that contains a silica membrane. Samples were centrifuged for 1 minute at 11, 000 x g and the flow-through discarded.

The silica membrane of the NucleoSpin® Tissue column was subjected to two washes. Firstly, 500 µL of buffer BW (containing guanidine hydrochloride and isopropanol) was added to aid in the precipitation of DNA from the solution to allow binding onto the silica membrane, followed by 1 minute centrifugation at the same conditions as mentioned above. The flow-through was discarded and the column subjected to a wash with 600 µL of buffer B5 (containing ethanol and low salt content) by centrifuging the sample for 1 minute at 11,000 x g. The silica membrane was subsequently dried to remove residual ethanol by 1 minute centrifugation at 11, 000 x g. A clean collection tube was used to collect the elution of DNA by adding 50 µL of Elution Buffer (BE, containing 10 mM Tris.Cl, 0.1 mM EDTA, pH 8.0, pre-warmed to 70°C) to the column and centrifuged for 1 minute at 11, 000 x g. This elution step was repeated with the same collection tube, hereby re-dissolving DNA in the elution buffer and resulting in a higher DNA yield and concentration. DNA samples were stored at 4°C until further analysis was performed.

DNA concentration was measured by the NanoDrop® ND100 spectrophotometer (NanoDrop Technologies, Thermo Fisher Scientific, USA) at a wavelength of 260 nanometers (nm). The purity of the DNA was evaluated by determining the ratio of the DNA absorbance to a measurement of protein absorbance at 280 nm. This is referred to as the A_{260}/A_{280} ratio and a value at ~1.8 indicates acceptable DNA purification. Equation 3.1 was used to calculate DNA concentration.

Equation 3.1 Calculation of stranded DNA concentration

$$[\text{dsDNA}]\text{ng}/\mu\text{L} = A_{260} \times (\text{dilution factor} \times 50 \text{ ng}/\mu\text{L})$$

([dsDNA] = double stranded DNA concentration. Adapted from Maniatis et al. (1982))

3.4 mtDNA copy number

The relative mitochondrial DNA copy number (RMCN) was estimated by relative quantification (RQ) of the mitochondrial DNA. RQ is the term used when referring to gene copy quantification in relation to that of

another gene. Both genes chosen for this study are located in highly conserved regions of the mitochondrial and nuclear genomes, respectively. In this study, beta-globin was used as the fixed copy nuclear gene to which the possibly variable mitochondrial DNA was compared. The ND2 gene of complex I, which is one of the 13 structural genes on mtDNA, was used as marker for mtDNA. The normal ratio is roughly found to be one beta-globin gene copy for every ten mitochondrial DNA copies, although it ranges greatly between different tissues (Davis *et al.*, 1996; Miller *et al.*, 2003). *Real-time* PCR was used as quantification method, together with the 7300 *real-time* PCR System analyser with 7300 System software and the TaqMan® chemistry from Applied Biosystems (Foster City, USA).

3.4.1 The Real-time PCR method

The *real-time* PCR method is based on the basic polymerase chain reaction (PCR) as first proposed by Kary B. Mullis *et al.*, (1986), allowing rapid *in vitro* enzymatic amplification of a specific DNA segment during successive thermal cycles (Mullis *et al.*, 1986; see also Section 3.5). PCR is accomplished by the interaction of the following components: DNA template, oligonucleotide primers which flank the desired amplification region on the DNA template, DNA polymerase, dNTPs, buffer and salts.

The PCR cycle programme consists of denaturing, annealing and extension stages at corresponding temperatures, incorporated into each cycle. In comparison with the DNA template, primers are added in excess. These primers hybridize on complementary positions on the single strands of the denatured DNA template, where the polymerase binds in order to catalyze the growth of a complimentary DNA strand using dNTPs. Strands of varying lengths are created and subsequently undergo this same process of elongation during successive cycles. Furthermore, each newly synthesised strand acts as a template in successive cycles, thereby amplifying the original target sequence.

During *real-time* PCR, data visualization of the increasing amount of template occurs in *real-time* by detecting fluorophore-based light emission. This is different to the usual light emission detection for PCR which occurs at the end of the run, with the use of gel-electrophoresis. Data visualization with *real-time* PCR occurs in "real time" during the PCR exponential phase and, depending on the type of chemistry used, may require a dual-labelled fluorogenic probe (TaqMan® probe) in addition to the primers used for PCR (Heid *et al.*, 1996).

Figure 3.1 shows the TaqMan® methodology. During the denaturing step of PCR, the double stranded DNA is subjected to high temperatures for separation of the two complementary DNA template strands. This opens primer and probe binding sites where primer and probe annealing takes place during the subsequent annealing phase (occurring at a lowered temperature). The TaqMan probe is a single-stranded

oligonucleotide, designed to be complementary to a ~40bp segment of the DNA template desired for amplification, with its annealing site located between the forward and reverse primer binding sites.

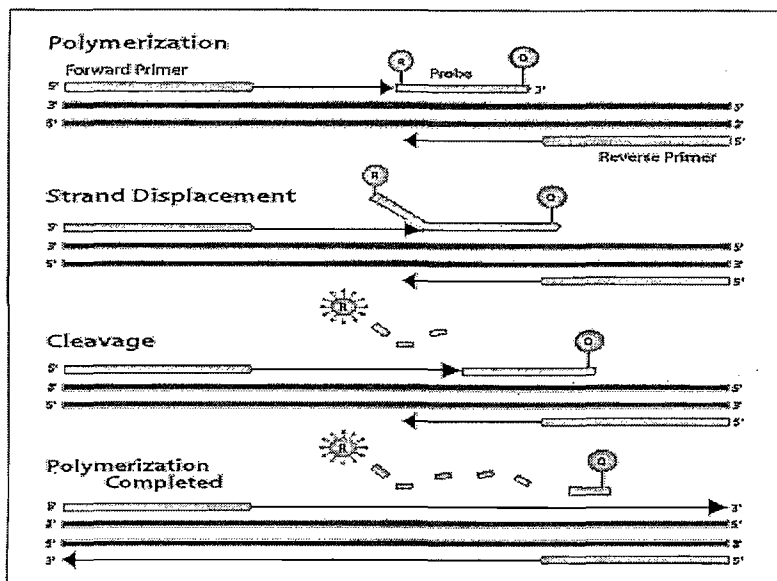


Figure 3.1 TaqMan®-based detection of PCR-amplified DNA.

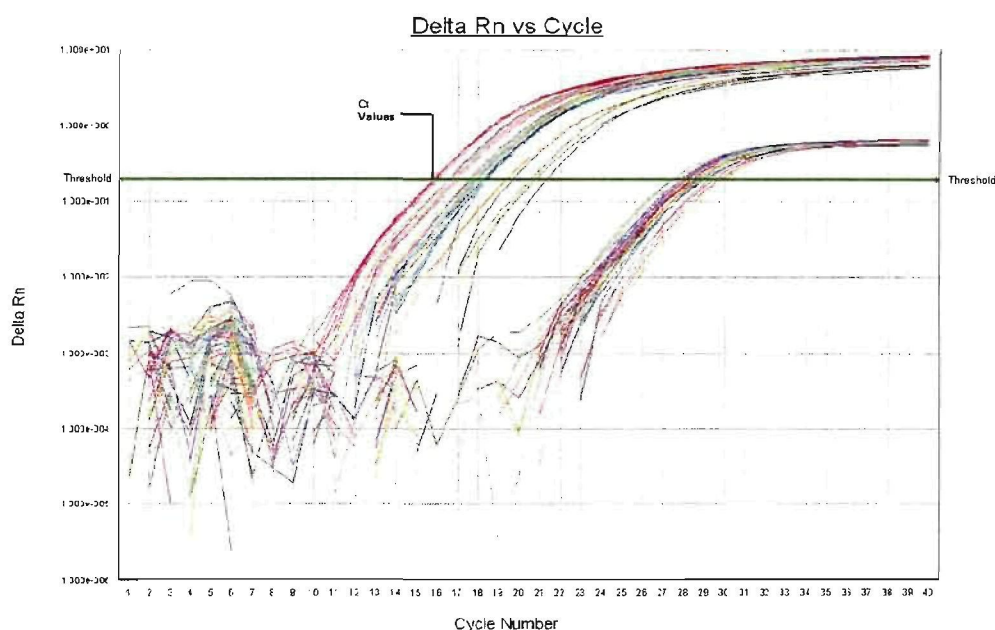
The figure illustrates the TaqMan® probe detection principle. Denatured dsDNA results in single stranded DNA templates for primer and probe annealing. Subsequent polymerization takes place until the Taq DNA polymerase initiates probe displacement by its 5' exonuclease activity, thereby releasing the reporter dye from the probe (step two). While the probe is still intact, the proximity of the quencher dye quenches the fluorescence of the reporter dye, by a process called fluorescence resonance energy transfer (FRET). However, when the reporter dye is cleaved, its short-wavelength fluorophore becomes detectable while the quencher is still attached to the template. Probe displacement continues until complete polymerization of the target strand is achieved. (Figure courtesy: Applied Biosystems)

The following final extension step occurs at a slightly raised temperature for optimal enzyme function, and allows polymerase extension of the complementary DNA strand. This extension starts at the primer binding site where the polymerase uses the original DNA strand as template until it reaches the probe binding site. Here it removes the TaqMan® probe from the DNA template with its 5'-3' exonuclease activity (Heid *et al.*, 1996). While the probe is unattached from the template, the fluorescence of the quencher dye quenches the fluorescence of the shorter wavelength colour of the reporter dye, due to their close proximity. However, when the reporter dye is released from the probe during the extension step, it emits light energy since the quencher dye is still attached to the probe and cannot quench the emitted light.

As this process is amplified during the PCR cycles, more light is emitted and detected, visualised and quantified by a cycler analyser and software converter. The cycler measures the fluorophore fluorescence with the use of laser beams or LEDs and displays the data in real time on a graph by its computer software. Figure 3.2 shows such an output from reactions performed in this study.

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The emitted fluorescence is directly proportional to the amount of fluorophore released from the probe and thus, to the amount of amplified DNA in the reaction. Fluorescence intensities for each PCR cycle is used to create the amplification plot (Figure 3.2) of ΔRn (normalised reporter, a background fluorescent detected signal) vs. the cycle number, to calculate the threshold cycle (C_T -value, the fractional cycle number at which the fluorescence passes the threshold). This C_T -value is used in further quantification of the amplified DNA product (or amplicon).



3.4.2 mtDNA copy number analysis using real-time PCR

Total cellular DNA was extracted from patient muscle homogenate and, as described in Section 3.3, was used for *real-time* PCR analysis. The DNA samples were firstly diluted to 2.5 ng/ μ L in order to achieve greater pipetting accuracy during reaction mixture preparations. The amount of DNA used in the *real-time* PCR reactions, were optimized by Dr O. Levanets, Division of Biochemistry, NWU, prior to this study.

Two separate reactions were prepared in an air-filtered hood: one reaction for the nuclear beta-globin gene and one for the mitochondrial ND2 gene. The 20 μ L *real-time* PCR mix for beta-globin analysis consisted of 10 μ L TaqMan[®] Gene Expression Master Mix (2X), that, according to the manufacturer, contains AmpliTaq

Gold® DNA polymerase, uracil-DNA glycosylase, dNTPs, a passive reference fluorescent dye called ROX™ and buffer components at optimized concentrations; 1 µL (0.5 µM) for the forward (5'-GTGCACCTGACTCCTGAGGAGA-3') and reverse (5'-CTTGATACCAACCTGCCAG-3') primers, respectively; 0.4 µL (0.2 µM) fluorescently-labelled probe (5'-FAM-AAGGTGAACTGGATGAAGTTGGTGG-TAMRA-3'); 4 µL of 2.5 ng/µL DNA and 3.6 µL PCR-grade H₂O (Bioline, Toronto, USA).

The primers and probe for the beta-globin gene reaction were obtained from Metabion International, (Martinsried, Germany). The reaction mix for the ND2 gene consisted of 10 µL TaqMan® Gene Expression Master Mix (2x), 1 µL of a primer-probe mix (20x, Applied Biosystems, catalogue number Hs 02596874g1), 4 µL of 2.5 ng/µL mtDNA and 5 µL PCR-grade H₂O, totalling a volume of 20 µL. The ND2 primers and probe were included in an optimized commercial reaction mix obtained from Applied Biosystems, who do not provide the detailed information of primer sequences and concentrations.

Samples were loaded in triplicate for each gene into separate wells in a 96-well *real-time* PCR plate. Reactions were performed using a 7300 *real-time* PCR System and Ct values were calculated by 7300 System Sequence Detection Software (version 1.4; Applied Biosystems). The cycling conditions started at 95°C for 10 minutes to initiate enzyme activation. Fluorescence was detected during the subsequent 40 cycles which consisted of [95°C for 15 sec. followed by 60°C for 1 minute]. The C_T-values were exported to a relative expression software tool (REST® 384) and used to calculate the RMCN.

3.4.3 Calculation of RMCN using the REST®384 expression software tool

C_T-values obtained from real-time PCR analyses of patient and control samples were exported to REST® software (Relative Expression Software Tool) (Pfaffl *et al.*, 2002). Relative quantification is usually applied to mRNA expression analyses. This determines changes in mRNA levels of the analyzed gene to multiple samples. It furthermore expresses these changes relative to the levels of an internal control RNA (housekeeping gene) which displays stable expression in experimental conditions. In this study this methodology, which is commonly used where absolute quantification is not possible (compare He *et al.*, 2002), was applied to mtDNA copy number analysis (RMCN), since mtDNA quantity per cell is variable in comparison with the nuclear genome (only a single copy per cell). The beta-globin gene (as the stable nuclear reference gene) and the ND2 mitochondrial gene (as the variable target gene) were used to calculate the RMCN of mtDNA quantity in patients' samples relative to those of healthy controls.

Different mathematical algorithms, based on *real-time* PCR efficiency and the C_T -value of an unknown sample versus a control, exist to calculate expression ratios. The mathematical model used in this study was the REST programme (Pfaffl *et al.*, 2002). The REST programme is based on the correction for exact PCR efficiencies and the mean C_T -value deviation between sample group(s) and control group(s) (Pfaffl *et al.*,

2002, <http://www.gene-quantification.de>). Subsequently the expression ratio results of the investigated transcripts are tested for significance by a Pair Wise Fixed Reallocation Randomization Test[®] and plotted on a graph, using standard error (SE) estimation, via a complex Taylor algorithm (<http://www.gene-quantification.de/rest-384.html>).

The final result of the REST calculations is an absolute gene expression value, indicating the relative level of analyzed gene expression compared to the controls and normalized by the used reference gene expression. In our case this value shows the relative quantity of mtDNA (RMCN) per cell for individual patients, compared to the average mtDNA content in the healthy controls (n = 18), normalized to the beta-globin gene. The final result of this calculation is a ratio value for the RMCN of each patient compared to the average of the healthy controls. For example, a value of 0.7 for a patient means that this patient has 70% of the mean mtDNA content of the healthy control group. Thus, this patient has 30% less mtDNA than the mean of the control group.

3.5 Molecular characterization of *POLG1* coding sequences (PCR)

In this study published methods for amplification of *POLG1* using genomic DNA were either directly used, or slightly modified (Lamantea et al., 2002; Nguyen et al., 2006). Figure 3.3 illustrates a schematic summary of the strategy followed for molecular characterization of *POLG1*. For amplification of the coding sequences of the *POLG1* gene, 22 exons (exon 2-23) of the gene were grouped into ten areas of ~200 - 2000 bp each that exclude amplification of non-coding sequences (introns, 5' and 3' un-translated regions) as far as possible (Table 3.3).

3.5.1 Primer design

At first, unmodified published primers and PCR conditions for *POLG1* amplification were used as indicated in the detailed strategy for this part of the study (Figure 3.3). PCR results generally gave clear, good bands when investigated on a 1% agarose gel (Figure 4.3 to Figure 4.5 Section 4.4.1), however subsequent sequencing of these amplicons revealed that, although these primers amplified the regions successfully during PCR, the primers were not optimal for the sequencing reaction, which used the same primers. Upon investigation, it was found that several of the published primers were positioned over known single nucleotide polymorphisms (SNPs) in the gene sequence. This may influence the primer binding affinity, resulting in primer dissociation especially in difficult sequencing areas with GC-rich content (Bai and Wong, 2005).

All primers from areas A1 to A8 were redesigned using the programme Sequencher 4.8 (Gene Codes Corporation, MI), to exclude any possible SNP's (see Table 3.3 for the primers and Appendix C for their alignment and positioning relative to the exon sequences). Primer design was done according to the

following principles: primers should preferably be 18 – 30 nucleotides in length (with 21 nucleotides as an optimum length); their GC-content should be between 50-60%, preferably without the incorporation of poly-G or poly-C regions; primer pairs must have equal or corresponding melting temperatures (T_m values) with annealing temperatures not more than 5°C lower than their T_m values (Equation 3.3 and 3.4); primers must be specific to the target amplification region, without the possibility of any internal secondary structure formation.

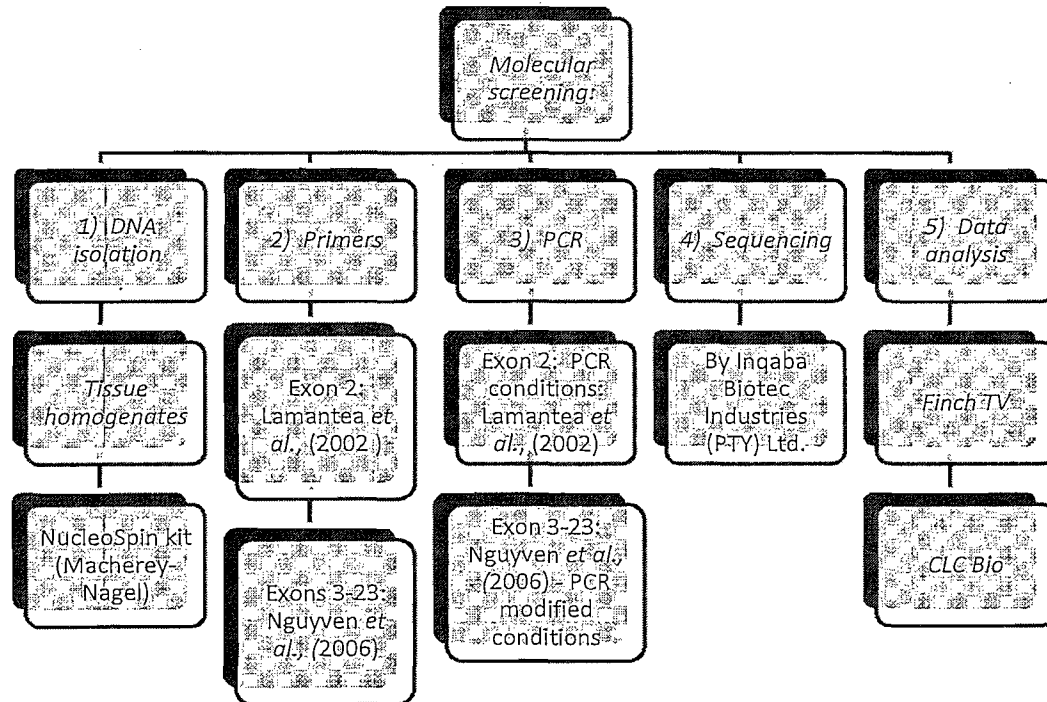


Figure 3.3 Strategy for molecular characterization of *POLG1*, depicting the five stages of analysis.

In order to run all the samples with the same PCR cycle protocol, all primers were designed to have melting temperature values (T_m values) at 64°C to 68°C. Equations 3.3 and 3.4 were used to determine the T_m of each primer and subsequently the annealing temperature (T_a) for the primer sets. Finally, Table 3.3 summarizes the primer sets that were used for amplification of *POLG1* coding sequences as well as during sequencing (additional sequencing primers are also indicated).

Equation 3.2 Primer melting temperature (T_m) calculation:

$$T_m = 2(A + T) + 4(C + G)$$

Equation 3.3 Calculation of primer sets annealing temperature

$$T_a = T_m (\text{mean of primer set}) - 5^\circ\text{C}$$

Estimated optimum primer annealing temperature = T_a

Table 3.3 Modified primer sequences for *POLG1* PCR and sequencing analyses (Lamantea *et al.*, 2002; Nguyen *et al.*, 2006).

Area	Exon range	Area Location on reference gene (bp position)	Size (bp)	Forward Primer (Fp) (5' to 3')	Reverse Primer (Rp) (5' to 3')
B1	Exon 2A	1483-1960	477	CTCCACGTCTTCCAGCCAGTAAAA	TTCTGCAGGTGCTCGACGCT
B2	Exon 2B	1890-2300	410	GGGCTGCACGAGCAAATCTT	AGCACGTAACAGGACCTCAGAAAA
A1 SP	Exon 3	5120-5315	196	GCT GTT TGA GTT AGG AGC ACC CTT TGG CTG CCA ATG CAC CAT C	GGG ACA TAC AAC AGC TAG GCA CAG AG
A2 SP	Exon 4-6	6285-6940	655	GAG GTC CAC ACC ACC AAG CAG CTG TTT GAG GAG CTG A	CAA GCA CAA CTC CAT GGC CCT C CTC CAT TCT TTC AGC C
A3 SP	Exon 7-9	8047-8785	738	GTG CCA TGT CAG TGG CTG GGT TG GCT AAA CCA GCT CAG G	CTG GAA GAC AGT GTG ACT GAA TGG C
A4 SP	Exon 10	9709-9946	237	GGC TAA AGC ATC CCA GAG GAG G ACA ACT GCC CTT ACC A	CAC TAG CCT GAG CTG ACC AGC
A5 SP	Exon 11-14	11169-12655	1486	GAA GGC ACT AGC AAG CTG CCT G GAG TGG GCA TCT GGT A GTG TCT GAG TAC CAT G	AGG CCT CTA GAC CAT AGT CAG GC
A6 SP	Exon 15-18	13380-14630	1250	CAC CTT GAG TCC AGT TAG TGA GGC GTA TAG CAG TCCTGG A CTG TCA CAC CAA TTG C	GGT CCT GGG TGT TAA AGT GGA TGG
A7 SP	Exon 19-21	16045-16855	810	GAG TCT TTG TCC TTT ATT GGG CTA C	ACA ATT CCC TCA ACC TCA CTG CTT C CTA ACCTCA CTG CTT C
A8 SP	Exon 22-23	17860-19055	1195	TGG GAG GTT AGG CTG CTG ATG G CTT TGC TCA CTT CTG AG	GGT CCT GCT ACT GAA AAA TGG CTG CTG GCC TTA GGC AAG C

Reference gene= mtDNA *POLG1* reference gene from Ensemble.org. (ENSG00000140521); SP = Sequencing primer.

3.5.2 PCR analysis

PCR amplification of each patient in the TPG for exons 2-23 of *POLG1* was performed using the modified primers (Table 3.3). Exon 1 of *POLG1* is not translated *in vivo* and was therefore not amplified and sequenced (Nguyen *et al.*, 2006). For the PCR reaction, modified protocols for exon 2 as described by Lamantea *et al.*, (2002) and for exons 3 to 23 as described by Nguyen *et al.* (2006) were used for amplification. Nguyen *et al.* (2006) could not successfully amplify exon 2 and the modified primers published by Lamantea *et al.*, (2002) for exon 2 were used.

The total PCR reaction volume of 25 μL consisted of 12.5 μL Lucigen EconoTaq Plus Green master mix (consisting of a high density 2X Master Mix containing EconoTaq DNA Polymerase, dNTPs, PCR enhancer & stabilizer, buffer with the magnesium concentration optimal for routine PCR, and gel loading & tracking dyes. (Lucigen, Middleton, WI), 1 μL of the previously isolated DNA at 125ng/ μL (Section 3.3), 1 μL of the respective forward and reverse primers (10 μM each) and 9.5 μL PCR-grade water. PCR cycle conditions for the B1, B2 and A1 to A8 fragments are summarized in Table 3.4 and Table 3.5 respectively.

Table 3.4 PCR steps for amplification of *POLG1* coding sequences: B1 and B2

PCR step	Number of cycles	Action	Temperature	Duration
1	1	Hot-start	95°C	10 min.
2	1	Denaturation	96°C	2min.
3	40	Denaturation	94°C	60 sec
		Annealing	57°C	30 sec
		Extension	72°C	60 sec
4	1	Hold	4°C	Indefinite

Table 3.5 PCR steps for amplification of *POLG1* coding sequences: A1 – A8

PCR step	Number of cycles	Action	Temperature	Duration
1	1	Denaturation	95°C	3min.
2	45	Denaturation	95°C	30 sec
		Annealing	60°C	30 sec
		Extension	72°C	2min.
3	1	Additional extension	72°C	10 min.
4	1	Hold	4°C	Indefinite

3.5.3 Agarose gel electrophoresis

To analyse the amplification of the various *POLG1* regions, PCR reactions were analysed using ethidium-bromide containing agarose gel-electrophoresis. After PCR amplification, 2 μL of each reaction mix was loaded onto a 1 % (w/v) agarose [TopVision LE GQ Agarose (low electro-endosmosis), Fermentas, Vilnius, Lithuania] gel containing 0.5 $\mu\text{g/mL}$ ethidium bromide (EtBr) and 1 x Tris-borate-EDTA (TBE) buffer (89.15

mM Tris.Cl, 88.95 mM boric acid, 2.5 mM ethylenediamine tetra-acetic acid (EDTA), pH 8.1). The samples were loaded along with loading buffer containing 5 % (v/v) glycerol and 0.4 % (w/v) bromophenol blue in 1 x TBE buffer. Gels were run for 15 minutes at a voltage (V) adjusted to the length of the gel (~7V/cm, 350 mA for 15 minutes). DNA fragment lengths were estimated by the Fast Ruler™ DNA ladder, Middle Range (Fermentas, Vilnius, Lithuania). Visualization of the amplicons was done using the ChemiGeniusQ® gel documentation system and GeneSnap® software (Syngene, Cambridge, UK) which records fluorescence of EtBr-intercalated DNA under UV light (254 nm). For reactions that appeared to produce the required size amplicons, the remainder of the reaction mix (not used on the gel) were sent to Inqaba Biotec Industries (PTY) Ltd., Pretoria, South Africa for gel-extraction, purification and sequencing. This was done since there was no sequencing apparatus available at this institution. Furthermore, Inqaba Biotec indicated that they prefer to perform gel-extraction and purification on site to ensure the best sequence results.

3.5.4 Gel-extraction and sample purification

PCR products were gel-extracted and purified by Inqaba Biotec Industries Pty. Ltd., Pretoria, SA, to remove all other components of the reaction from the target amplicons. Gel-extraction was done with a Zymoclean™ Gel DNA recovery kit (Zymo Research, Orange, CA, USA). DNA fragments were cut from the agarose gel using a scalpel. The fragments were transferred to a 1.5 ml microcentrifuge tube with the addition of three volumes agarose dissolving buffer (ADB) for each volume of excised agarose (e.g. for 100 µl (mg) of agarose gel slice 300 µl of ADB was added). Samples were incubated at 37-55 °C for 5-10 min. until the gel fragments dissolved completely.

The melted agarose was immediately transferred to a Zymo-Spin™ I Column (Zymo Research, USA) and centrifuged at 10,000 x g for 30-60 sec. The column was subsequently washed with 200 µl *Wash Buffer* and centrifuge at 10,000 x g for another 30 sec. This step was repeated, after which 6 µl of water was added to the column and centrifuged at 10,000 x g for 30-60 sec. to elute DNA. This DNA was subsequently purified with the SAP (shrimp alkaline phosphatase, Fermentas, Vilnius, Lithuania) protocol. Cleanup (purification) aids in the removal of excessive nucleotides and unincorporated primers. Extracted PCR product (0.5 µl) was added to 0.5 µL (10 U) exonuclease I and 1 µL (1 U/µl) SAP. Samples were incubated at 37°C for 15 min. and the reaction was terminated by heating the samples at 85°C for 15 min.

3.5.5 Cycle sequencing

The method used for automated cycle sequencing was originally developed by Sanger *et al.*, (1977), and was called the "dideoxy" DNA sequencing method. This method still forms the basis of today's sequencing methods, which use fluorescent dye terminators. For sequencing reactions, template (PCR product),

sequencing primers, dNTPs, enzyme (polymerase) and fluorescently labelled dideoxynucleotides (ddNTPs) are necessary. ddNTPs for each of the four nucleotide basis are labelled with a fluorescent dye, each emitting an unique wavelength. After primer annealing and during strand elongation, dNTPs and ddNTPs are incorporated into the strand at random. However, when a ddNTP is incorporated, strand synthesis terminates, resulting in product fragments with varying lengths. These sequenced fragments are then transferred from the reaction tube to a lane in a polyacrylamide gel, for fragment separation and detection. Shorter fragments emerge first at the end of the gel, where a laser beam detector detects light emitted from the incorporated ddNTP. This signal is analyzed by computer software and an electropherogram output is generated, displaying the sequence in base pair order.

In this study, purified PCR amplicons were sequenced in both directions with designed PCR and/or sequence primers (Table 3.3), using an AB1 3130XL genetic analyser (Applied Biosystems, Foster City, CA), incorporating the ABI Big Dye Terminator Cycle Sequencing kit version 3.1 (Applied Biosystems, Foster City, CA). The exact composition of the kit is not disclosed by the company. Sequencing was performed by Inqaba Biotech Industries Pty. Ltd., Pretoria, South Africa. Electropherograms of the sequences generated were inspected with FinchTV software (version 1.4; Geospiza Inc., Seattle, WA) as described in the following section.

3.5.6 Sequence data analysis

The following programmes were used for data analysis and interpretation of sequence results for our TPG: BlastN (NCBI database), CLC Bio Sequence Viewer (CLC Bio, USA) and Finch TV version 1.4 (Geospiza Inc., Seattle, WA). Obtained sequences were compared to existing databases (Ensembl.org) for detection of all known SNPs and to establish whether possible deletions occurred.

Sequences obtained for individual exons of all the patients were aligned with each other and the *POLG1* reference gene, ENSG00000140521 (Ensembl.org), using CLC sequence viewer. This enabled detection of any change in sequence, including known polymorphisms, pathogenic mutations, insertions and deletions of each patient of the TPG. Finch TV was used to inspect the quality of the electropherograms of the obtained sequences. Variations, obtained in results for the particular exon(s) of the relevant patient(s), were confirmed by re-sequencing the particular genetic variation in both directions, by the above mentioned procedures (Sections 3.1.5.1 – 3.1.5.5). This was done to establish whether a particular variation might be due to technical error or a genetic characteristic to the patient.

With detection of a deletion or possible mutation in an exon, the particular exon sequence would be translated to its corresponding protein sequence using CLC Bio (should these changes occur). This would have been done to investigate possible frame-shifts (in the case of a deletion or insertion), amino acid

substitutions or initiation of a premature stop codon. Since no mutations or deletions were found in our patient group, translation was not necessary during the study.

4 Results and Discussion

4.1 Introduction

38 Patient and 18 control muscle biopsies were collected before the onset of this study. Based on clinical and biochemical information, eight patients (TPG) presenting with clinical phenotypes indicative of possible POLG involvement were identified for *POLG1* gene assessment as indicated in Table 3.2, Section 3.2. The 22 coding exons of the *POLG1* genes of the TPG were amplified, sequenced and analysed for potential mutations and/or deletions. Due to the amount of data generated, only an example of each type of analysis will be presented here. However, results displaying SNP's, mutations or deletions of each patient will be presented and discussed. In addition to sequencing of the *POLG1* gene, relative mtDNA copy numbers were determined for all 38 patients and not only the TPG. This value may help to identify aberrations of mtDNA copy number, which may or may not be attributed to POLG deficiency.

4.2 Results for DNA isolation of patients

As previously mentioned, DNA (containing both mtDNA and nDNA) was isolated from muscle tissue homogenates of the TPG. Tissue sample quantities were often limited, yet DNA with sufficient quality could be isolated for subsequent PCR and *real-time* PCR analysis. Table 4.1 summarizes the quantity and quality of DNA isolated from the nuclear pellets and homogenates respectively (also see Figure 2.8, Section 2.5). DNA from muscle tissue of the remaining 30 patients was also isolated for mtDNA copy number analysis, although results are not shown here.

As can be seen from the table, the DNA concentrations and quality of the various DNA isolations varied slightly among samples. The DNA concentration was more than sufficient for mtDNA copy number, which required approximately 15 ng (~2.5 ng/μL), as well as for *POLG1* sequencing, which required approximately 500 ng (~125 ng/μL). The quality of all samples, as indicated by the A_{260}/A_{280} ratio, was also acceptable with ratio values slightly above the target value of 1.8 (Maniatis *et al.*, 1982) (A_{260} and A_{280} are indications of DNA and protein content, respectively).

Table 4.1 Quantity and quality estimation of DNA isolated from TPG samples.

Patient number	DNA from homogenate (ng.µL ⁻¹)	A ₂₆₀ /A ₂₈₀ ratio	DNA from nuclear pellet (ng.µL ⁻¹)	A ₂₆₀ /A ₂₈₀ ratio
P4	82.2	1.92	191	1.98
P7	124.5	1.93	341.1	2.00
P9	98.1	1.98	236.2	2.00
P12	127.7	1.97	139.9	2.00
P14	56.9	1.94	371.3	1.99
P16	80.5	1.94	80.6	1.98
P21	41.1	1.90	75.5	1.97
P27	55.2	1.93	237.6	1.97

P4 - P27 = TPG; ng.µL⁻¹ = nanogram per microlitre; A₂₆₀/A₂₈₀ ratio = ratio of light absorbance as an indication of DNA purity (DNA absorbance at 260nm and protein absorbance at 280 nm).

4.3 mtDNA copy number

4.3.1 Results

Factors that could influence the quality and accuracy of *real-time* PCR are contamination of the reaction mixture, mis-priming, primer-dimer artefacts and the combination of fluorescent and quencher dyes for the two analysed genes. *Real-time* PCR was performed in triplicate on the isolated DNA for 18 controls and 38 patients as described in Section 3.4.1.

Real-time PCR data, as illustrated in the example in Figure 3.2 (Section 3.4.1), showed that fluorescence increase was first detected for ND2 gene PCR reactions of each DNA sample. The relative abundance of the ND2 gene in the cell is about ten times higher than the abundance of the nuclear-encoded beta-globin gene. From the *real-time* PCR data set, C_T values were generated and imported into the REST programme. Subsequently, absolute gene expression values of patients and controls were generated and subsequently referred to as the relative mtDNA copy number (RCMN). Both RCMN and standard deviations (SD) for the triplicate results were calculated by the REST program for each individual (patients and controls, respectively) and are listed in Table 4.2.

Firstly, to visually illustrate the variations among the samples, the box plot shown in Figure 4.1 compares the relative distribution of the patient and control RCMN's.

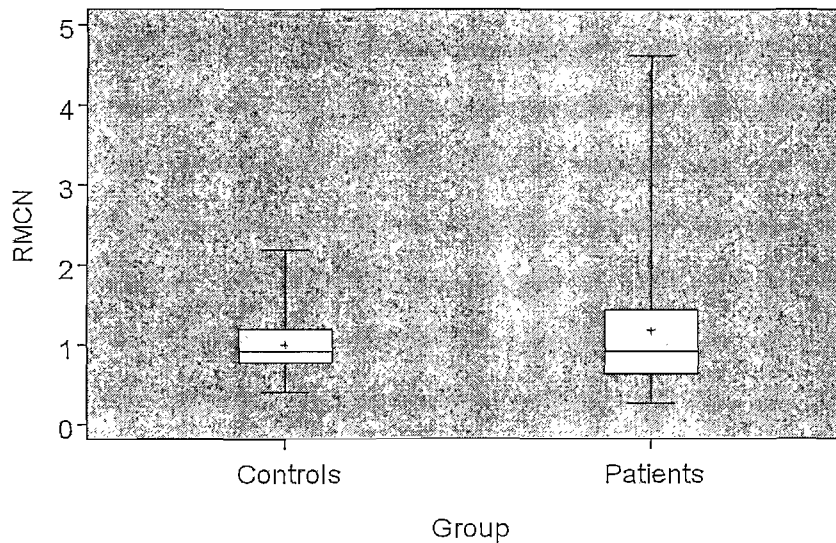


Figure 4.1 RMCN for patients and controls.

The boxes show the median (horizontal line) as well as mean (red cross) and 75th and 25th percentile of the RMCN (y-axis). The ranges are also indicated as whiskers in the plot. The mean of the control was arbitrarily set at 1.0.

Each box plot gives an idea of the distribution of the RMCN, around the mean and median of the specific group. The median is defined as the middle value of the value range of each group. The top and bottom extended line ends of each box plot indicate the group's range of values. The top of each box represents the 75th percentile of the data and the bottom the 25th percentile.

From the plot it can be observed that the distribution of the patient group was over a much wider range (0.26 to 4.6) than the control group (0.5 to 2.72). The mean of the RMCN for the patient group was slightly higher (1.17) compared to the control group mean, set at a value of 1.0. However, the opposite was true for the median values (patient median = 0.91; control median = 1.13).

Table 4.2 further gives the individual RMCN values for 18 controls and 38 patients. Samples for each patient were done in triplicate in order to verify reproducibility and accuracy of the analysis. The rule of thumb was that the SD for the triplicate samples had to be lower than 20% of their mean, in order to conclude that the reproducibility and the accuracy of generated data were acceptable. Further graphical verification of the accuracy was evident in Figure 3.2, where triplicate samples closely represented a single line on the graph. The RMCN analyses of these samples were also repeated by another person in a related study and comparable values were obtained (results not shown).

Table 4.2 RMCN of patients and controls as generated by the REST programme.

Control	RMCN	Rescaling*	Standard deviation	Patient	RMCN**	Standard deviation	RMCN (%)
C1	2.72	2.18	0.40	P1	0.30	0.04	30.27
C2	1.61	1.29	0.23	P2	0.47	0.07	46.55
C3	1.13	0.91	0.16	P3	1.10	0.15	109.89
C4	0.59	0.47	0.08	P4	0.84	0.11	83.61
C5	1.64	1.32	0.30	P5	0.47	0.07	46.99
C7	1.20	0.96	0.20	P6	0.91	0.13	91.13
C8	1.89	1.52	0.26	P7	0.52	0.07	52.37
C9	1.49	1.20	0.21	P9	1.28	0.18	127.65
C10	0.98	0.79	0.13	P10	0.91	0.13	91.21
C11	0.68	0.55	0.10	P11	0.60	0.08	59.87
C12	1.07	0.86	0.15	P12	1.84	0.26	184.37
C13	1.27	1.02	0.17	P13	0.51	0.07	51.46
C14	0.92	0.74	0.14	P14	0.85	0.12	84.59
C15	0.96	0.77	0.13	P15	1.67	0.24	166.53
C16	1.10	0.88	0.15	P16	2.19	0.31	219.10
C17	1.42	1.14	0.20	P17	0.84	0.12	84.08
C18	0.50	0.40	0.07	P18	0.26	0.04	25.56
Mean	1.25	1.00	0.18	P19	0.62	0.09	62.24
				P20	0.79	0.11	79.03
				P21	1.20	0.16	120.30
				P22	0.81	0.11	81.21
				P23	1.43	0.20	142.54
				P24	0.99	0.14	99.20
				P25	0.60	0.08	59.60
				P26	1.82	0.25	181.96
				P27	0.87	0.12	86.91
				P28	0.93	0.13	93.39
				P29	2.14	0.86	214.33
				P30	0.55	0.09	54.79
				P31	1.10	0.42	109.50
				P32	0.65	0.12	64.71
				P33	4.60	1.12	460.02
				P34	2.72	0.90	272.11
				P35	0.69	0.14	69.13
				P36	2.29	0.45	228.51
				P37	1.17	0.23	117.42
				P38	1.81	0.31	181.39
				Mean	1.17	0.22	117.12

*Calculations done by the REST program arbitrarily rescaled control values to have a mean of 1.0

**Patient RMCN values are generated as a proportion of the rescaled RMCN control group mean.

Figure 4.2 shows the RMCN values in a bar graph in order to give a better indication of the RMCN values and patients displaying notable changes in the RMCN. In this graph possible outliers, as defined by Eriksson and

Wang (2008), are also indicated. According to Eriksson and Wang (2008), RMCN values below 30% of the mean value for the controls (indicated as a light blue horizontal line), could possibly have a mitochondrial depletion syndrome. This graph as well as Table 4.2 illustrate two patients, P1 (at 30.3%) and P18 (25.6%) with values near and lower than 30% of the control mean, respectively.

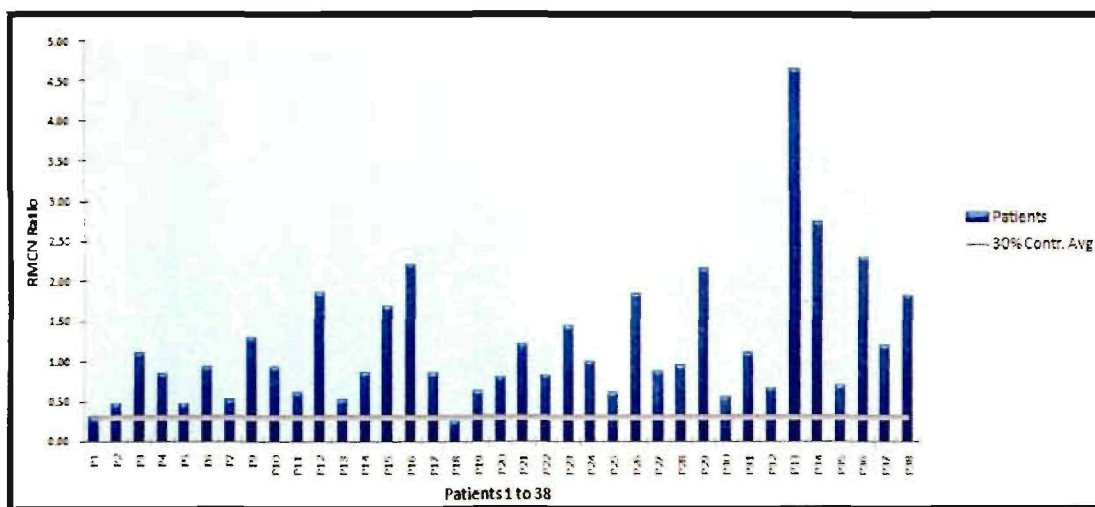


Figure 4.2 Graphical presentation of RMCN values for the 38 patients.

The thin horizontal blue-grey line indicates 30% of the control group's mean value. Patients below this line might have mitochondrial depletion syndrome (Eriksson and Wang., 2008).

4.3.2 Discussion

The *real-time* PCR method used in this study to determine RMCN was essentially based on commonly applied protocols. However, the application and use of RMCN data to gain additional insight into our TPG was still a new approach, and required a critical consideration of its accuracy.

Although little information is found in literature to aid the interpretation of RMCN data, there were some indications, acquired from the RMCN data itself that allowed assessment of the quality and accuracy of our methods.

Firstly, as indicated from the raw data presented in Figure 3.2 (Section 3.4.1), a greater abundance of the ND2 gene (mtDNA) was observed relative to that of the nuclear beta-globin gene (with only two copies per cell). This was also reflected in the lower C_T values for ND2 real-time data.

Unless very low levels of mtDNA exist, which would indicate a possible mitochondrial depletion syndrome, this result is expected. Furthermore, as described in the previous section, the standard deviations were

acceptably low in triplicate analyses and an additional repeat of all samples in a separate study gave similar results.

From these indications it was concluded that the method was sufficiently accurate to measure RMCN. Eighteen healthy control samples were available to compare patient data with and to generate a RMCN scale for comparison. The comparative distribution of the values were shown in Figure 4.1, where it was clear that in both groups variation in the values existed, although more so in the patient group. As mtDNA replication regulation is likely to be more affected in patients with mitochondrial disorders, this varied distribution in the patient group is expected.

As further illustrated in Figure 4.1 and Table 4.1, it was observed that on average, the RMCN's of patients were slightly higher than control values. It is not clear if there is any significance to these observations as there are several RMCN values that are notably high (e.g. over 200%) and others that are notably low (e.g. lower than 50%) that greatly affect these averages.

Similar findings were made by Bai and Wong, (2005) for 18 patients with clinical and laboratory findings consistent with mitochondrial respiratory chain disorders. Three of their 18 patients (investigated by *real-time* PCR analyses) illustrated severe mitochondrial depletion (3 to 10% of the mean). Four of the 18 patients had a 2 to 6.4 fold mtDNA amplification (205 to 672%). No deleterious deletions were detected by *real-time* PCR for any of the 18 patients, and investigation of the entire mtDNA genome delivered no pathogenic mtDNA mutations. Numerous reported SNPs were, however, detected. Consequently, they attributed their findings to the possibility that the mitochondrial depletion and over replication could be a secondary influence due to primary molecular defects in nuclear genes involved in mtDNA replication.

In accordance with the findings of Bai and Wong, (2005), the possibility remains that a deleterious mtDNA deletion could up-regulate nuclear genes involved in mtDNA replication. The resultant up-regulation of mtDNA could then compensate for malfunctioning OXPHOS and thereby meet cellular energy demands. However, this does not explain the down regulation of mtDNA in patients with OXPHOS deficiencies.

P12 of the TPG has been identified to contain an mtDNA deletion (results not indicated) and in accordance with an up-regulation hypothesis displayed a notable up-regulation of mtDNA replication of 200% (Figure 4.2). There are at least six potential patients (see Figure 4.2) not originally included in the TPG with similar RMCN profiles to P12. In view of this result and the lack of mtDNA mutations in the group reported by Bai and Wong, (2005) the aetiology of up-regulation of mtDNA replication can clearly originate from nuclear and/or mitochondrial mutations.

Limited indications in literature exist to aid interpretation of RMCN values. As mentioned before, a patient with a RMCN value lower than 30% of the control average has a high possibility to have a mitochondrial depletion syndrome (Eriksson and Wang, 2008). Although a few patients had RMCN values lower than the control average, only two patients (P1 and P18) had values close to or lower than 30% of the control (31% and 26% respectively). In these cases the lower values could point to a deficient POLG function and neither of these two patients was initially considered in the TPG.

Although no clear indications exist in the literature as to what RMCN values can be considered as high, it is clear that several patients have values that are markedly higher than the mean of the controls. If samples that are ~three-fold (30%) lower than the mean are considered pathologically significant (Eriksson and Wang, 2008), then it could be argued that values which are ~three-fold higher than the control mean, in this case P33 (460%) and P34 (270%), could be pointed out as possible candidates for significantly high values.

However it should be kept in mind that many factors, of which some aspects are poorly understood, control mitochondrial biogenesis and, more specifically, mtDNA replication. Nuclear and mitochondrial genes, intra- and intercellular cell signalling, oxidative stress (ROS and free radicals), ATP levels, hormones and second messengers, elements involved in mitochondrial replication, and nucleotide pools could all influence mtDNA content (Reinecke *et al.*, 2009). Any element that could activate or de-activate genes involved with mtDNA replication could therefore potentially influence mtDNA copy numbers.

Furthermore, the type of mutation, its genetic location and tissue distribution together with its level of heteroplasmy (mutant load – Bai and Wong, 2005; Rossignol *et al.*, 2003) can greatly influence the extent of mtDNA up- or down regulation. (Reinecke *et al.*, 2009; Di Mauro and Schon, 2001; Zeviani and Di Donato, 2004).

Unlike mtDNA depletion, there are no indications from literature if over replication has any detrimental effect on mitochondrial function. More importantly to this study, there is also no evidence to suggest that over replication of mtDNA may be a consequence of POLG dysfunction. However, this cannot be ruled out, since theoretically, changes in the structure of an enzymes' active site may increase its catalytic efficiency. Nevertheless, over replication of mtDNA is mostly considered to be a consequence of induced intergenomic (nucleus-to-mitochondria) signalling to compensate for dysfunctional mitochondria (Bai and Wong, 2005 Reinecke *et al.*, 2009).

Therefore, when considering Figure 4.1 and the RMCN data, only superficial interpretations can be made from it.

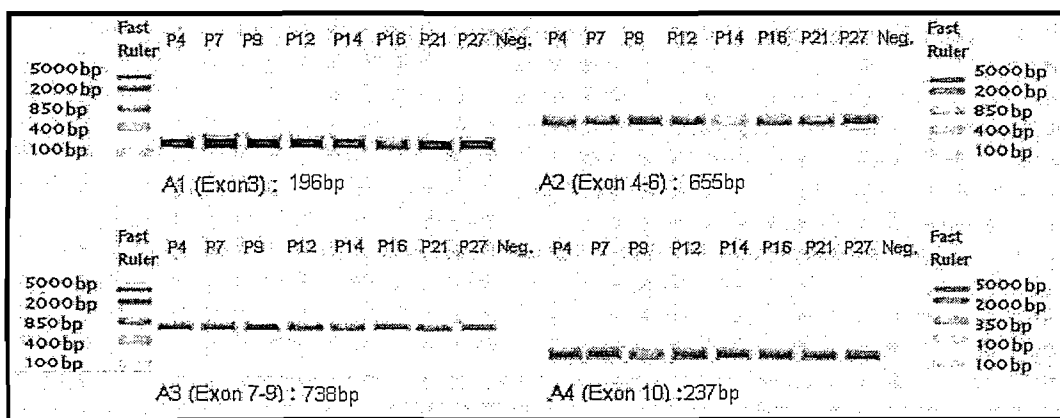
4.4 PCR amplification of *POLG1* coding sequences and gel-electrophoresis

Isolated DNA from the nuclear enriched pellet for each of the target group patients was used for subsequent PCR of the 22 coding regions (exons) of *POLG1*. As mentioned in Section 3.5.1, the published primers (Nguyen *et al.*, 2006) were redesigned for areas A1 - A8 of the gene, with a correspondingly modified PCR cycle programme (Table 3.4 and Table 3.5).

4.4.1 Results

PCR amplicons for the TPG coding sequences were evaluated using agarose gel electrophoresis with ethidium bromide staining. An example of a typical result is shown in Figures 4.3 - 4.5, where the amplicons for each region (B1 - A8) of all the patients are shown. As can be seen in these figures, clear single DNA bands can be observed for individual patients. As expected, the smaller fragments of the A1 and A4 areas migrated faster in the gel than the larger fragments (e.g. A2), which are seen closer to the top of each gel. The negative controls were utilised to confirm that no contamination occurred during the PCR preparation.

In some cases, as shown for P14, amplicon A2, some smearing was detected. In these cases it was concluded that the obtained result was not due to the DNA quality, as other amplifications for the same patient were good. The likely reason is that loading of the PCR amplicon into the well of the gel was not always constant, considering that only 2 µl was loaded. Gel-electrophoresis was only used to confirm the presence of the amplicon and the remaining volume of amplicon was more than sufficient for sequencing. Therefore in cases such as amplicon A2 for P14, the remaining PCR amplicon product was used for sequencing instead of gel-extracted amplicons. Amplicon sizes for each region are indicated on the gel (see also Table 3.3, Section 3.5.1).



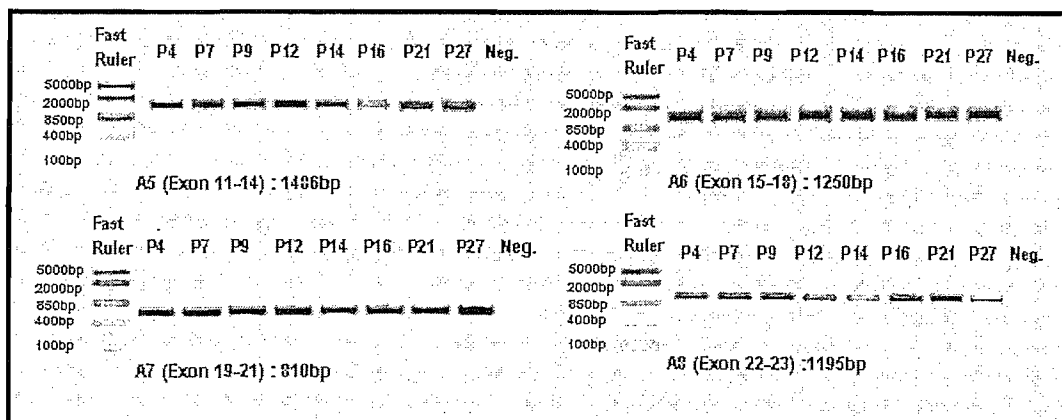


Figure 4.4 Photographic representation of a 1% (w/v) agarose gel electrophoresis analysis. The gel was run at 100V for 15min. in 1 x TBE buffer. Fermentas Fast Ruler middle range ladder was used and four (A5, A6, A7, A8) of the ten amplicons for eight patients in the TPG, as well as a negative control (Neg) containing no template is shown. bp: base pairs.

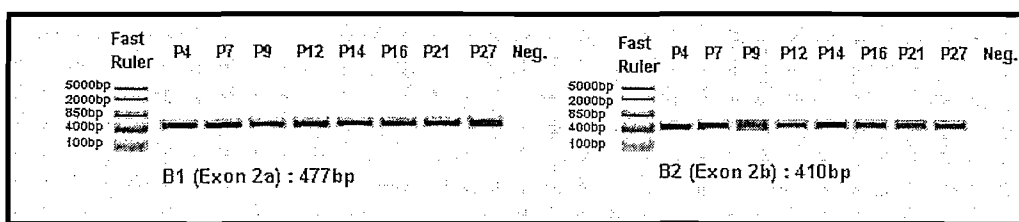


Figure 4.5 Photographic representation of a 1% (w/v) agarose gel electrophoresis analysis. The gel was run at 100V for 15min. in 1 x TBE buffer. Fermentas Fast Ruler middle range ladder was used and two (B1 and B2) of the ten amplicons for eight patients in the TPG, as well as a negative control (Neg) containing no template is shown. bp: base pairs

4.4.2 Discussion

Newly designed primers and subsequently modified PCR conditions (Section 3.5.2) yielded clearly visible PCR amplicons with the correct sizes as illustrated by the example in Figures 4.3-4.5. After gel-extraction and purification (done at Inqaba Biotec Industries, Pty. Ltd., South Africa), these amplicons were further used for cycle sequencing.

4.5 Cycle sequencing

4.5.1 Introduction

Cycle sequencing was done at Inqaba Biotec Industries Pty. Ltd. South-Africa, on gel-extracted and purified amplicons generated through PCR, for each of the eight patients in the TPG. Purified amplicon (~1 µL) was used for sequencing, although the volume varied according to the purified amplicon concentration (~125 ng/µL). PCR primers were used for sequencing of all regions.

The first round of sequencing was done with forward primers for each selected region, where after these results were confirmed by sequencing the same area with reverse PCR primers. For selected areas consisting of longer DNA stretches (more than 600bp), additional sequence primers, which were designed to overlap sequences, were used (Section 3.5.1). This insured that all the exons in a particular region were sequenced. However, after redesigning primers for area A8 and after several attempts to sequence exon 23, a limited amount of DNA prevented the elucidation of this exon (Table 4.3).

4.5.2 Results – Data processing

The quality of the PCR and the success of its gel-extraction and purification greatly affect the quality of sequencing. Excess template generates broad peaks, complicating the reading of individual peaks, whereas insufficient template with low amplitude peaks are easily overshadowed by background peaks. If amplicon samples are contaminated by non-specific PCR products, background or “noise” is generated which interferes with the generated sequence, leading to insufficient amplitude identification with non-specific base calling.

Figure 4.6 illustrates the raw data generated as an electropherogram during cycle sequencing. Different bases are indicated with different colours which are generated by the four different fluorescent dyes (attached to the ddNTPs). The bases are represented by the following colours: G = yellow, although illustrated in black for better legibility; C = blue; A = green and T = red. Similar electropherograms were generated for each of the 22 exons of each patient.

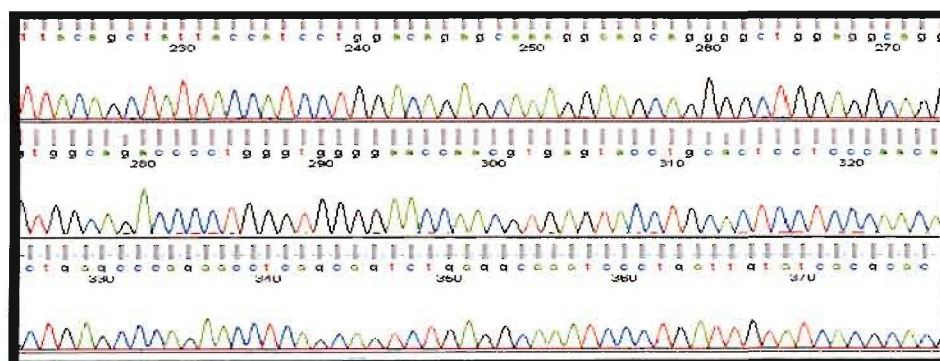


Figure 4.6 Cycle sequencing output 1: example of an electropherogram using Finch TV software.

The figure shows the electropherogram (chromatogram) where ddNTP-terminated fragments elute during the capillary chromatography. The ddNTPs are: a =Adenine; c = Cytosine; g = Guanine; t = Thymine. The deduced sequence is shown in the top row along with a visual indication of the quality of the read (relative length of the vertical grey bar above the sequence read) and numbered according to the generated sequence's length.

Peaks are clearly visible and distinguishable from adjacent peaks, therefore aiding to sequence elucidation. There was little background “noise” in most sequences, which indicated that PCR, gel-extraction and cleanup procedures were of good quality.

Finch TV and CLC BIO are user-friendly programs that are especially suitable for sequence viewing and data interpretation. Sequences were therefore exported from Finch TV, the electropherogram viewer, into CLC BIO’s sequence viewer. Sequences of corresponding areas were aligned to the Ensembl *POLG1* SNP reference sequence and also to corresponding sequences of the TPG.

As an example of the processing done with CLC Bio, Figure 4.7 illustrates the Ensembl reference gene and TPG sequences aligned to it for a specific sequence in PCR region A6. The pink bars below the sequences are indicative of the sequence base pair conservation. The more an individual base pair at a specific position corresponds with that of other sequences in the alignment, the higher the level of conservation of that base pair, therefore, creating a correspondingly higher pink bar.

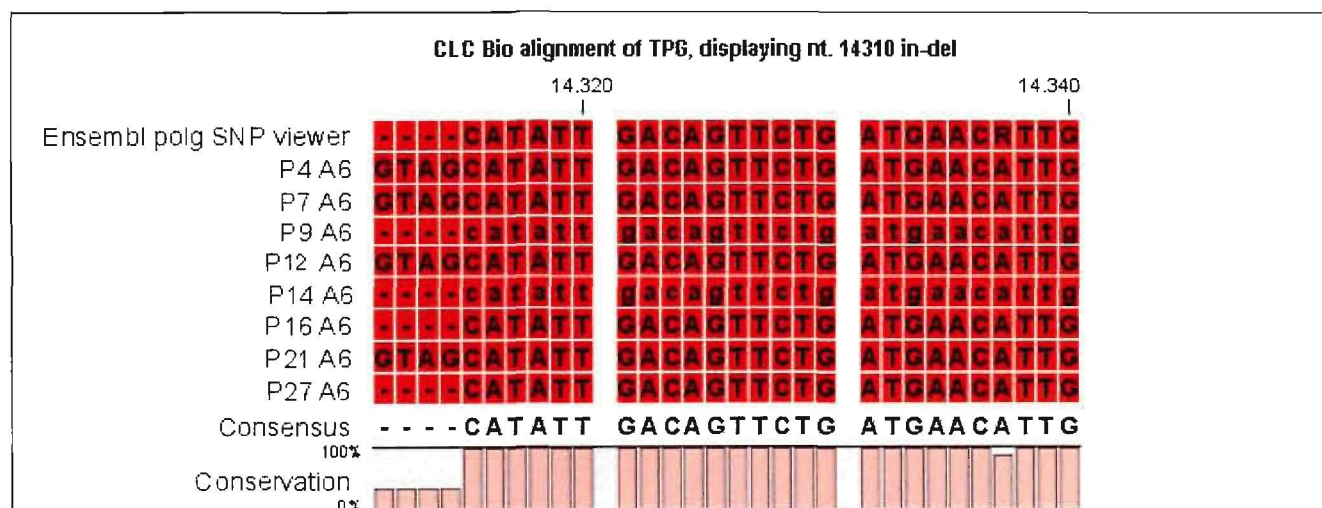


Figure 4.7 Cycle sequencing output 2: example of the sequence alignment using CLC-BIO software.

The reference gene sequence for *POLG1* in coding area A6 (exon 15-18), obtained from the genome data base, Ensembl, and is displayed in the top row. Sequence data is shown for the TPG in the same region and a consensus sequence is given in the bottom row. The software’s SNP viewer displays an “in-del” (insertion-deletion) in this area (14310 _14314delinsGTAG). The pink bars at the bottom of the sequences indicate the conservation of the sequences. Thus it indicates how well each sequence is aligned with the other at that particular base pair position. As seen at 14 388-51A/G, a SNP is indicated in the reference gene by the letter R (R could be an A or a G). All the patients are homozygous for this particular SNP, but the inconsistency of the conservation at base pair R (as opposed to all the A’s) is indicated in a lowered pink bar.

All the sequences generated were aligned to the *POLG1* SNP Ensembl reference gene in CLC Bio sequence viewer as shown in Figure 4.7. This allowed detection of genomic variations (SNPs, mutations and/or insertions/deletion) and is a fast and effective tool for sequence analysis and interpretation.

Several nucleotide variations in the TPG *POLG1* genes were observed in this way and are subsequently discussed.

4.5.3 Results and Discussion

4.5.3.1 Terminology used

For the purpose of describing and interpretation of the results, it is important to consider the terminology used. Various large and small scale genomic variations occur in genes. Those small scale variations that are of particular importance to this study include the single nucleotide polymorphisms (SNPs), mutations ("silent", "missense" and "nonsense"), and insertion-deletions (in-dels).

As noted by Condit *et al.*, (2002), there are varying opinions concerning nomenclature used to distinguish between "mutations" and "polymorphisms". Both forms can present in a coding or non-coding region or in intronic regions between exons. In order to clarify their use in this study, they are subsequently discussed.

A single nucleotide polymorphism (SNP) is defined as the variation of a single nucleotide in the genome of two individuals of the same species, provided that it occurs in more than 1% of the population (Human genome Project information, 2008; Stoneking, 2001).

Most SNPs functional effects are not directly observed in the phenotype, although they cause a predisposition toward certain disorders (Oliver, 2004) and can therefore be used as genetic markers. Because they cause no evident phenotypic change, SNPs are mostly considered to be "neutral" or "harmless" genetic variations (Cotton and Scriver, 1998).

Intronic SNPs don't seem to have a direct impact on the subsequent protein sequence. However, it could still influence RNA splicing, transcription factor binding and gene regulation (Oliver, 2004; Conde *et al.*, 2004).

An exonic SNP could cause an amino acid substitution, in which case the protein function might be altered leading to the expression of a pathogenic phenotype. This emphasizes the nomenclature issue at hand, given that this particular illustration would mostly be classified as a "missense" point mutation.

A mutation, although not always deleterious, generally refers to a genetic sequence change with pathogenic consequences visible in the patient's phenotype. To distinguish between the natural occurrence of a genetic variation and its unnatural form, the terms "wild type" and "mutant" are used respectively.

In a coding region, single nucleotide mutations (point mutations) are classified as synonymous or non-synonymous mutations. A synonymous (also called a silent) mutation occurs when both forms (wild type or mutant) of a nucleotide variation generate the same amino acid during translation. Thus, the amino acid sequence is unchanged with a synonymous point mutation.

A non-synonymous point mutation could result in a "missense" or "nonsense" mutation. "Missense" mutations result in a change in the amino acid sequence, which often result in pathogenic proteins. "Nonsense" mutations on the other hand, are characterised by the insertion of a premature stop-codon at the site of the nucleotide variation, resulting in incomplete peptides. (Antonarakis, 1998; Dunnen and Antonarakis, 2000)

Insertions and deletions are one or more nucleotides that are inserted into or deleted from a genetic sequence. Their effect and consequences range according to their location of insertion or deletion in the genome. When they occur in exons they alter the amino acid sequence and could severely influence the function of the subsequently formed protein. Their effect in intronic regions are mostly regarded the same as that of a SNP. "Insertion-deletions", commonly abbreviated as "in-dels", are characterised by a deletion directly followed by an insertion.

To conclude, the term SNP is mostly used to indicate "neutral" or "harmless" genetic variations with no visible phenotypic influence. The term "mutation" is mostly used to describe deleterious genetic variations with evidence in phenotypic expression.

4.5.3.2 SNP's found in the POLG genes of the TPG

During this study, no known pathogenic mutations ("missense", "nonsense", "insertions" or "deletions") were detected by using the approach described in Section 4.5.2. Only eight different SNP's were detected, which include two in-dels, all in intronic regions. All of these intronic SNP's are summarized in Table 4.3.

Table 4.3 SNPs and in-dels detected in the POLG1 gene introns of the TPG

A	Bp position	Patients								Known SNP	Comment
		P4	P7	P9	P12	P14	P16	P21	P27		
A3	8229+51C/T	C/T								C/T	Heterozygous
A5	11495-22T/C						T/C		T/C	T/C	Heterozygous
A5	11581+92T/C	T/C					T/C		T/C	T/C	Heterozygous
A5	12493-164TG 12333_12336 delinsTGTG; 12336_12343 delinsGCGTGC		in-del TGTG TGCG							In-del	Heterozygous TGTGCG**
A6	14135-122C/T	C/T	C/T							C/T	Heterozygous
A6	14271+39CT 14310_14314 delinsCTAC			in-del CTAC		in-del CTAC	in-del CTAC		in-del CTAC	In-del	P16, P28 Heterozygous P9 and P14 Homozygous for deletion
A7	16297-36A/G						A/G			A/G	Heterozygous
A7	16297-11T/C						T/C			T/C	Heterozygous

A = area/region Known SNP: Ensembl.org SNP viewer (POLG (ENSG00000140521));

** = The color coded base pairs for P7 indicates the in-del with respect to the corresponding base pairs of the two known in-dels. P7's in-del starts two nucleotides ahead of the first in-del at 12331_12334delinsTGTG, thereby overlapping the first known in-del (indicated in yellow). P7's in-del also overlaps the first three base pairs of the second known in-del at 12336_12343 delinsGCGTGC and is indicated in green. (Note: the last basepair of the first known in-del overlaps with the first basepair of the second known in-del – indicated in yellow).

The following electropherograms illustrate the presence of the SNPs found in the TPG (Table 4.3) respective to Ensembl's POLG1 reference gene (Ensembl.org):

Figure 4.8 indicates the intronic SNP 8229+51C/T of patient 4. This patient is the only patient of the TPG to have this SNP.

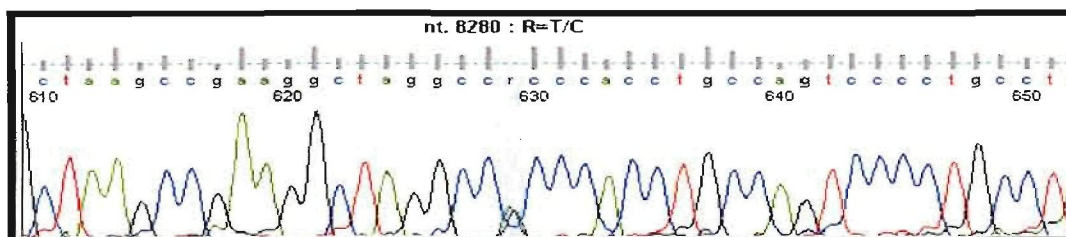


Figure 4.8 Heterozygous SNP on nucleotide position 8229+51C/T for patient 4 (the reverse sequence is displayed). R could be a T or a C.

12331_12338delinsTGTGTGCG as illustrated in Figure 4.11. It precedes the known in-del at 12333_12336delinsTGTG by two positions, and overlaps with the first three nucleotides of the second in-del at 12336_12338delinsGCGTGCGC. Since this deletion is intronic, it doesn't affect the amino acid sequence of *POLG1*. The extent to which intronic deletions could possibly affect the protein formation or function is mostly unknown and no direct link could be established between these findings and biochemical and enzyme analysis done for this patient.

This patient is heterozygous for this deletion as can be seen in Figure 4.11 where the double peaks start. The reason for the double peaks is that during PCR both alleles with the region of interest are amplified. One contains the deletion and the other is without the deletion. The deletion is small enough not to have a significant affect on the size of the sequence, thereby "masking" the detection of the deletion beforehand, during electrophoresis. When these amplicons are subsequently sequenced, they are essentially the same, up unto the deleted nucleotides.

The cycle sequencer reads both alleles since their amplification is the same, but it cannot interpret that a deletion occurred in one allele and make subsequent adjustments. Therefore, the sequences of the two alleles overlap each other, creating double peaks. The part of the sequence occurring after the deletion was elucidated with a reverse primer, which gave a good, clear sequence of the 3' end of this exon. In this case the same double peaks, this time for the 5' area, were generated where the in-del occurred. With the alignment of the forward and reverse sequences in CLC Bio, it was evident that a deletion occurred and at which position it occurred. (This is also displayed in Appendix C.)

Patients 4, 7, 12 and 21 generally displayed similarities in the variations of their sequences. Figure 4.12 illustrates the SNP occurring at nucleotide position 14135-122C/T of patients 4 and 7. Both patients display similarities in developmental delay and dysmorphisms.

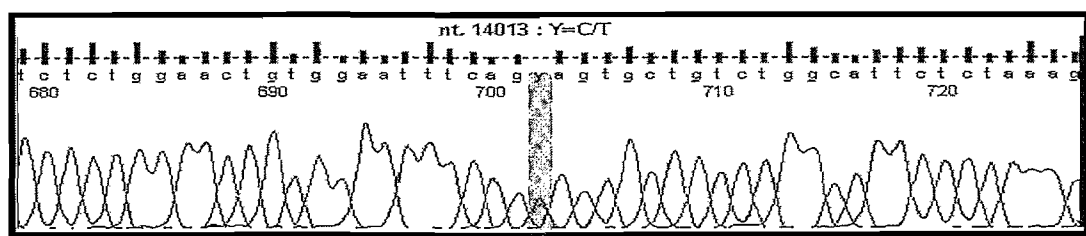


Figure 4.12 Heterozygous SNP at nucleotide position 14135-122C/T for P 4 and P 7. Y could be a C or a T

The in-del at 14310_14314delinsCTAC can be seen in Figure 4.13 to Figure 4.15. Patients 4, 7, 12 and 21 did not have the deletion, whereas patients 16 and 27 were heterozygous for the in-del (Figure 4.14). Patients 9 and 14 were homozygous for the in-del (Figure 4.15).

The same procedure was followed as mentioned previously to elucidate the 3' end of the sequence following the deletion. Figure 4.7 also indicates the alignment of these sequences in CLC Bio at this particular in-del. It would be interesting to further investigate these seemingly unimportant in-dels and SNPs to verify whether a specific combination of these could contribute to a patient's clinical and biochemical profiles (Oliver, 2004).

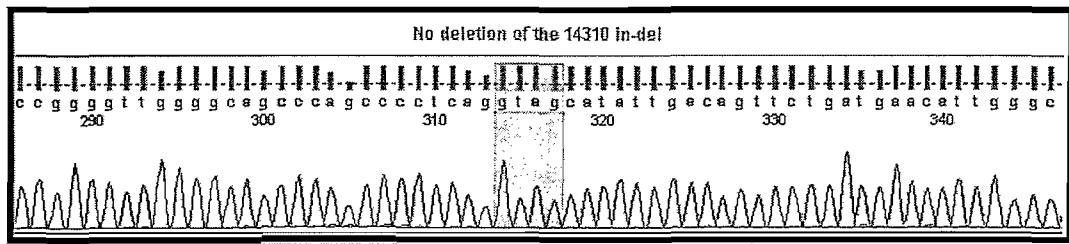


Figure 4.13 Patients 4, 7, 12 and 21 do not have the insertion-deletion at position 14310_14314delinsCTAC. The highlighted area indicates the area without the deletion.

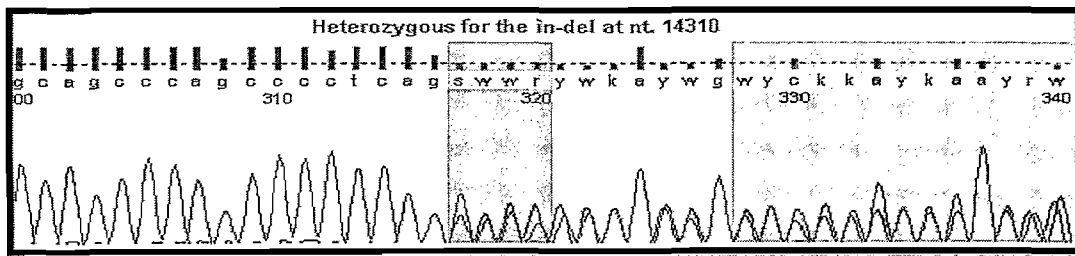


Figure 4.14 Heterozygous insertion-deletion at nucleotide position 14310_14314delinsCTAC for P16 and P27. This can be seen where the double peaks start. The highlighted area indicates the area with the deletion.

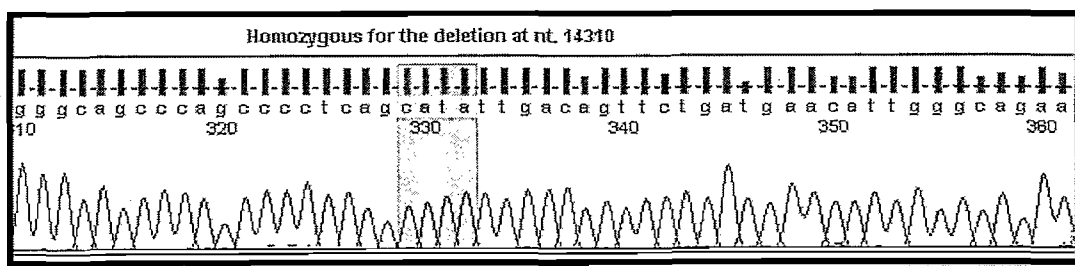


Figure 4.15 Homozygous insertion-deletion at nucleotide position 14310_14314delinsCTAC for P9 and P14. The missing nucleotides usually precede the highlighted area. The deleted nucleotides (GTAG) are deleted on both alleles of these two patients and therefore, no double peaks occur in Figure 4.11.

Figures 4.16 and Figure 4.17 illustrates two heterozygous SNPs for P16. Both SNPs are found within 100 nucleotides from exon 20 at nucleotide positions 16297-36A/G and 16297-11T/C respectively. Patient 16 is a

20 year old male with complex III deficiency. His clinical phenotype consists of extreme tiredness, exercise intolerance and visual impairments.

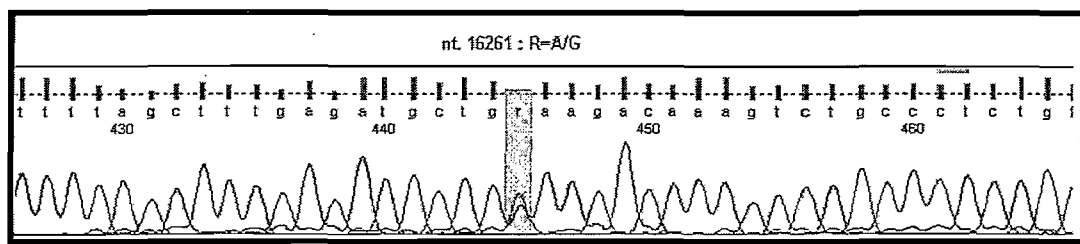


Figure 4.16 Heterozygous SNP at nucleotide position 16297-36A/G for P16. R could be an A or G.

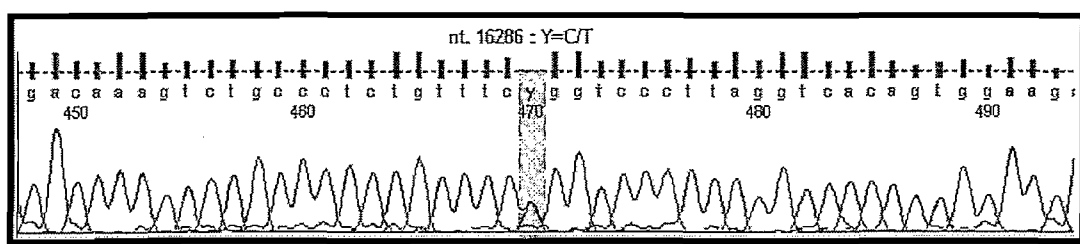


Figure 4.17 Heterozygous SNP at nucleotide position 16297-11T/C for P16. Y could be a C or T.

All the indicated in-dels and SNPs found in this study are located in intronic regions of the *POLG1* gene. Current understandings of the effect of these variations on the *POLG1* gene are limited at best. As such, no conclusive indications for the correlation of the clinical and biochemical phenotype with the genetic sequence of *POLG1* were revealed during the sequencing of the TPG.

4.5.4 Conclusions on sequencing of the *POLG1* gene in the TPG

Although cycle sequencing is a powerful analyzing tool, we experienced a few difficulties during elucidation of the required sequences. Table 4.3 illustrates the final results obtained for sequencing of the TPG in the course of this study. It also gives an indication of the areas where sequencing did not give data for *POLG1* coding sequences.

Primers were designed to be used at the same PCR conditions for all amplifications. However, although amplifications of all areas were possible, some exons were difficult to sequence after amplification as summarized in Table 4.4. Sequencing of the longer DNA stretches, in particular, were problematic even though additional intronic sequence primers were designed.

Although sequencing of 1000bp is possible, quality sequence of ~700bp could be expected. Primers were therefore designed to anneal to the DNA template at ~100bp to 150bp before (Fwd. primer) or after (Rev. primer) the desired region. This was necessary since the first ~100bp and the last ~100bp of an amplified

region did not give useful sequence data. This occurs because of too much initial background noise, causing poor resolution. Because of the loss of accurate sequences for the beginning and ends of some exons, a few nucleotides in these regions couldn't be elucidated notwithstanding the effort to design primers to prevent this as far as possible. To solve this problem, primers were redesigned and sequencing was repeated with these primers. Unfortunately, even after repetitive PCR and sequencing of these exons, and with a limited amount of DNA available, the sequences of these areas could not be elucidated.

The insufficient sequencing information for these areas may be corrected by designing primers at different positions with different T_m-values. Thereby preferably shorten the required sequence length. Amplification of shorter areas is recommended for future investigations. This approach should be investigated in a future study since it could possibly lead to less repeats of the sequencing reaction for these exons, thereby saving patient DNA as well as cost. Alternatively, for all primers used a sequence tag could be added, e.g. M13 sequence primer tags that would allow similar conditions for annealing during sequencing.

Obtained sequences were finally aligned to each other and the reference gene respectively, with the use of CLC Bio sequence viewer. This program proved to be very effective to analyse and interpret the sequence data of the TPG as described in Section 4.5.3.

Table 4.4 Final summary of areas of the POLG1 gene that were effectively sequenced in the TPG.

Area	Patients							
	4	7	9	12	14	16	21	27
B1	✓	✓	✓	✓	✓	✓	✓	✓
B2	✓	✓	✓	✓	✓	✓	✓	✓
A1	✓	✓	✓	✓	✓	✓	✓	✓
A2	✓	✓	✓	-46 bp (beg Ex 4)	✓	✓	✓	✓
A3	✓	✓	✓	✓	✓	✓	✓	✓
A4	✓	✓	✓	✓	✓	✓	✓	✓
A5	✓	✓	✓	-34 bp (end Ex 14)	✓	✓	-19 bp (beg Ex 11)	✓
A6	✓	✓	-86 bp (end Ex 18)	✓	✓	✓	✓	✓
A7	✓	✓	✓	✓	✓	✓	✓	✓
A8	-Ex 23	-Ex 23	-Ex 23	-Ex 23	-Ex 23	-Ex 23	-Ex 23	-Ex 23

bp = base pairs ; '-' = missing ; beg = beginning ; Ex = exon.

In conclusion, automated cycle sequencing is a fast and effective technique, allowing accurate analysis of large amounts of data. Despite some difficulties experienced with the sequencing of exon 23, sequence elucidation for the TPG was successful. Overall, eight SNP's in intronic regions were successfully detected although no mutations, that could be indicative of a POLG related disorder, were identified in any of the TPG. This technique gave quality sequences and is recommended for mutation screening analysis.

5 Conclusion

5.1 Aims of this study:

The study presented in this dissertation forms part of a broader project initiative in which the aetiology of mitochondrial disorders of 38 South African paediatric patients with confirmed mitochondrial disorders, was investigated. The main aim of this study was to investigate the mtDNA *POLG1* gene sequence in a selected subset of 38 South African mostly paediatric patients, together with RMCN analyses. The broader objective was to investigate *POLG* gene involvement in mitochondrial disorders of South African paediatric patients through which possible *POLG* genetic markers, that are associated with mitochondrial disorders, could be found. Genetic markers could simplify diagnostic screening of *POLG* related mitochondrial disorders and contribute to the understanding, diagnosis and possible treatment of patients with mitochondrial disorders. A methodology was devised for this investigation, as discussed earlier (Figure 2.8, Section 2.5).

5.2 Clinical selection:

Eight patients (TPG) were clinically selected based mainly on eye involvement from the 38 patients, previously diagnosed with mitochondrial disorders, for *POLG1* sequencing. The *POLG1* gene for the TPG was subsequently sequenced, and the relative mitochondrial copy number (RMCN) for the group of 38 patients and 18 controls was estimated.

According to literature, a relatively large number of mitochondrial disorders may be attributed to *POLG* deficiency (Chinnery and Zeviani, 2008). This was substantiated by initial reports that these patients present with similar phenotypes. Due to limited resources and these initial findings, a selection of eight patients was made from the available 38 patients to conduct *POLG1* sequencing. Patients were clinically selected based on phenotypes that seemed more likely to be linked with *POLG* deficiency. These selected patients had defective eye features, which included ptosis, nystagmus, strabismus and visual impairment or disturbances.

However, this approach for patient selection might present some obstacles, as recently reported by Wong *et al.*, (2008). They stated that patients identified with a single mutant *POLG* allele, displays broad clinical phenotypes with no apparent genotype-phenotype correlation. Clinical heterogeneity, time-dependent development and display of symptoms, variable phenotypic expressions and inconsistent muscle pathology findings, complicate the interpretation and selection of patients. In the area of mtDNA replication (*POLG* function), Bai and Wong, (2005) found that a patient's phenotype is sometimes not representative of conditions on a molecular level, due to compensatory mechanisms such as mtDNA over-replication. They also identified the proportion of heteroplasmy and the total mtDNA content of a sample as important factors that influence the molecular diagnosis of these patients.

It is clear that mutant load and a threshold effect of mutated vs. wild type mtDNA ultimately have an important effect on clinical phenotypes. This threshold is highly variable between different affected tissues and different individuals (Chabi *et al.*, 2003). Therefore, a patient with large proportions of mutant load could present with mild clinical symptoms and *vice versa* (Bai and Wong, 2005). These conditions give rise to clinical phenotypes that are not necessarily representative of the cause and severity of the disorder on the molecular level.

POLG related mtDNA disorders might furthermore present with unique phenotypic expression in the South African population, adding to the complex clinical selection.

This study confirmed, albeit in a limited number of patients, that genotype-phenotype relations are not accurately represented in clinical phenotypes. As observed during this study, POLG1 sequencing, excluding exon 23, of the clinically selected patients did not produce *POLG1* mutations. However, RMCN data of the 38 patients indicated that other patients, who were not originally included in the TPG, may be better candidates for POLG malfunctioning. From this it is clear that selection of patients is complex and if required, may have to include more specific alternative approaches.

5.3 Alternative approaches for pre-sequence patient selection:

5.3.1 RMCN

In this study, the generated RMCN mean for the 38 patients, as compared to the control group mean, indicated that patients' RMCN values were on average slightly higher than those of the controls. With limited data, comparative statistical analysis could however not be performed to indicate whether our findings were statistically significant. With the exception of a few outliers, the raised copy number could indicate a possible up-regulation of POLG, since POLG is the only polymerase responsible for mtDNA replication. Likewise, the patients with a severe down-regulation in their copy numbers could indicate a POLG down-regulation. However, it is important to consider that other factors (regulated or unregulated) are also involved during mtDNA replication which might also influence mtDNA copy number (Moraes, 2001). This includes factors involved in mtDNA transcription which could result in marked changes in RMCN. In fact, it is clear that POLRMT, TFAM and mtSSBP are better associated with changes in mtDNA *content* (not fidelity) than POLG itself and that several other secondary factors, including ROS, Ca²⁺ and ATP/ADP, impact on the signalling mechanisms that regulate mtDNA replication and transcription (Reinecke *et al.*, 2009).

RMCN analysis (using *real-time* PCR) indicated two patients (patient 1 and 18) that displayed very low copy number values when compared to the control group mean. This could indicate a possible mitochondrial

depletion syndrome. Their relative expression ratios were close to or less than 30% of the control-group average, which is suggested by Eriksson and Wong, (2008) to be indicative of a mitochondrial depletion syndrome. Limited information exists regarding the aetiology of mitochondrial depletion syndromes, while the interpretation of high RMCN values is still not completely clear. He *et al.*, (2002) and Bai and Wong, (2005) previously observed different patterns of abnormalities for patients with single or multiple mtDNA deletions. Bai and Wong, (2005) found that patients with single deletions tend to undergo mtDNA over-replication as a compensatory mechanism for malfunctioning mtDNA. Yet, they couldn't establish a direct link between the mutation site or size and the ratio of mitochondrial up-regulation. Their research provided no explanation for mtDNA down regulation.

During this study, P12 displayed an mtDNA over replication of about 200% which seemed to be in accordance with the previously confirmed mtDNA deletion. At least six of the 38 patients in this study displayed RMCN profiles similar to that of P12. They had severe mtDNA up or down-regulation (more than three times the control average) but were excluded from the TPG because they did not comply with the criteria used during the clinical selection. Although no direct relationship between RMCN and POLG activity can be made, a possibility does exist, that severe up or down-regulation of RMCN could indicate malfunctioning POLG activity. This preliminary investigation seemed to indicate that, if selection is required, patient selection based on RMCN values in addition to clinical phenotype, could prove to be a valuable approach in identifying patients prone to display POLG malfunctioning. Although no direct link between up-or down regulated mitochondrial copy number and POLG malfunctioning exists yet, it would be worth while to investigate the *POLG* sequence of these patients in order to confirm whether a possible RMCN – POLG mutation relationship exists.

In order to properly utilize RMCN data, factors such as tissue sample and patient age should be taken into consideration. Bai and Wong, (2005) found that muscle mtDNA content was ~10 times higher than blood mtDNA content. Also, newborn muscle samples contained only ~20% to 30% of adult muscle mtDNA. Furthermore, research done by Van Goethem *et al.*, (2004) demonstrated that traditional symptoms associated with defective POLG activity were not mandatory features for the identification of POLG related disorders. These symptoms include myopathy, ragged red fibres and structural abnormalities of muscle mtDNA as displayed by Southern blot analysis. This stresses the complexity of POLG related disorders and its investigation. It might therefore be advisable to follow a systematic approach for POLG investigation by performing various analyses on different tissue types of individual patients.

In this study all RMCN analyses were performed on muscle biopsies. RMCN information for South African patients with mitochondrial disorders was inadequate and subsequently no age-matched means or normal values existed for this patient group. Since our patient and control groups were not large enough to provide normal age-matched means for the South African patients, RMCN results were interpreted according to the mean of a group of 18 control patients (as opposed to a set of age-matched mean values). For accurate RMCN quantification and utilization, future experiments should take the age of their patients into consideration since this could explain much of the RMCN variation found in this study. It is suggested that age-matched means of other population groups be used in future studies concerning RMCN analyses, until a South African patient group dataset is established.

RMCN analysis provided supporting data to aid in the interpretation of the molecular diagnosis of our TPG. Patient data variation was observed but due to insufficient age-group corrected RMCN data, it was not possible to statistically analyse these data in order to find statistically significant differences between the control and patient groups or amongst individuals of the patient group. Nevertheless, RMCN analysis seems a promising technique if all factors that influence RMCN data interpretation are taken into consideration by the onset of the analysis.

5.3.2 Supporting molecular genetic analyses

The complex aetiology of POLG related mitochondrial disorders emphasizes the necessity of transdisciplinary research during molecular diagnosis of mitochondrial disorders (Reinecke *et al.*, 2009; Zeviani and Di Donato, 2004; Smeitink, 2003).

Other possibilities of patient selection include mtDNA structure integrity analysis using Southern blotting or long template PCR. Southern blotting is not very sensitive to minor deletions, mutations or even copy number and must consequently be used in combination with other analysis such as quantitative *real-time* PCR (Chabi *et al.*, 2003). Southern blot analysis as well as long-template PCR was done in a parallel investigation on the 38 patients of this study which revealed a novel mtDNA deletion in P12. As previously mentioned, during the study presented here, P12 displayed an mtDNA over replication of about 200% which indicate upregulation of mtDNA replication through a mechanism seemingly unrelated to POLG deficiency. Thus, no direct link was established between P12's *POLG1* gene sequence and the mtDNA deletion. However, unless all the exons and the *POLG2* gene are also investigated, POLG-involvement cannot be ruled out conclusively. This follows from the fact that *POLG2* encodes the accessory subunit of the POLG heterotrimer, which enhances polymerization and POLG's nucleotide binding affinity (Longley *et al.*, 2006; Farge *et al.*, 2007).

Investigations of POLG activity through enzyme analyses or any functional test are other possible approaches that could also contribute to pre-sequence patient selection. The combination of these analyses could contribute to the accurate pre-sequence patient selection illustrative of POLG-related disorders. Taking these factors into account, Bai and Wong, (2005) concludes that it is important to perform quantitative and qualitative analysis when investigating the molecular diagnosis of mitochondrial disorders.

Accurate pre-sequence patient selection could, in principle, avoid unnecessary costs involved during *POLG* sequencing. Considering the aspects mentioned in the past few sections, the feasibility of a pre-patient selection approach to identify patients with *POLG* mutations, is however highly questionable. The great number of factors and the high degree of variability related to the manifestation of these factors in patients with mitochondrial disorders make accurate selection nearly impossible. Due to these reasons Wong *et al.*, (2008) stated that molecular investigation through *POLG* gene sequence is inevitably the only definitive diagnosis of POLG related mitochondrial disorders. In practical terms this means that, until any accurate selection procedure is found, and irrespective of additional approaches and clinical evaluations mentioned here, sequencing of *all* patients with diagnosed mitochondrial disorders is currently the best approach in identifying POLG involvement in mitochondrial disorders.

5.4 Sequencing:

Some methodological conclusions regarding sequence analysis were also made during this study. In general sequencing proved to be a fast and effective technique to identify possible *POLG1* mutations, although a few minor difficulties were present during *POLG1* sequencing in this study. In order to reduce sequencing costs and to simplify the sequencing process, the 22 coding exons of *POLG1* were grouped into 10 regions which could be sequenced accurately.

PCR and subsequent sequencing of long DNA stretches proved to be difficult and additional sequencing primers had to be synthesised to accommodate the longer DNA stretches (Chapter 3 and 4). These difficulties could possibly have been eliminated by amplification of shorter DNA stretches together with the use of additional primers. This approach was not followed due to resource restrictions.

An alternative approach includes the use of uniformly tagged primers (e.g. using M13 tag sequences) for all amplifications and subsequent sequencing. This approach would increase specificity and consolidate the conditions of the sequencing reaction. Greater primer specificity could prevent possible mis-priming or premature primer dissociation during sequencing, which is especially important for the sequencing of longer stretches of DNA.

Unfortunately, even after repetitive PCR and sequencing of area A8, the sequence data of exon 23 could not be obtained. Primers for this region were firstly designed according to published data (Nguyen *et al.*, 2006) but when required PCR amplicons were not obtained, PCR primers were redesigned. These primers gave good amplification of the A8 region, which was in accordance with the expected size of the amplified region. However subsequent sequencing delivered no results for the final exon. This was the case in all eight patients of the TPG and it is highly unlikely that all of the TPG has a defect in exon 23 that could cause poor sequencing results. A more plausible explanation for the problem encountered with exon 23 sequencing, seems to be poor primer hybridization or a secondary sequence structure that disrupts chain elongation. No repetitive sequences occur in this exon that would also result in poor sequencing.

DNA of the patients in this study was limited and indefinite trial and error sequencing of this exon was not possible. When it is difficult to sequence a specific exon and sample DNA is limited (such as in the case of DNA isolated from muscle biopsies), it is advisable to use more readily accessible blood samples for DNA isolation. By following this approach re-adjustment of PCR and sequencing conditions can be repeated, until the exon is analysed. Once these conditions readily produce the required exon, the process can be repeated with the original limited DNA samples if necessary. In the case of any nuclear gene, such as *POLG*, this approach would usually not be required. However, since *POLG* analysis was performed on muscle biopsies during this study, and it is advisable to perform all analysis on the same tissue type for more accurate data comparison, this study could have benefitted from the use of additional blood samples.

Intronic regions were generally not amplified by PCR for subsequent sequencing during this study. Nevertheless, due to the grouping of exons into regions (clusters), introns between exons that lay within close proximity of each other, were inevitably amplified and sequenced.

Most of the genome consists of introns previously thought to be "junk DNA". However recent studies suggest the importance of these intronic sequences in the genome (Crosio *et al.*, 1996; Nakaya *et al.*, 2007). Because the intronic regions are not translated into protein sequences, mutations in these regions do not cause amino acid substitution during translation, as is the case with missense or non-sense point mutations in exons.

Very little is known about the effect of mutations or deletions in the intronic regions of the genome and single nucleotide polymorphisms (SNPs) found here are generally not pathogenic. Recent research done by Zhang and Gutterman, (2008) suggests that mutations occurring in intronic sequences in certain regions of the genome might alter the gene splicing of the exons around the specific intron. The translation of the mis-incorporated exons could lead to malfunctioning proteins and ultimately disorders.

No mutations were obtained during this study to conclusively indicate *POLG1* mutations as cause for these patients' mitochondrial disorders. However, SNPs in the amplified intronic regions were discovered for the TPG during genetic sequencing of *POLG1*, but not in splicing regions

All eight discovered SNPs, which include two intronic insertion-deletions, are known polymorphisms and are currently not known to cause any disorders. It was interesting to note similarities in SNP variations between the patients of the TPG. Patients 4, 7, 12 and 21 generally had the same variations on known SNPs, whereas patients 16 and 27 and patients 9 and 14 showed great similarities. Although it is too early to indicate possible disorder causality between these patients, these similarities could be indicative of a common origin for the disorder amongst the individual patients. Additional research is necessary in order to conclusively establish a causal relationship. (Preliminary research in this regard has been reported by Wong et al., (2008).)

5.5 Future prospects

In South Africa inadequate research funding and access to costly apparatus such as DNA sequencers and qualified personnel to perform and interpret analysis, are limiting factors that could profoundly affect research of this nature. At the moment, South Africa has limited high throughput genetic analysis capabilities such as GS 20 sequencers with the so-called 454 technology. Very few companies in SA, notably Inqaba Biotech Industries, PTY. Ltd., provide this service which comprises of extremely fast, accurate and sensitive genome sequencing. Europe or North America has many more of these resources to their disposal and this greatly influences costs involved for GS 20 sequencing as well as conventional Sanger sequencing. GS 20 is the latest in sequencing technology and surpasses conventional Sanger sequencing, however due to the relatively high costs involved, its use is most beneficial for larger genomic studies that span several hundred mega bases.

Sanger sequencing is currently still very expensive in comparison with overseas conventional sequencing due to the availability thereof. However, a trial and error approach with pre-sequence patient selection might very well be more expensive and yield less conclusive results than direct sequencing of the *POLG* gene. It is hereby suggested that the costs and effectiveness of direct sequencing of both *POLG1* and *POLG2* genes of all patients with mitochondrial disorders be evaluated, as opposed to inconclusive pre-sequence patient selection.

For the study presented in this dissertation, the specific aims were to evaluate the mtDNA copy number in the whole patient group and to determine whether *POLG1* mutations exist in a clinically selected target patient group, presenting with visual impairment. RMCN revealed interesting, but inconclusive results and

no mutations were found in the TPG. It was concluded that pre-sequence patient selection, based solely on clinical phenotypes, was not adequate to identify patients with pathogenic mutations. Should pre-sequence patient selection be required in the future, preliminary data gathered during this study indicated that RMCN analysis may provide an additional selection parameter for more accurate pre-sequence patient selection than selection based only on clinical phenotypes. As suggested before, the approach to select patients before subsequent sequencing is, however, not recommended. It can be argued that efficiency of direct sequencing of the *POLG* genes surpasses possible inaccuracy of a patient selection approach. This seems to justify an initial high cost for sequencing which might, in the long run, even prove to be less costly than pre-sequence patient selection approaches.

Although no *POLG1* mutations were identified in this study, it provided a good foundation for the future approach of molecular genetic studies that involve the occurrence of *POLG* gene mutations. Primers and protocols necessary for such research are now in place for further research to commence. Until both *POLG* genes of the 38 patients have been sequenced, a final conclusion on the occurrence of *POLG* mutations in South African paediatric patients with mitochondrial disorders cannot be made.

6 Bibliography

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7 Annexure

7.1 Annexure A: A molecular model of the mtDNA POLG protein structure.

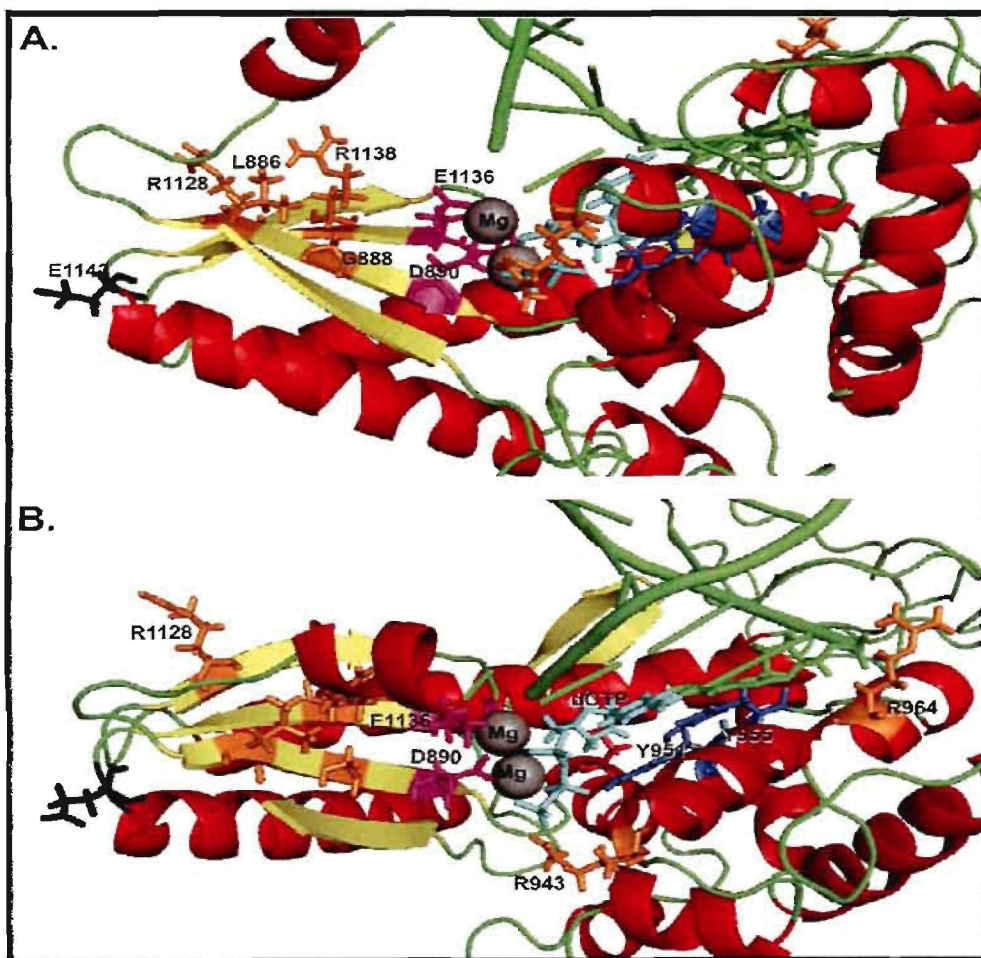


Figure 2. Molecular modeling of the human DNA polymerase gamma active site with amino acids of interest indicated. Critical amino acids important for catalysis are colored in. Those residues modified by *POLG* mutations are decorated: magenta for those carboxylic residues involved in chelating the active site Mg²⁺ ions (grey spheres); blue for those amino acids critical in dNTP recognition. Amino acid side chains colored in orange represent the sites of substitutions caused by novel disease mutations identified in *POLG* in the study by Wong *et al.*, 2008. An incoming dNTP molecule is shown in turquoise. **A.** The polymerase active site viewed with the palm domain labeled. **B.** The polymerase active site rotated to view the fingers domain. (From: Molecular and clinical genetics of mitochondrial diseases due to *POLG* mutations. Wong *et al.*, 2008. Reprinted with permission of John Wiley & Sons, Inc.)

WONG, L.J., NAVIAUX, R.K., BRUNETTI-PIERRI, N., ZHANG, Q., SCHMITT, E.S., TRUONG, C., MILONE, M., COHEN, B.H., WICAL, B., GANESH, J., BASINGER, A.A., BURTON, B.K., SWOBODA, K., GILBERT, D.L., VANDERVER, A., SANETO, R.P., MARANDA, B., ARNOLD, G., ABDENUR, J.E., WATERS, P.J. & COPELAND, W.C. 77

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7.2 Annexure B: Informed assent and consent forms for patients and controls.

Ethical approval for this study had previously been obtained from the University of Pretoria (UP):
Approval number 91/98. Titled: "Mitochondrial disorders in the South African context."

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria comply with ICH-GCP guidelines and has
US Federalwide Assurance. PWA 00002567, Approved dd 22 May 2002 and Expires 24 Jan 2009.
RB 0000 2235 IORG0001762 Approved dd Jan 2006 and Expires 21 Nov 2008.



Universiteit van Pretoria
University of Pretoria

Faculty of Health Sciences Research Ethics Committee University of Pretoria
Soutpansberg Road Private Bag x 385
MRC-Building Pretoria
Room 2 - 19 0001

Date: 4/04/2007

PROTOCOL NO.	91/98
AMENDMENT	K: Addition of Blue Native polyacrylamide gel-electrophoresis (PAGE) L: Addition of urine specimen in control population M: Revised Informed Consent for Control N: Revised Assent for control
PROTOCOL TITLE	Mitochondrial disorders in the South African context."
INVESTIGATOR	Dr I Smuts; Dept Pediatrics; Prof. Van der Westhuizen, North West University.
STUDY DEGREE	None.
CONTACT DETAILS	T:012-354 5296 F: 012-354-5275 E:izelle.smuts@up.ac.za
COMPLETE POSTAL ADDRESS	Pretoria Academic Hospitals; Pretoria
ORDER / CONECT NUMBER	None
MEETING DATE	31/01/2007

This Amendment has been considered by the Faculty of Health Sciences Research Ethics Committee, University of Pretoria on 28/03/2007 and found to be acceptable.

*Advocate AG Nienaber	(female)BA(Hons) (Wits); LLB; LLM (UP); Dipl.Datametrics (UNISA)
*Prof V.O.L. Karusseff	MBChB; MFGP (SA); M.Med (Chr); FCS (SA): Surgeon
Prof M Kruger	(female) MB.ChB.(Pret); Mmed.Paed.(Pret); PhD. (Leuven)
Dr N K Likibi	MB.BCh.; Med.Adviser (Gauteng Dept of Health)
Mrs. E.L. Nombe	(female) B.A. CUR Honours; MSC Nursing – UNISA (Lay Member)
Snr Sr J. Phatoli	(female) BCur (ELA); Senior Nursing-Sister
*Dr L Schoeman	(female) Bpharm, BA Hons (Psy), PhD
*Prof J.R. Snyman	MBChB, M.Pharm.Med; MD: Pharmacologist
*Dr R. Sommers	(female) MBChB; M.Med (Int); MPhar.Med;
*Prof TJP Swart	BChD, MSc (Odont), MChD (Oral Path) Senior Specialist; Oral Pathology
*Dr A.P. van Der Walt	BChD, DGA (Pret) Director: Clinical Services of the Pretoria Academic Hospital
Prof C.W. van Staden	MBChB; Mmed (Psych); MD; FTCL; UPLM; Dept of Psychiatry

[Signature]

PATIENT: ASSENT FORM

The investigation of the biochemical profiles of patients with possible mitochondrial disorders

We are going to explain the study to you. If you understand and want to take part, we ask you to sign the form.

WHAT DO WE WANT TO DO IN THE STUDY?

Mitochondrial disorders are diseases that may cause a problem in many parts of the body, because too little energy is made. You were born with the problem. The aim of the project is to measure if your body makes enough energy. We hope to be able to have a centre one day in South Africa where patients with these problems could be helped.

HOW LONG WILL THE STUDY BE?

This project will start in 2007 and carries on.

WHAT ARE WE GOING TO DO?

We will explain to you what we are going to do and give you time to ask your questions.

We are going to ask your parents about other people in your family and whether they are ill. We will examine your body.

If we think that you have a this possible problem, we will do some test to see if there are other parts of your body that is also ill and to see if the problem gets worse. It can be sore if we do some of the test, but then we will give you medicine. It will help you then to sleep and lie very still. It will also help to take away the pain.

Less than 15 ml blood will be taken. It may be necessary to take some of the fluid from your back. Afterwards you may have a sore head, and then you will have to lie in bed for a while. If your muscle or nerves are weak we are going to test it with a machine. It is sore for a short while. If we really do not get an answer from those tests it may be necessary to do a small operation to get a small piece of muscle. The little operation may cause bleeding, pain and leave a small mark on your e.g. leg. Your urine will also be sent to be tested in a big machine, but that is not sore.

Sometimes we need a picture of your brain. That is not painful at all you just have to lie very quiet in the machine that we call a scanner. It is like taking photos of your brain.

If you can not see or hear well, we may also test your eyes and or ears. It is not sore.

WILL ANYBODY KNOW ABOUT YOU?

Only the doctors that know you and the people in the laboratory that work with your blood, urine or muscle see the answers and your hospital number and name. If we find some answers we would like to write about it in a medical journal, but nobody that will read the article will be able to know that you took part, only the answers will be given.

WHAT HAPPENS IF YOU DO NOT WANT TO TAKE PART?

The occurrence of mitochondrial DNA polymerase gamma gene mutations in mitochondrial deficiencies in a selection of South African paediatric patients

You or your parent may at any time tell us if you do not want to take part any further at any time, but your parent must please write us a letter. We will not be cross with you and we will still carry on to treat you as good as possible.

THE ABOVE MENTIONED PROJECT WAS THOROUGHLY EXPLAINED AND THE FOLLOWING EXTRA POINTS WERE TOLD TO ME.

The blood, urine and muscle are given for research and that I will not be paid for it.

You will let us know if you find the answers.

I may decide not to take part any further in the study

WHO SHOULD WE CONTACT IF WE HAVE QUESTIONS?

Dr I. Smuts, Paediatric Neurology Unit, Department of Paediatrics and Child Health, University of Pretoria, P.O. Box 667, Pretoria, 0001.

Enquiries regarding ethical aspects of the project can be addressed to the: Chairperson of the Ethics Committee, Pretoria Academic Hospital, Private Bag X169, Pretoria, 0001.

That I will receive a copy of assent form.

DECLARATION OF ASSENT

I, _____

(Print full name and surname)

Hereby assent to take part in the project.

I understand that I take part in the research project as a **patient**.

I confirm that I understand everything.

Signed at _____ on _____

(Place)

(Date)

(Signature of a minor)

Witness 1: _____

(Signature)

Witness 2: _____

(Signature)

(Signature of responsible who explained the project and assent to the participant.)

CONTROL: ASSENT FORM

The investigation of the biochemical profiles of patients with possible mitochondrial disorders

We are going to explain the study to you. If you understand and want to take part, we ask you to sign the form.

WHAT DO WE WANT TO DO IN THE STUDY?

Mitochondrial disorders are diseases that may cause a problem in many parts of the body, because too little energy is made. It is difficult to tell when people have these diseases. One way to find out is to do special tests on their muscle and urine. The tests are very difficult. We hope to be able to have a centre one day in South Africa where patients with these problems could be helped. To be able to do so, we need muscle of patients who do not have this disease. The people in the laboratory can then compare the ill children's muscle to the healthy muscle and urine and then we can tell them what their problem is.

HOW LONG WILL THE STUDY BE?

This project will start in 2007 and carries on.

WHAT ARE WE GOING TO DO?

We will explain to you what we are going to do and give you time to ask your questions.

Your muscle is healthy, but you have a problem with your legs and the surgeons are going to operate your legs. We would like to have a tiny piece of the big muscle that the surgeon is going to cut. The people from the laboratory will then do special tests on your muscle. Next time when there is an ill patient we can compare their muscle tests to yours and then we can tell them what their problem is, although you are not going to benefit.

You are not going to feel anything, because you are going to sleep very nicely. The piece of muscle that you give is not going to make you weak later on.

We are also asking you to give us a bit of urine in a small bottle so that it can be tested.

WILL ANYBODY KNOW ABOUT YOU?

Only the doctors that know you and the people in the laboratory that work with muscle see the answers. Your hospital number and name will not be given to anybody. If we find some answers we would like to write about it in a medical journal, but nobody that will read the article will be able to know that you took part, only the answers will be given.

WHAT HAPPENS IF YOU DO NOT WANT TO TAKE PART?

You or your parent may at any time tell us if you do not want to take part any further at any time, but your parent must please write us a letter. We will not be cross with you.

THE ABOVE MENTIONED PROJECT WAS THOROUGHLY EXPLAINED AND THE FOLLOWING EXTRA POINTS WERE TOLD TO ME.

The muscle and urine are given for research and that I will not be paid for it.

The occurrence of mitochondrial DNA polymerase gamma gene mutations in mitochondrial deficiencies in a selection of South African paediatric patients

I may decide if I do not want to participate anymore.

WHO SHOULD WE CONTACT IF WE HAVE QUESTIONS?

Dr I. Smuts, Paediatric Neurology Unit, Department of Paediatrics and Child Health, University of Pretoria, P.O. Box 667, Pretoria, 0001.

Enquiries regarding ethical aspects of the project can be addressed to the: Chairperson of the Ethics Committee, Pretoria Academic Hospital, Private Bag X169, Pretoria, 0001.

That I will receive a copy of assent form.

DECLARATION OF ASSENT

I, _____

(Print full name and surname)

Hereby assent to take part in the project as a control.

I confirm that I understand everything.

Signed at: _____ **on** _____

(Place)

(Date)

(Signature of a minor)

Witness 1: _____ **Witness 2:** _____

(Signature)

(Signature)

(Signature of responsible who explained the project and assent to the participant.)

PATIENTS: INFORMED CONSENT FORM

The investigation of the biochemical profiles of patients with possible mitochondrial disorders

AIM OF THE STUDY

Mitochondrial disorders are a group of disorders that may affect the brain, muscle and several other organs, because a defect exists in the energy production of the cell. It is caused by a genetic defect. The aim of the project is to measure respiratory function in your child's muscle in conjunction with the enzyme activity of the energy producing system as your child is identified as having a possible mitochondrial disorder. The urine will also be examined for chemical compounds that may be excreted in the urine if such a disorder is present. The long-term goal of this project is to establish a diagnostic service in which mitochondrial disorders could be diagnosed properly in South Africa.

EXPECTED DURATION OF THE PROJECT

This project will initially include patients from 2007 and evolve into a long term project.

PROCEDURES FOLLOWED DURING THIS PROJECT

The study will be explained to you and you will be allowed time to ask questions and decide whether you would prefer to give consent for your child to take part.

A family history will be taken and a complete physical examination will be performed.

In the case of a patient with a suspected disorder appropriate special investigations will be performed to confirm the diagnosis of a mitochondrial disorder, to determine involvement of other organ systems and to monitor the course of the disease.

Not more than 15 ml of blood will be taken, depending on the age of the child. This blood will be used for appropriate biochemical and haematological investigations and a small volume will be stored away to do genetic studies when an appropriate test comes available. If it would be applicable in future, you will be contacted again for a follow up blood sample to establish cell lines to ensure a continuous supply of DNA for further analyses.

In some instances where the nervous system symptoms are unexplained it will be necessary to do a lumbar puncture. It implies that fluid surrounding the brain will be obtained through a thin needle in the back. A maximum of 5 ml cerebrospinal fluid will be needed.

Nerve conduction studies and electromyography in which the electrical functioning of the nerves and the muscle will only be done if it has not been done before and it seems to be affected.

A muscle biopsy will be done in some cases if the blood tests were inconclusive. The muscle biopsy will be taken under general anaesthetics by a qualified paediatric surgeon. A 3-4 cm incision will be made on the thigh and a small piece of muscle about 1 cubic cm will be removed for the biochemical analyses and an additional smaller piece of muscle will be taken for histology. The wound will be sutured. The muscle biopsy taken for biochemical analyses will be processed and the excess solution will be frozen for possible future use if better biochemical or genetic tests come available.

A specialised type of X-Ray, e.g. computerised tomography (CT) scan or magnetic resonance imaging (MRI) of the brain will be done to obtain a photographic picture only if it has not been done before and your doctor is of opinion it might be helpful to clarify some of the symptoms and signs.

A formal hearing and/or vision test will be performed if deafness or visual impairment is suspected.

RISKS AND POSSIBLE DISCOMFORT FOR THE PATIENT / INDIVIDUAL

Blood sampling is causing mild pain, but the risks are minimal.

A lumbar puncture will cause moderate pain and discoloration at the puncture site. The patient may suffer from a headache afterwards.

The EMG and nerve conduction study may cause pain, but the greatest care will be taken to assure the comfort of the patient as far as possible.

The muscle biopsy has the following risk factors: possible bleeding, discomfort including pain, possible scarring, and a remote risk of infection.

DECLARATION OF CONFIDENTIALITY

All the Information provided for the purpose of this study will be treated as highly confidential. Only individuals of the research group and officials, who are entitled to it by law, will have access to information. Data published in a medical journal will include no information that could identify a patient or his/her family.

WITHDRAWAL CLAUSE

I understand that I am entitled to withdraw from the project at any time without penalty or loss of benefits. The participation of my child, in this project is therefore on a voluntary basis until I request withdrawal in writing.

THE ABOVE MENTIONED PROJECT WAS THOROUGHLY EXPLAINED AND THE FOLLOWING ADDITIONAL INFORMATION POINTED OUT TO ME

- That the entire blood sample and muscle biopsy is used for research purposes and no compensation for this will be provided.
- That I will be kept informed of significant developments in the project.
- That the withdrawal clause was explained to me and that I understand its implications.

ENQUIRIES ABOUT THIS PROJECT SHOULD BE DIRECTED TO:

- General enquiries should be directed to: Dr I. Smuts, Paediatric Neurology Unit, Department of Paediatrics and Child Health, University of Pretoria, P.O. Box 667, Pretoria, 0001.
- Enquiries regarding ethical aspects of the project can be addressed to: The Chairperson of the Ethics Committee, Pretoria Academic Hospital, Private Bag X169, Pretoria, 0001.

That I will receive a copy of this informed consent form.

DECLARATION OF CONSENT

I, _____

(Print full name and surname)

Hereby consent to the participation of my child,

The occurrence of mitochondrial DNA polymerase gamma gene mutations in mitochondrial deficiencies in a selection of South African paediatric patients

_____ in the above-mentioned project.

(Print full name and surname or stick a sticker)

I confirm that I am fully aware of the content of this form and that by signing it I give the necessary consent.

Signed at: _____ on _____

(Place)

(Date)

(Signature of a parent/guardian)

Witness 1: _____

(Signature)

Witness 2: _____

(Signature)

(Signature of responsible who explained the project and informed consent from to the participant.)

CONTROL: INFORMED CONSENT FORM

The investigation of the biochemical profiles of patients with possible mitochondrial disorders

AIM OF THE STUDY

Mitochondrial disorders are a group of disorders that may affect the brain, muscle and several other organs, because a defect exists in the energy production of the cell. It is caused by a genetic defect. We are currently doing a project to measure respiratory function in muscle in conjunction with the enzyme activity of the energy producing system. It is absolutely necessary to be able to compare the values that we get of the ill children to the values of normal children. We can not use values of children elsewhere in the world as every laboratory has specific conditions and has to have a set of controls. **As your child is not affected by this condition** and has to go to theatre for an operation on his legs we would like to ask your consent to have a small piece of muscle and a urine specimen. The long-term goal of this project is to establish a diagnostic service in which mitochondrial disorders could be diagnosed properly in South Africa.

EXPECTED DURATION OF THE PROJECT

This project will initially include patients and controls from 2007 and evolve into a long term project.

PROCEDURES FOLLOWED DURING THIS PROJECT

The study will be explained to you and you will be allowed time to ask questions and decide whether you would prefer to give consent for your child to take part.

A family history will be taken and a complete physical examination will be performed to confirm that it is highly unlikely that your child has a mitochondrial disorder.

A small piece of muscle about one cubic centimetre will be removed under general anaesthetics from an appropriate muscle group while your child is being operated on for the planned orthopaedic operation on his legs to correct the abnormal rotation or angle. It is not an additional surgical procedure and it will not affect your child neither will he benefit directly from the process.. This muscle sample will be used for biochemical analyses and the measurement of respiration. The excess solution derived from the analyses in the laboratory will be frozen and might be used in future if more biochemical or genetic analyses come available in order to improve the quality of the analyses and diagnosis.

Additional we ask for 20 ml of urine as well that will be collected in a sterile bottle and frozen.

OR in the scenario of children not being operated on and only recruited for urine control specimens for the purpose of the metabolomics no muscle biopsy will be taken.

RISKS AND POSSIBLE DISCOMFORT FOR THE PATIENT / INDIVIDUAL

The risks of anaesthetic and the planned operation as explained to you in the consent process of the operation by your orthopaedic surgeon are applicable, but there are no additional risks by having the muscle biopsy done. The collection of the urine is also not invasive or harmful to your child.

DECLARATION OF CONFIDENTIALITY

All the Information provided for the purpose of this study will be treated as highly confidential. Only individuals of the research group and officials, who are entitled to it by law, will have access to information. Data published in a medical journal will include no information that could identify a child or his/her family.

WITHDRAWAL CLAUSE

I understand that I am entitled to withdraw from the project at any time without penalty or loss of benefits. The participation of my child, in this project is therefore on a voluntary basis until I request withdrawal in writing.

THE ABOVE MENTIONED PROJECT WAS THOROUGHLY EXPLAINED AND THE FOLLOWING ADDITIONAL INFORMATION POINTED OUT TO ME

- That the entire muscle biopsy and urine specimen are used for research purposes and no compensation for this will be provided.
- That I will be kept informed of significant developments in the project.
- That the withdrawal clause was explained to me and that I understand its implications.

ENQUIRIES ABOUT THIS PROJECT SHOULD BE DIRECTED TO:

- General enquiries should be directed to: Dr I. Smuts, Paediatric Neurology Unit, Department of Paediatrics and Child Health, University of Pretoria, P.O. Box 667, Pretoria, 0001.
- Enquiries regarding ethical aspects of the project can be addressed to: The Chairperson of the Ethics Committee, Pretoria Academic Hospital, Private Bag X169, Pretoria, 0001.

That I will receive a copy of this informed consent form.

DECLARATION OF CONSENT

I, _____

(Print full name and surname)

Hereby consent to the participation of my child,

_____ in the above-mentioned project.

(Print full name and surname or stick a sticker)

I confirm that I am fully aware of the content of this form and that by signing it I give the necessary consent.

Signed at: _____ on _____.

(Place)

(Date)

(Signature of a parent/guardian)

Witness 1: _____

(Signature)

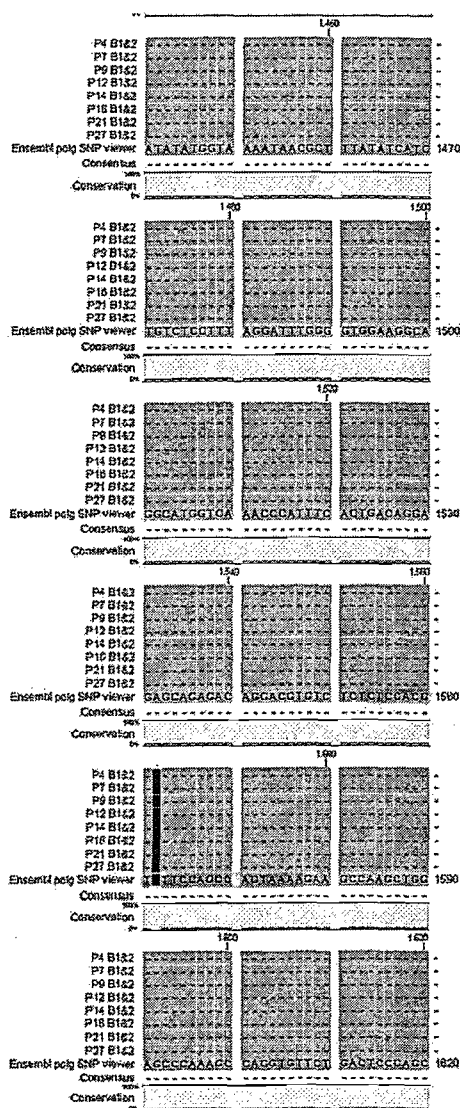
Witness 2: _____

(Signature)

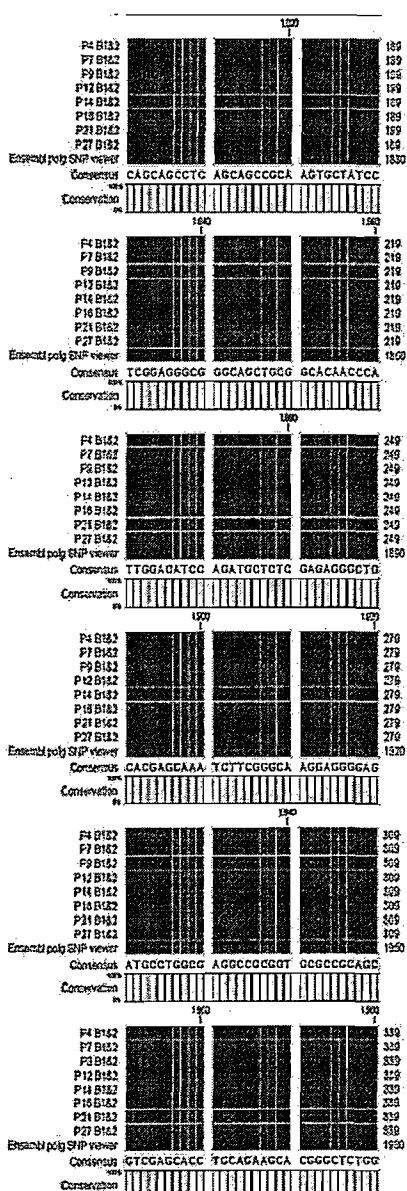
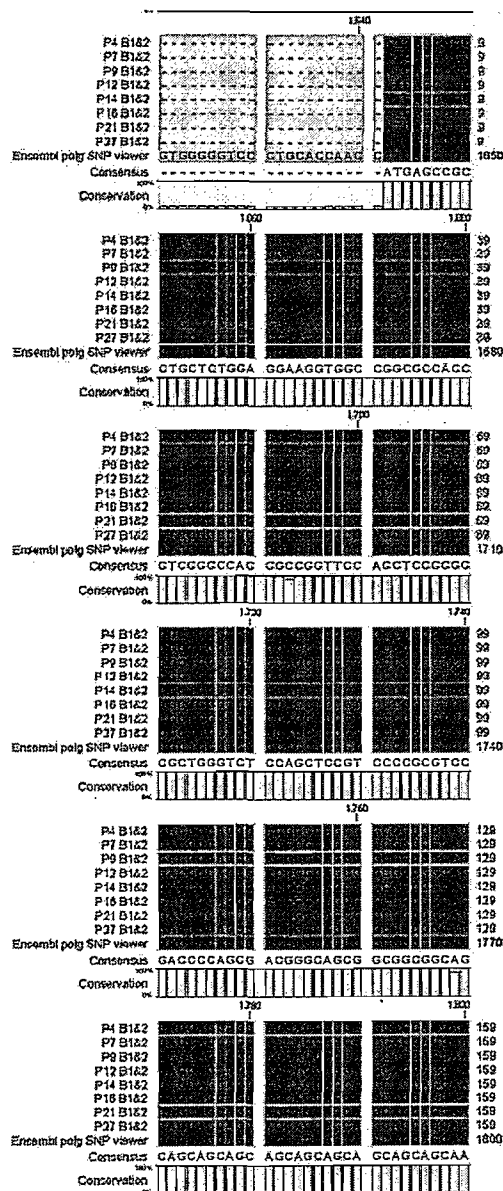
(Signature of responsible who explained the project and informed consent from to the participant.)

7.3 Annexure C: TPG sequence alignments of POLG1 exons 2-23 with corresponding primers.

Note: Only areas B1-A8 are illustrated and primers for these areas are also indicated. Each area is started with the first base pair of the corresponding exon and also ended with the last base pair of the corresponding exon. Intronic regions in between exons are included in the sequence alignment.



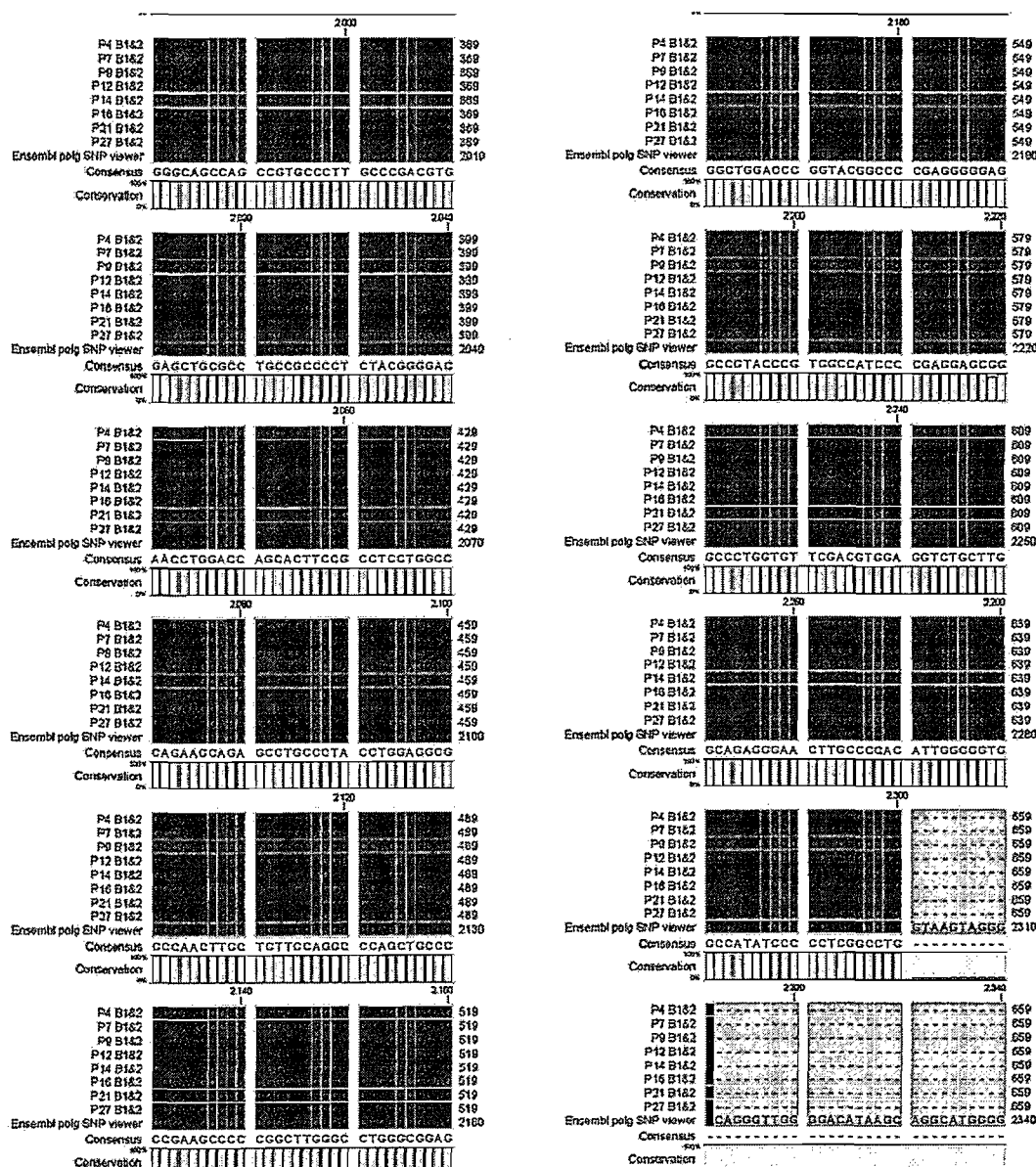
The occurrence of mitochondrial DNA polymerase gamma gene mutations in mitochondrial deficiencies in a selection of South African paediatric patients



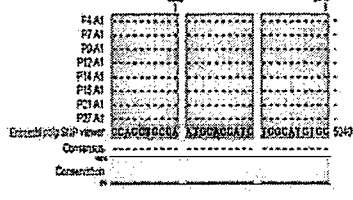
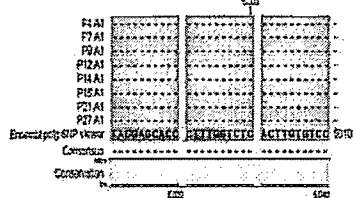
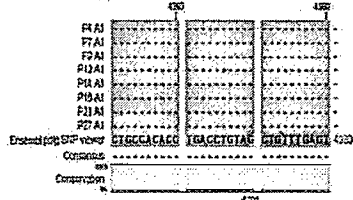
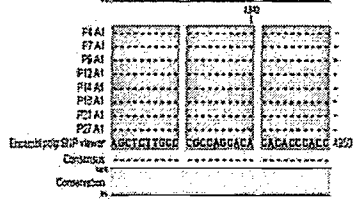
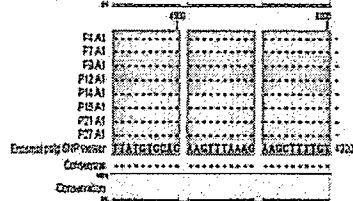
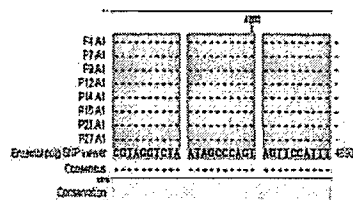
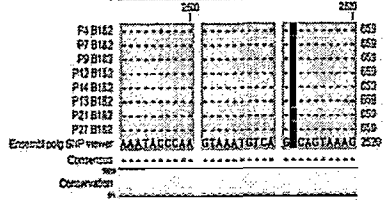
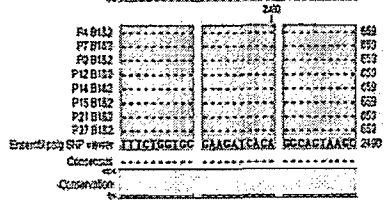
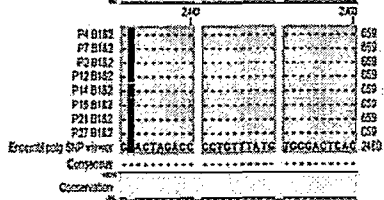
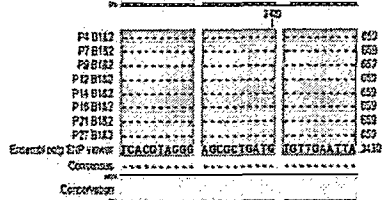
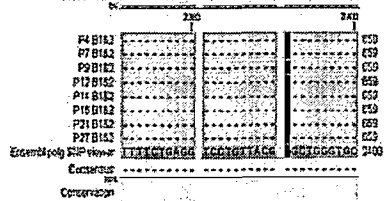
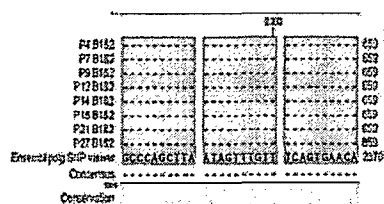
Forward Primer: B2

Reverse Primer: B1

The occurrence of mitochondrial DNA polymerase gamma gene mutations in mitochondrial deficiencies in a selection of South African paediatric patients



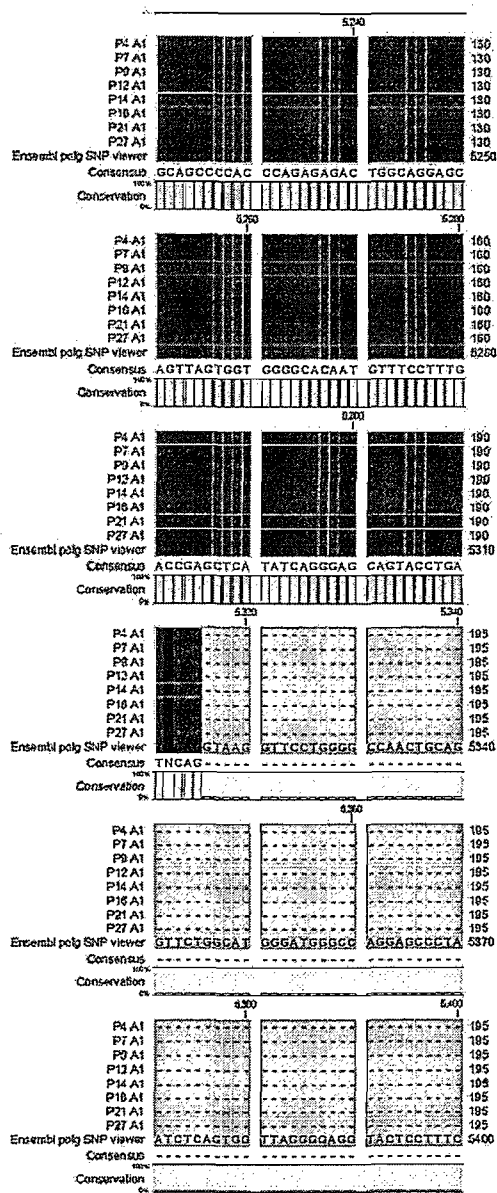
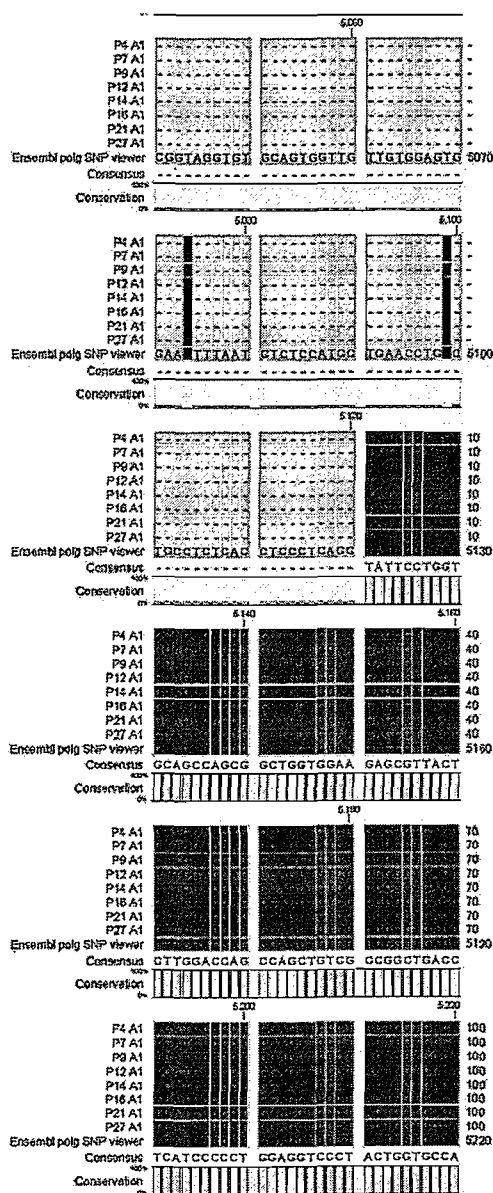
The occurrence of mitochondrial DNA polymerase gamma gene mutations in mitochondrial deficiencies in a selection of South African paediatric patients



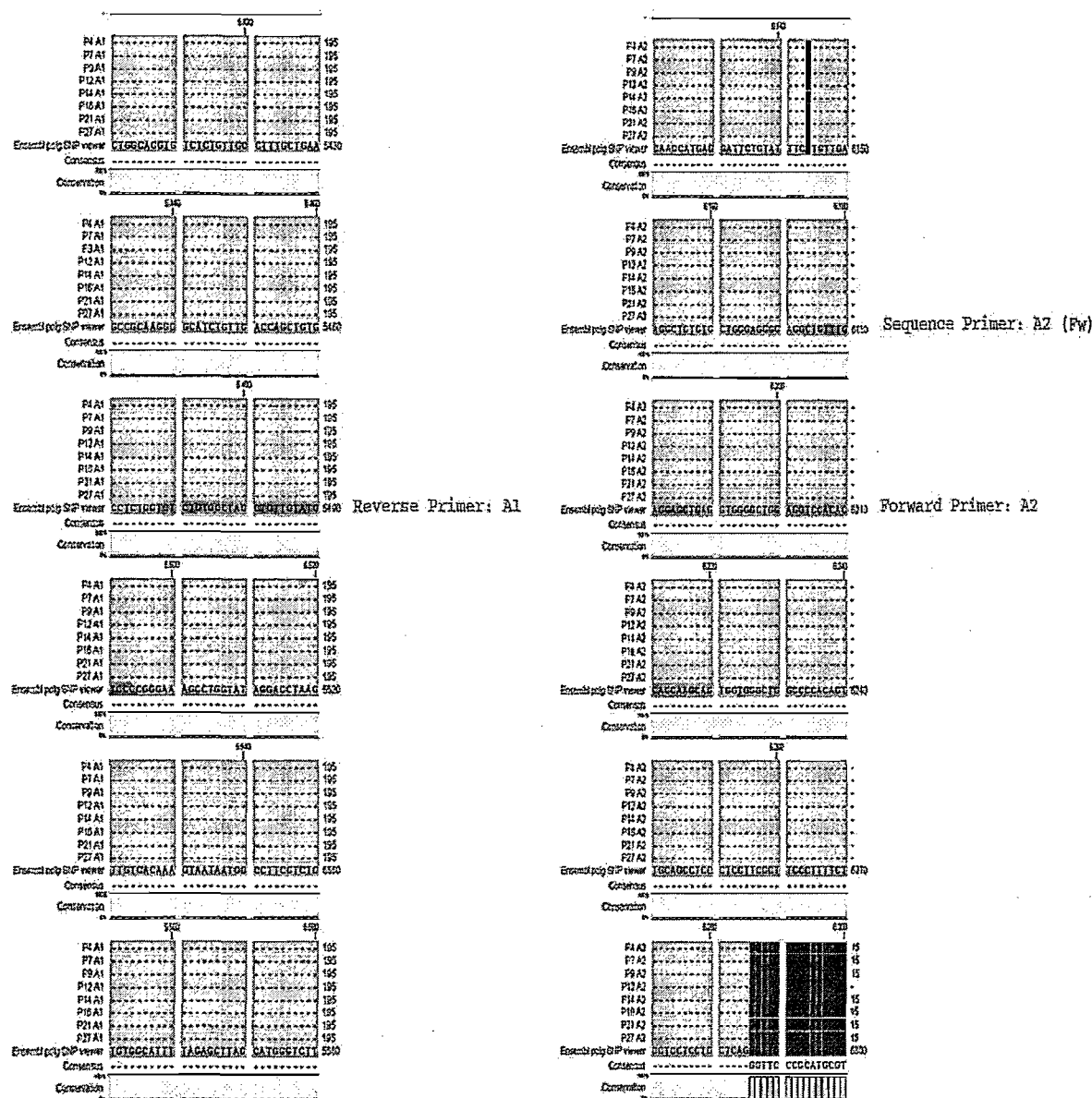
Forward Primer: A1

Sequence Primer: A1

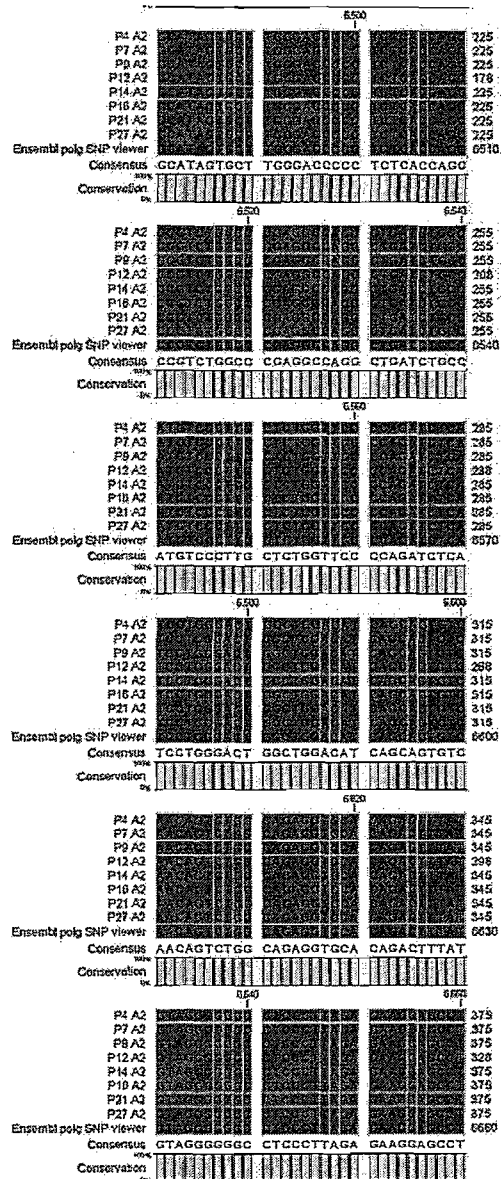
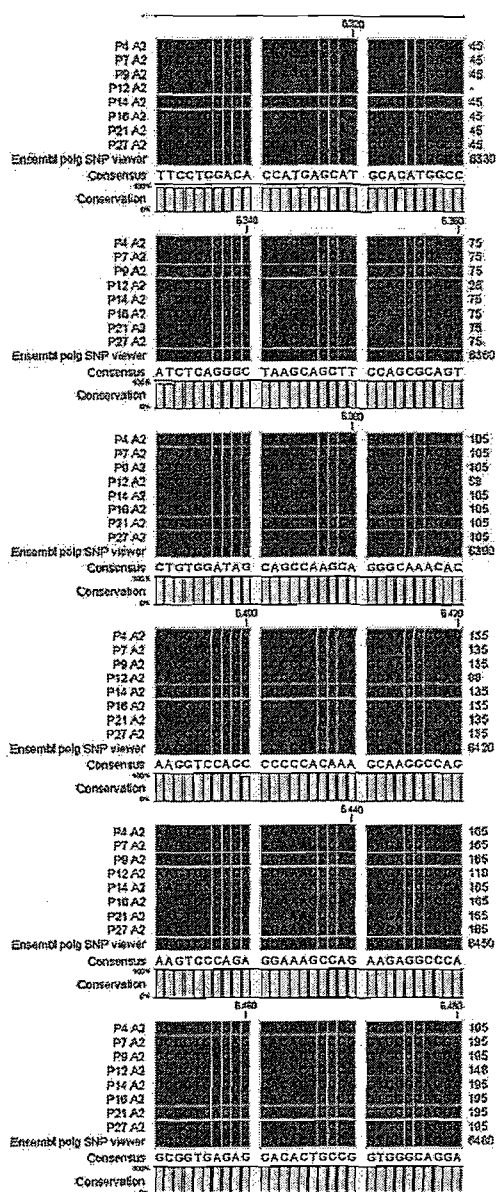
The occurrence of mitochondrial DNA polymerase gamma gene mutations in mitochondrial deficiencies in a selection of South African paediatric patients



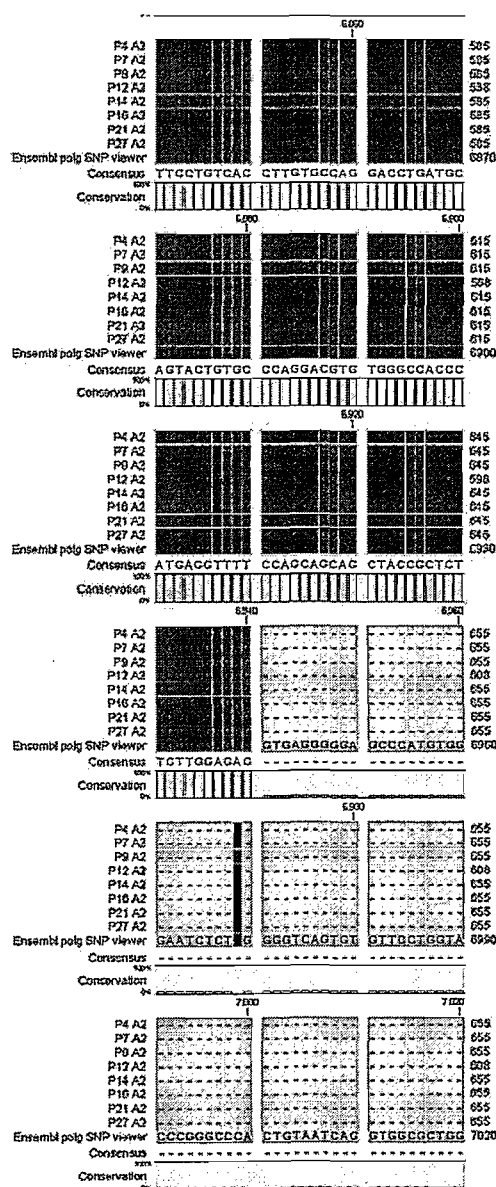
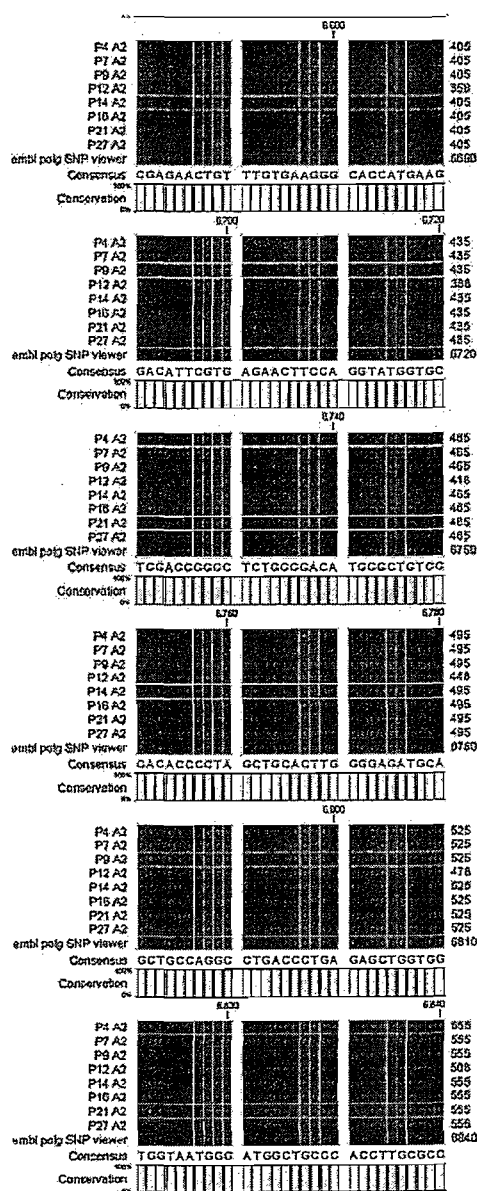
The occurrence of mitochondrial DNA polymerase gamma gene mutations in mitochondrial deficiencies in a selection of South African paediatric patients



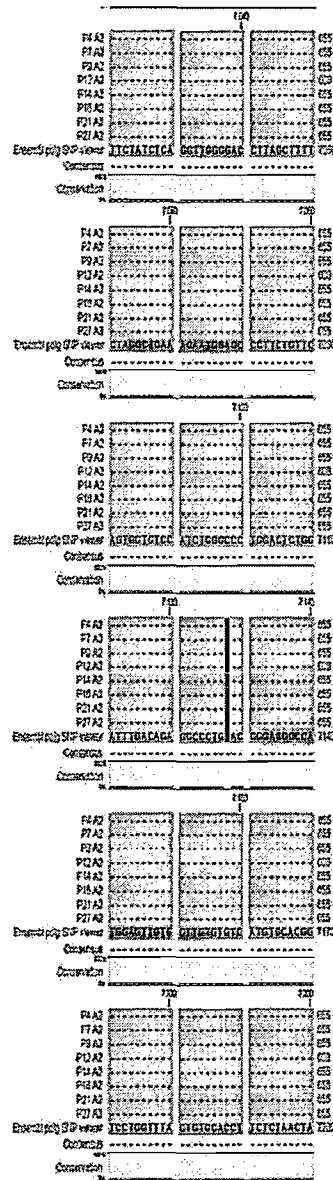
The occurrence of mitochondrial DNA polymerase gamma gene mutations in mitochondrial deficiencies in a selection of South African paediatric patients



The occurrence of mitochondrial DNA polymerase gamma gene mutations in mitochondrial deficiencies in a selection of South African paediatric patients

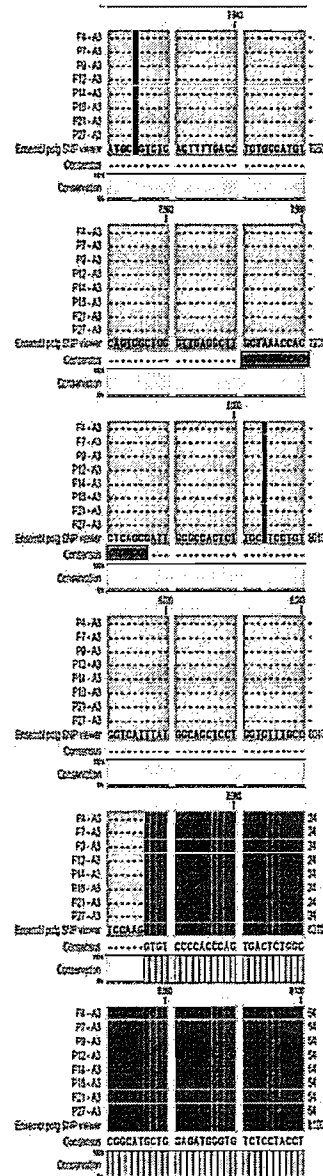


The occurrence of mitochondrial DNA polymerase gamma gene mutations in mitochondrial deficiencies in a selection of South African paediatric patients



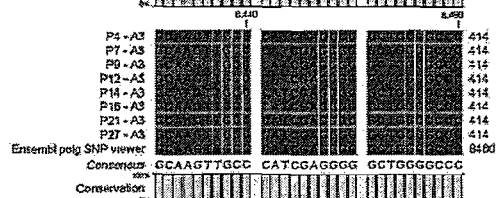
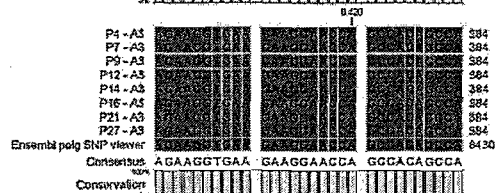
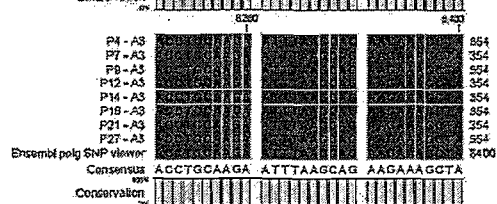
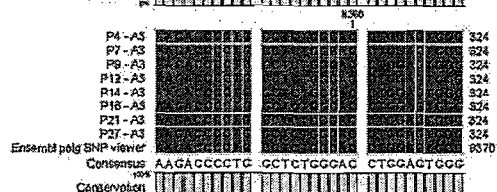
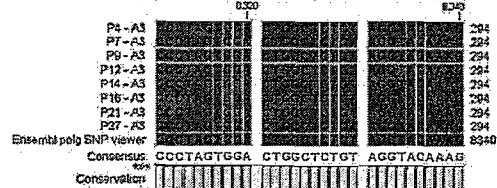
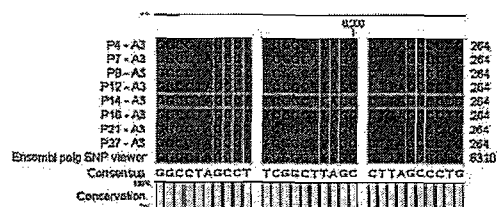
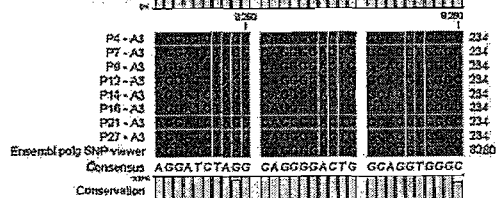
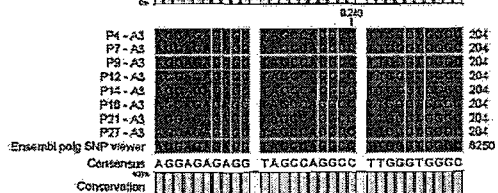
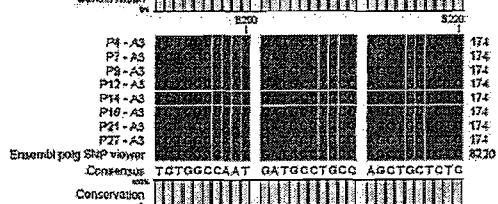
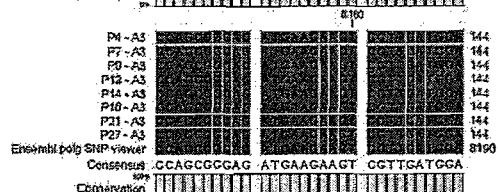
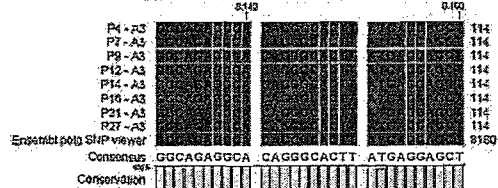
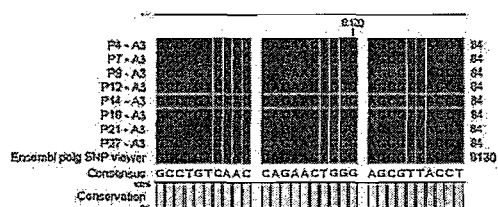
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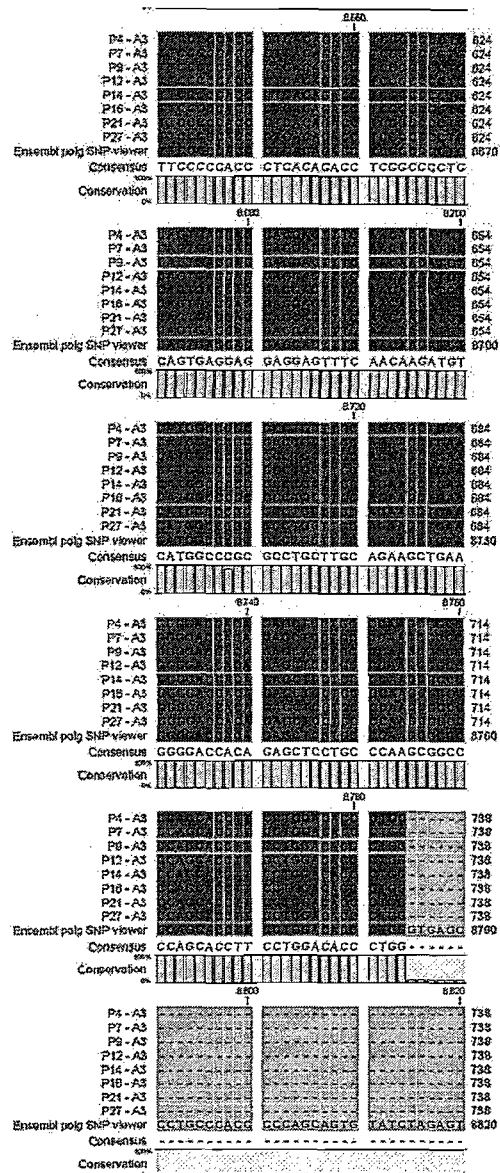
Reverse Primer: A2



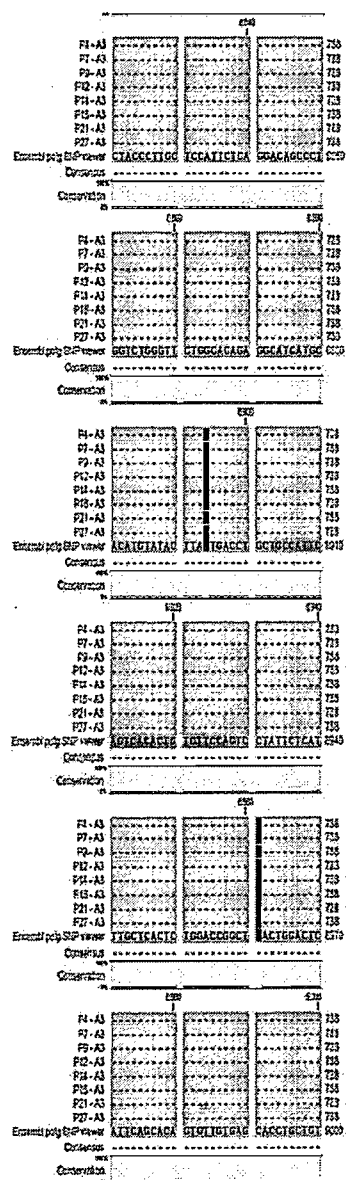
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Sequence Primer: A3 (Fw)

The occurrence of mitochondrial DNA polymerase gamma gene mutations in mitochondrial deficiencies in a selection of South African paediatric patients

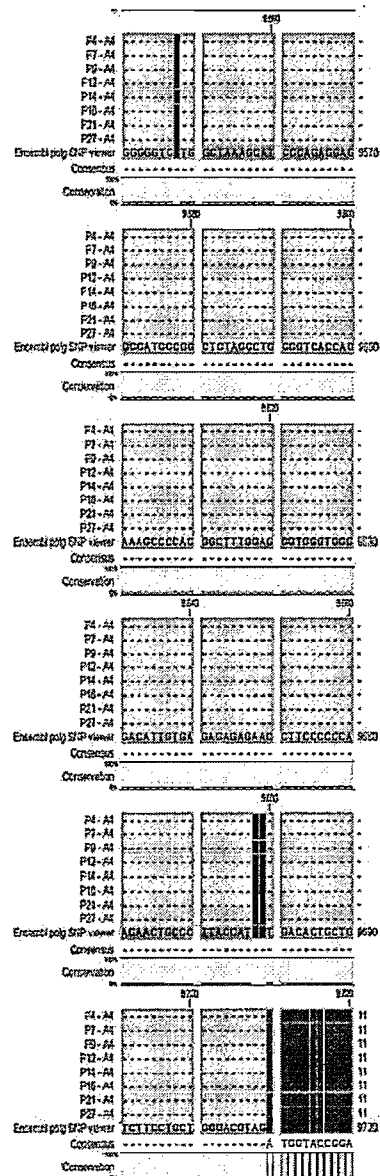




The occurrence of mitochondrial DNA polymerase gamma gene mutations in mitochondrial deficiencies in a selection of South African paediatric patients



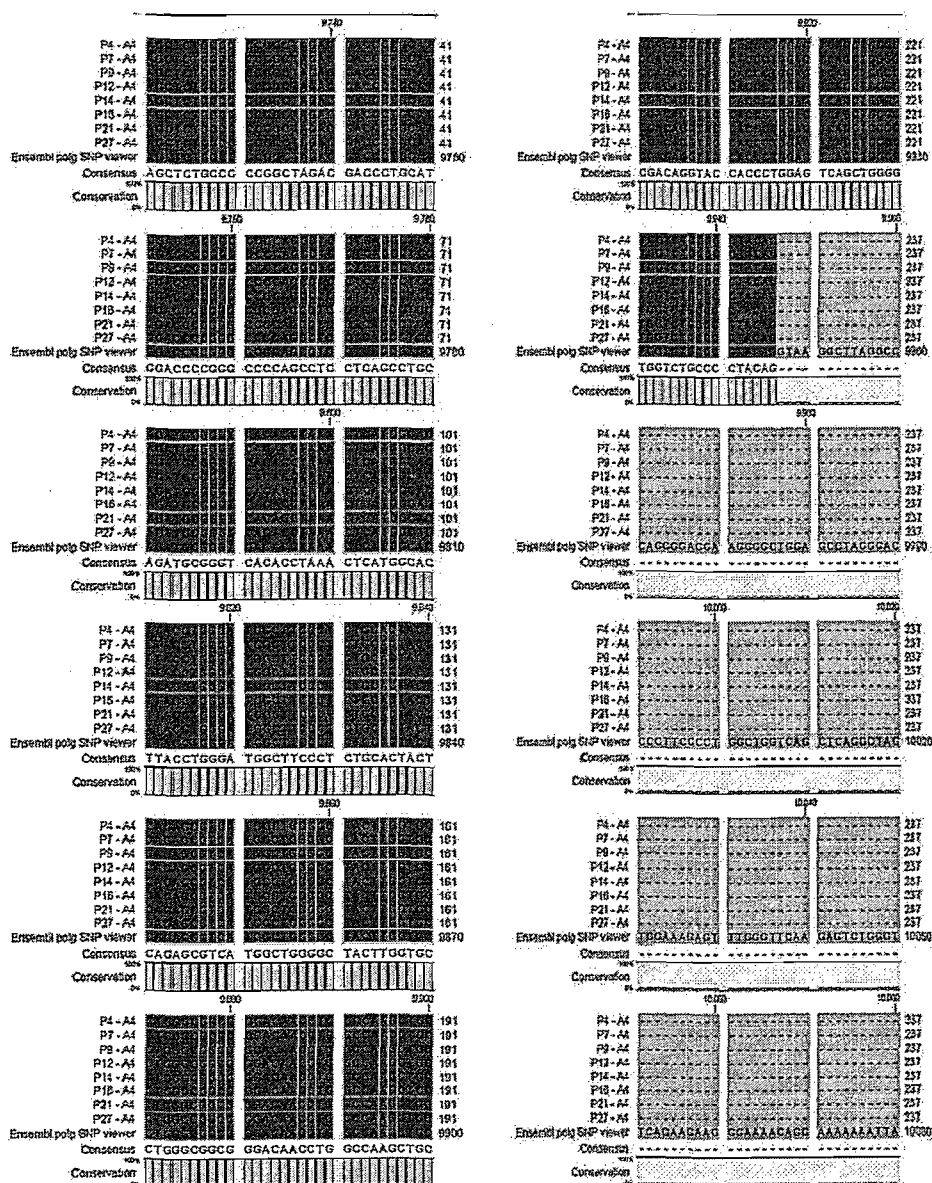
Reverse Primer: A3



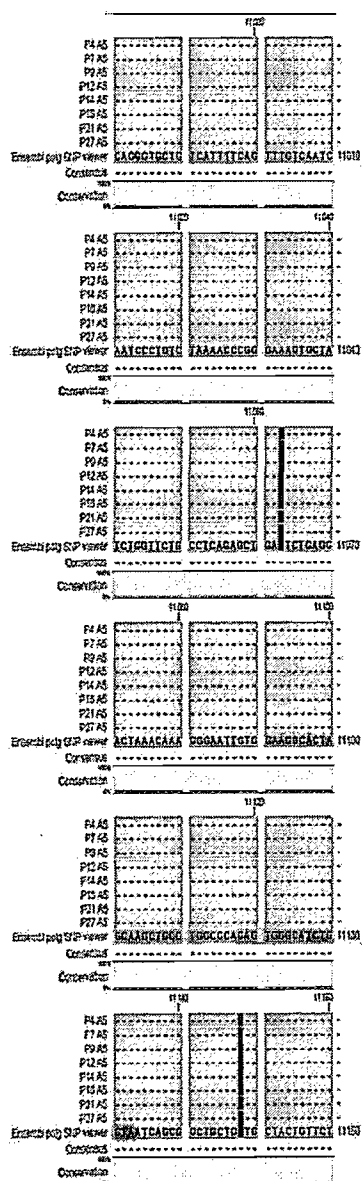
Forward Primer: A4

Sequence Primer: A4

The occurrence of mitochondrial DNA polymerase gamma gene mutations in mitochondrial deficiencies in a selection of South African paediatric patients

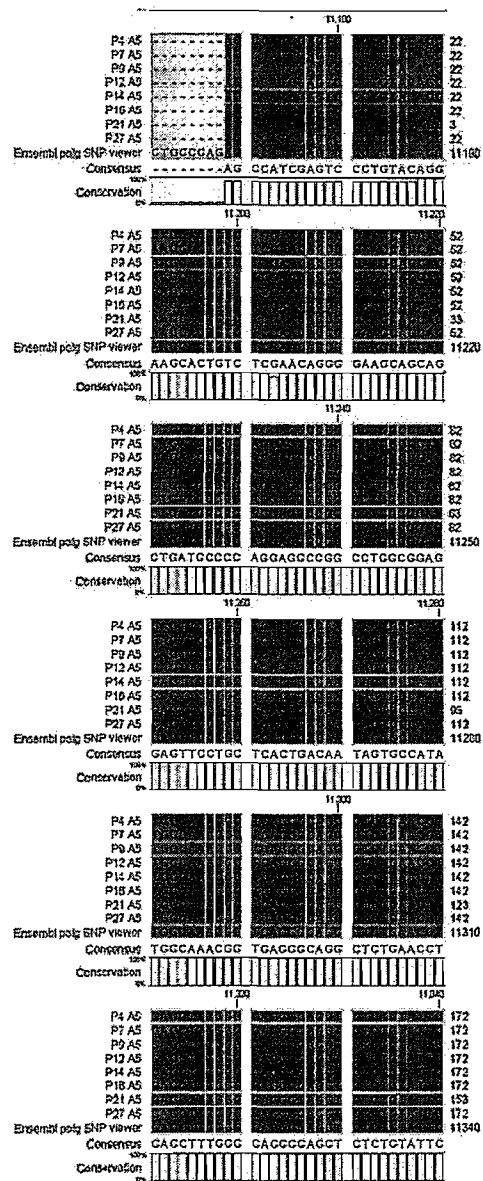


The occurrence of mitochondrial DNA polymerase gamma gene mutations in mitochondrial deficiencies in a selection of South African paediatric patients

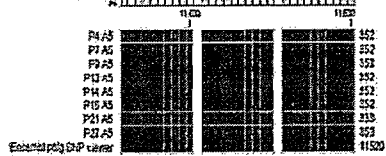
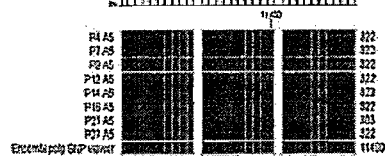
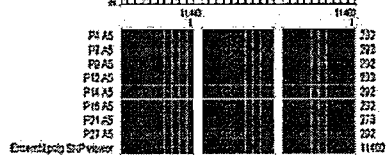
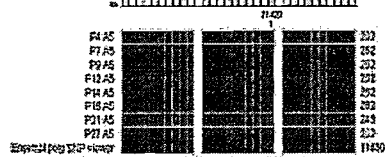
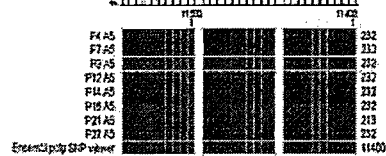
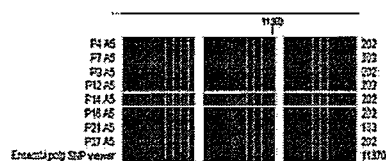


Forward Primer: A5

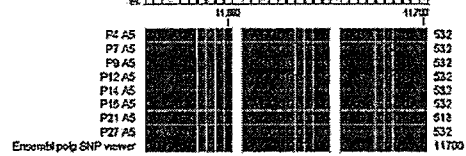
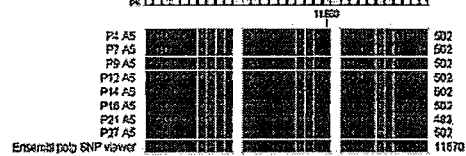
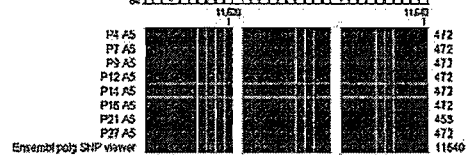
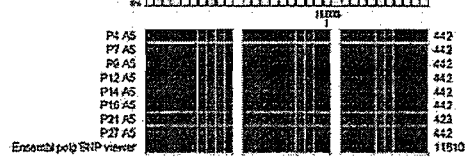
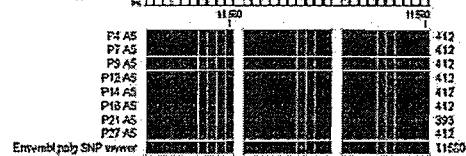
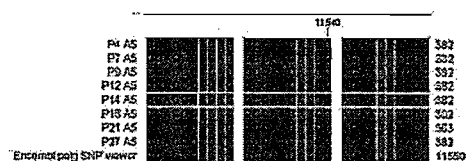
Sequence Primer: A5 (Fw)



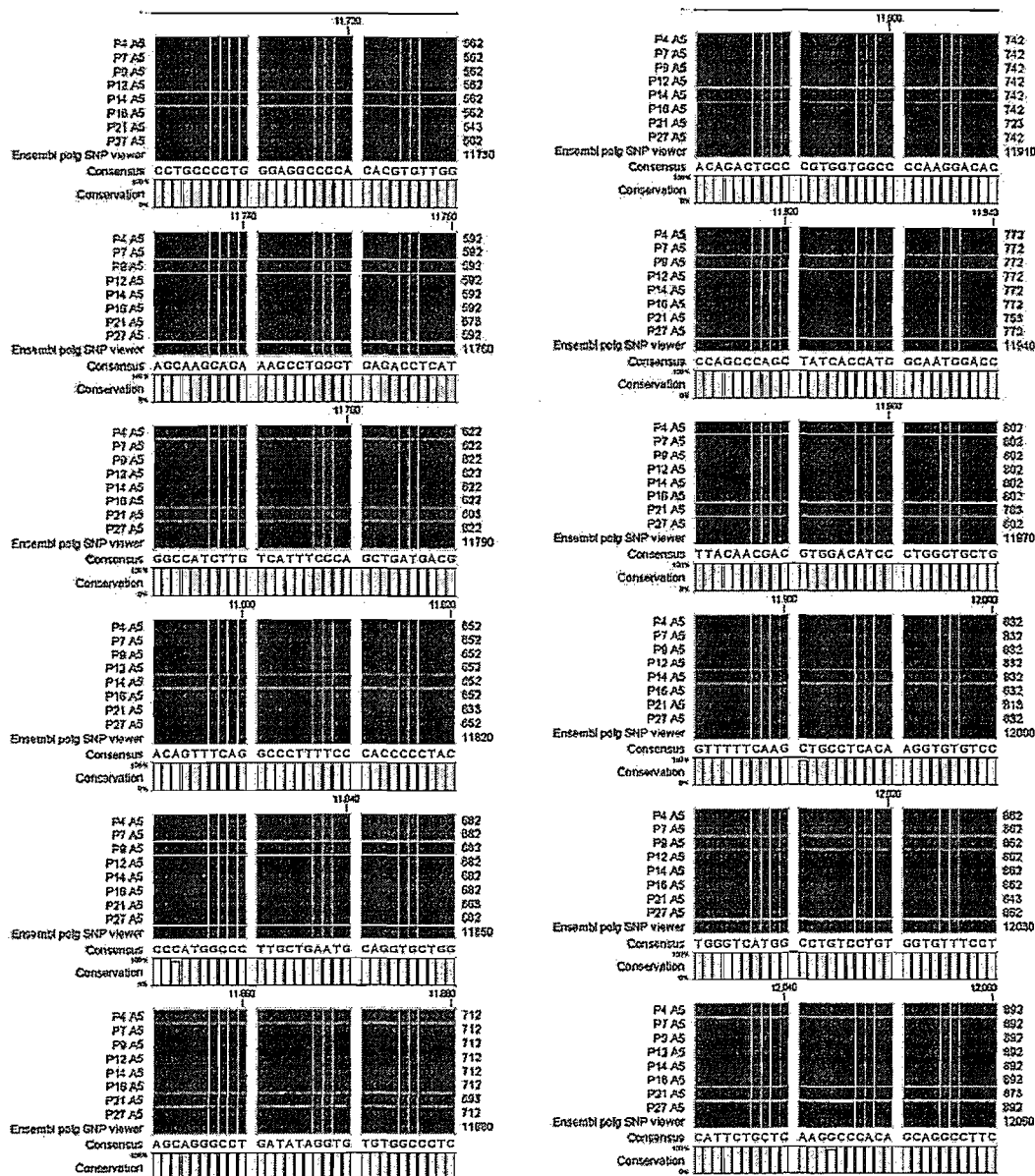
The occurrence of mitochondrial DNA polymerase gamma gene mutations in mitochondrial deficiencies in a selection of South African paediatric patients



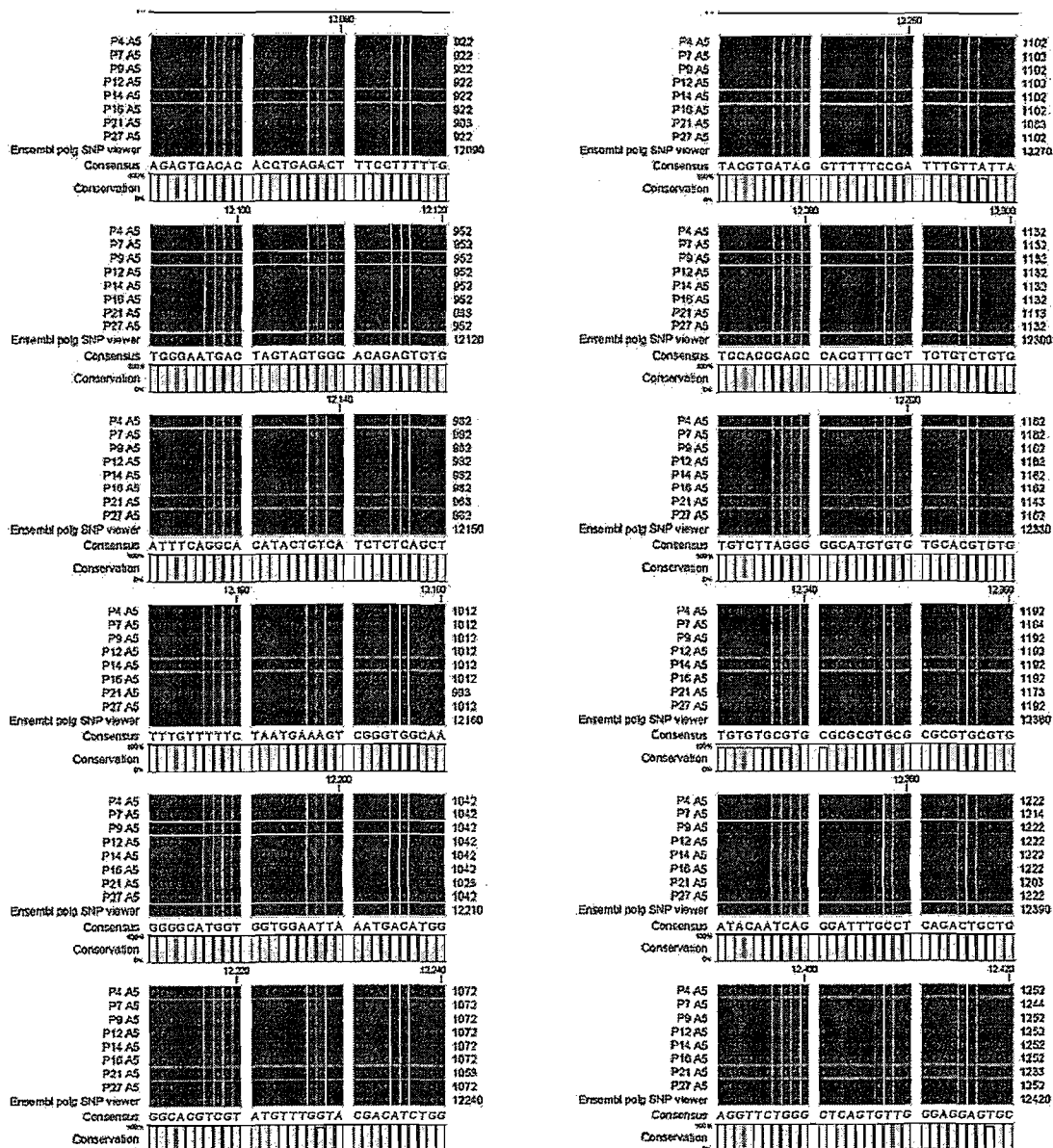
Sequence Primer: A5 (Fw2)



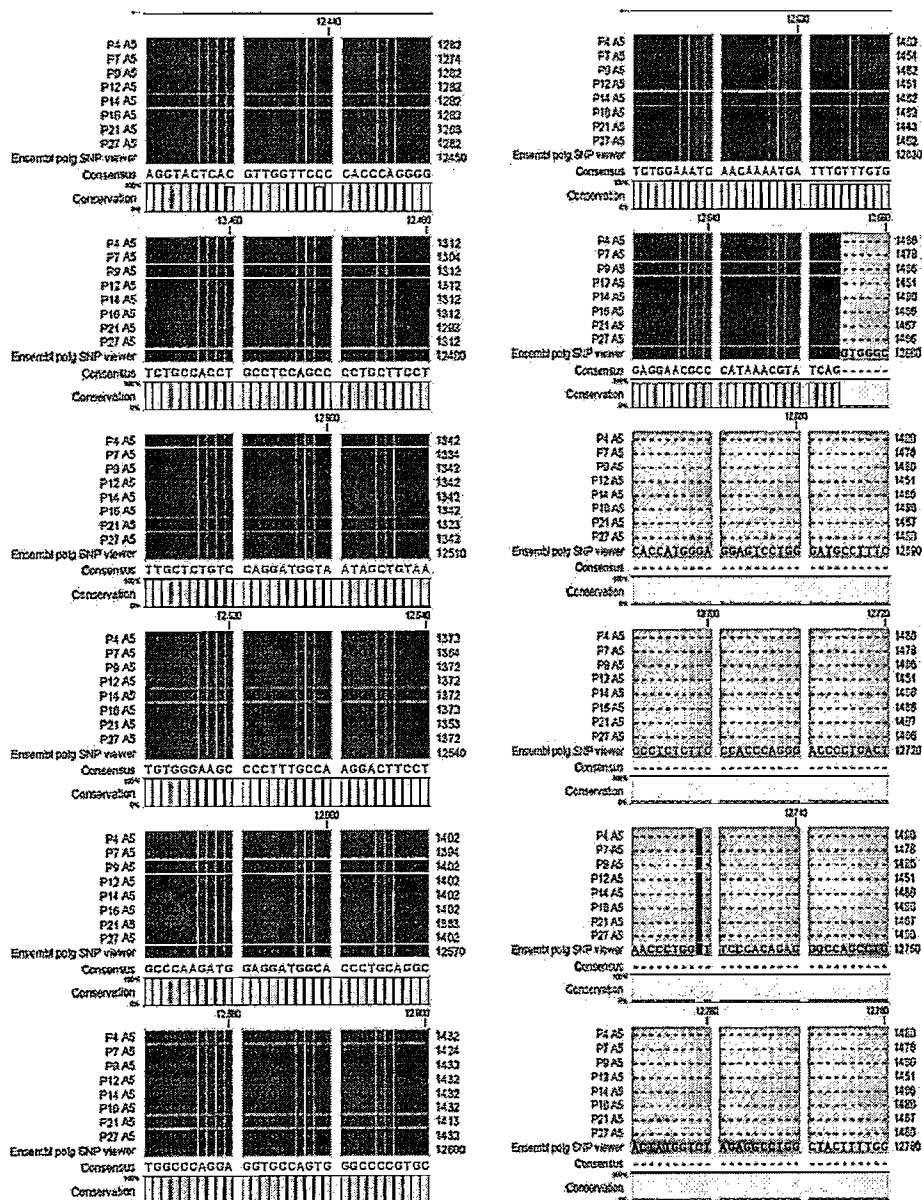
The occurrence of mitochondrial DNA polymerase gamma gene mutations in mitochondrial deficiencies in a selection of South African paediatric patients



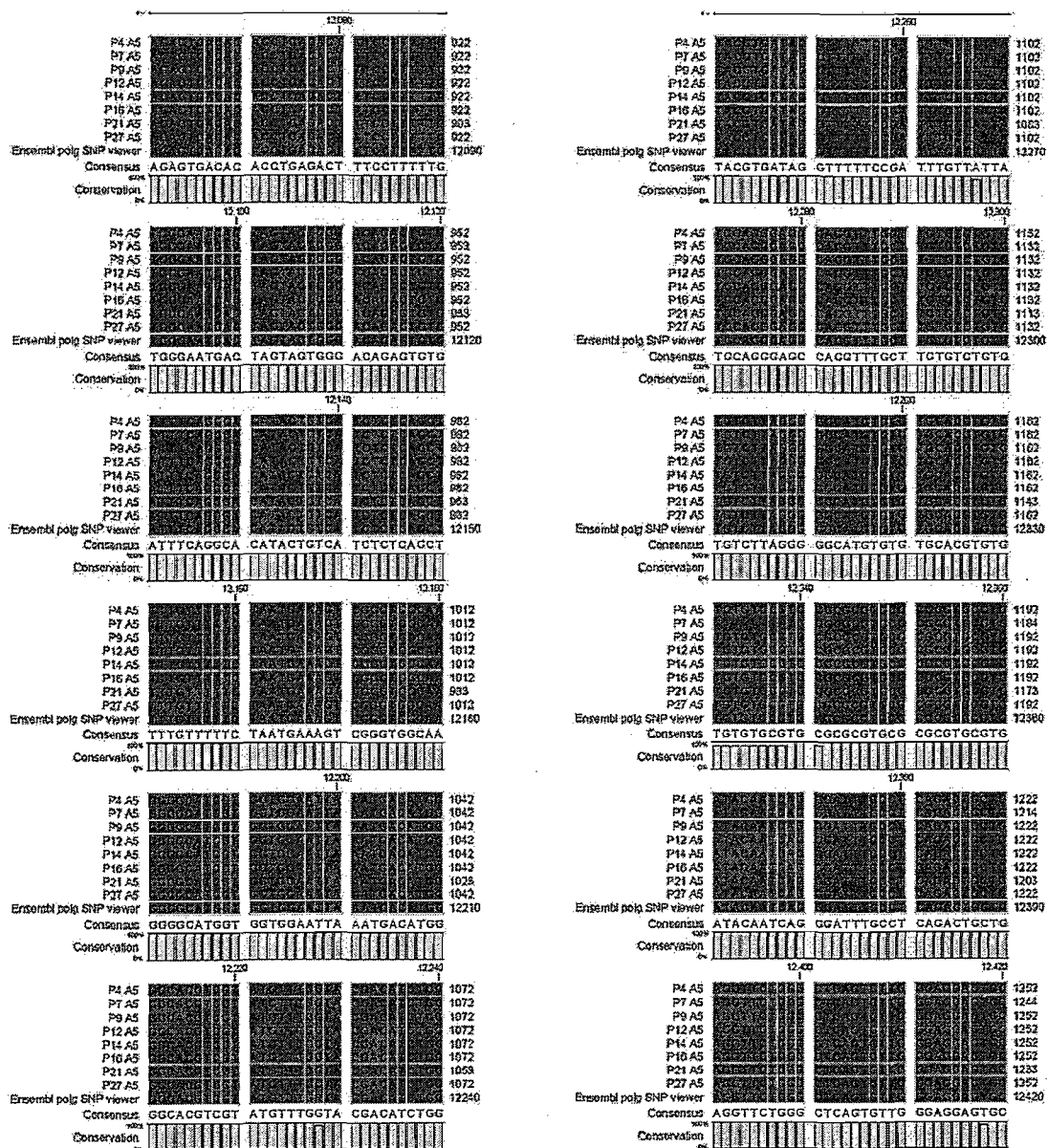
The occurrence of mitochondrial DNA polymerase gamma gene mutations in mitochondrial deficiencies in a selection of South African paediatric patients



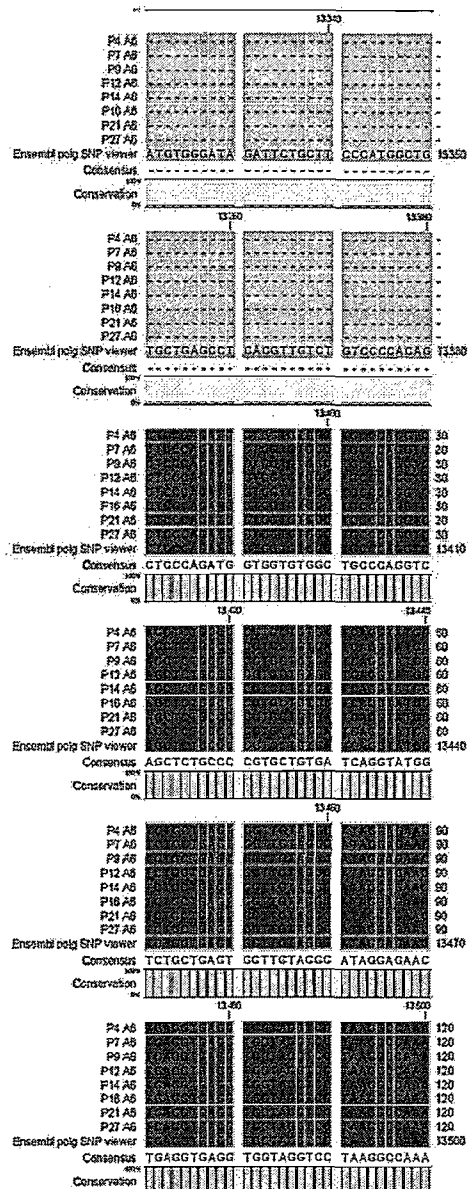
The occurrence of mitochondrial DNA polymerase gamma gene mutations in mitochondrial deficiencies in a selection of South African paediatric patients



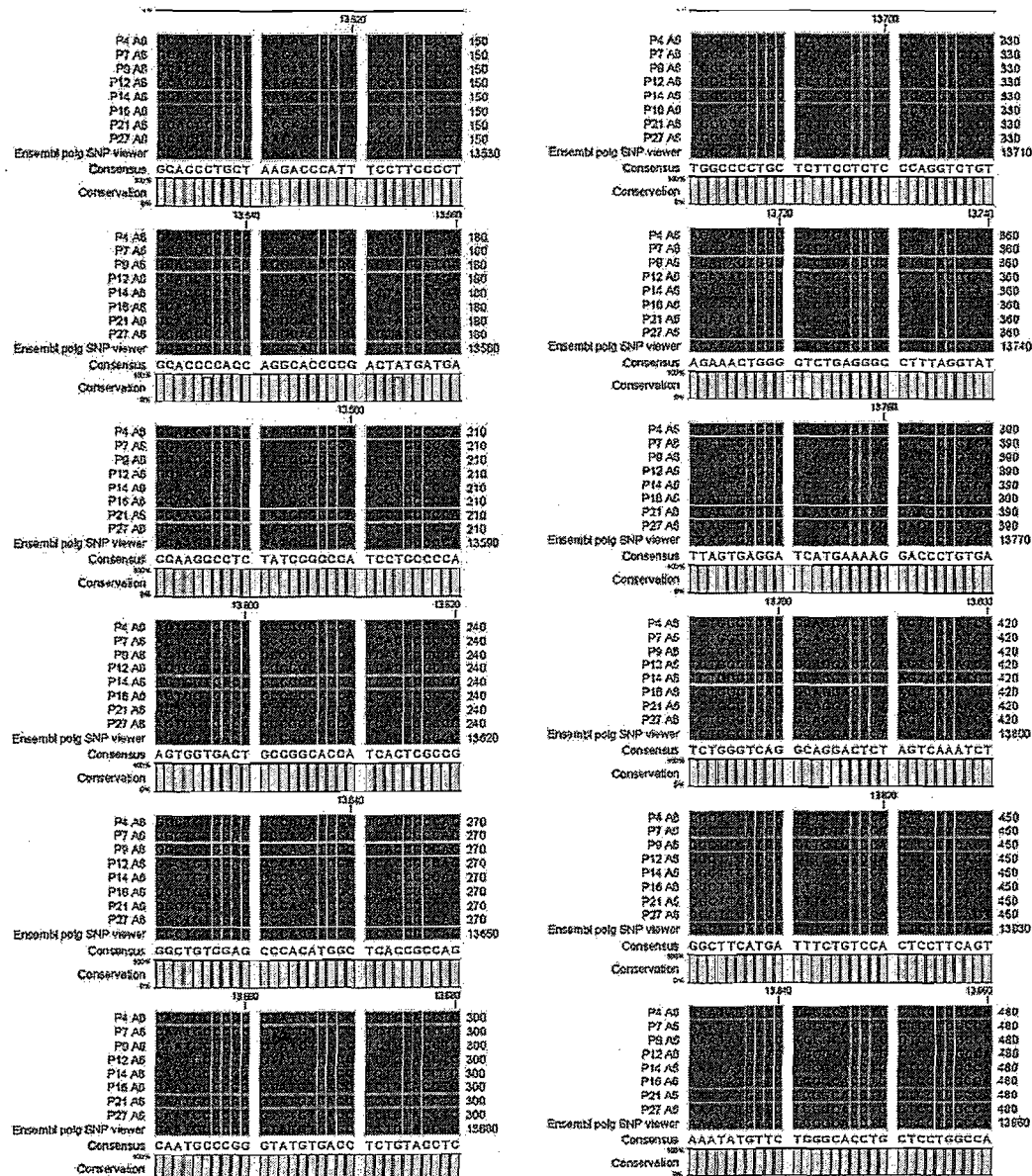
The occurrence of mitochondrial DNA polymerase gamma gene mutations in mitochondrial deficiencies in a selection of South African paediatric patients



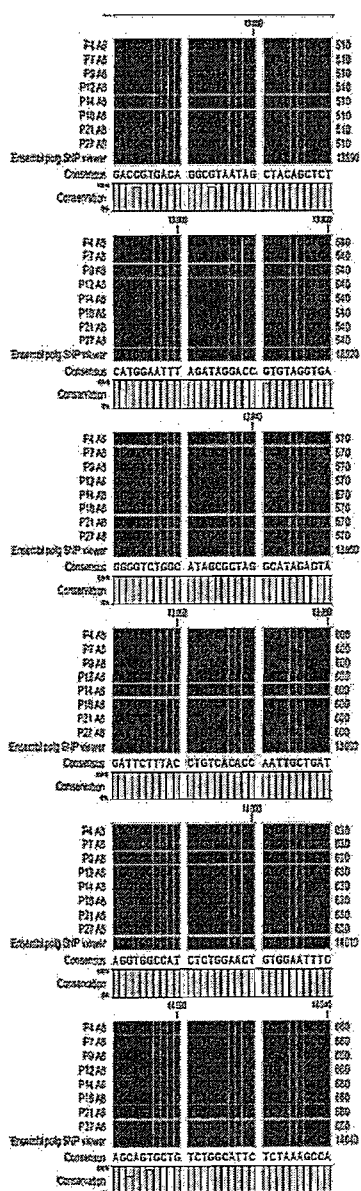
Sequence Primer: AG (Fw)



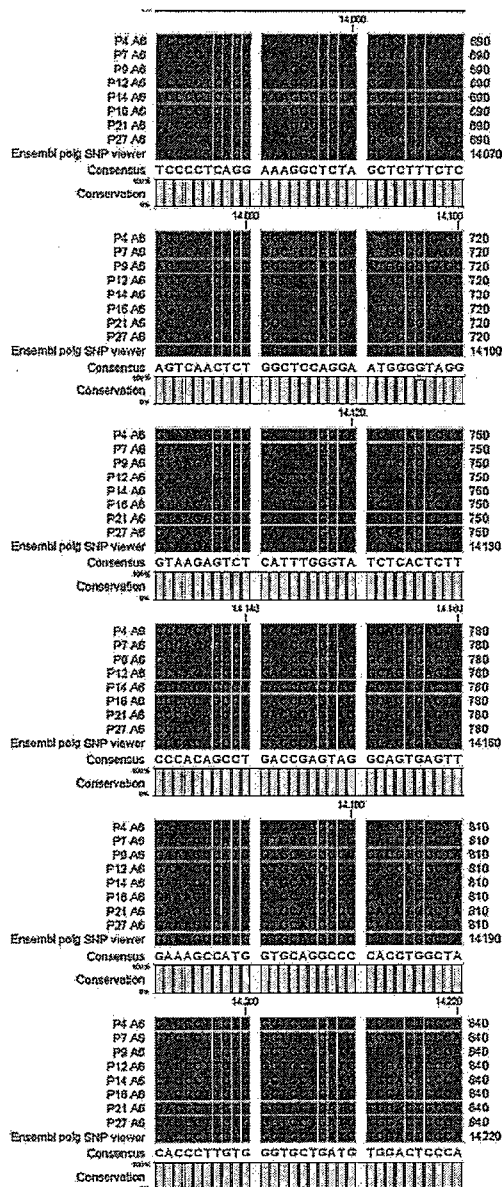
The occurrence of mitochondrial DNA polymerase gamma gene mutations in mitochondrial deficiencies in a selection of South African paediatric patients



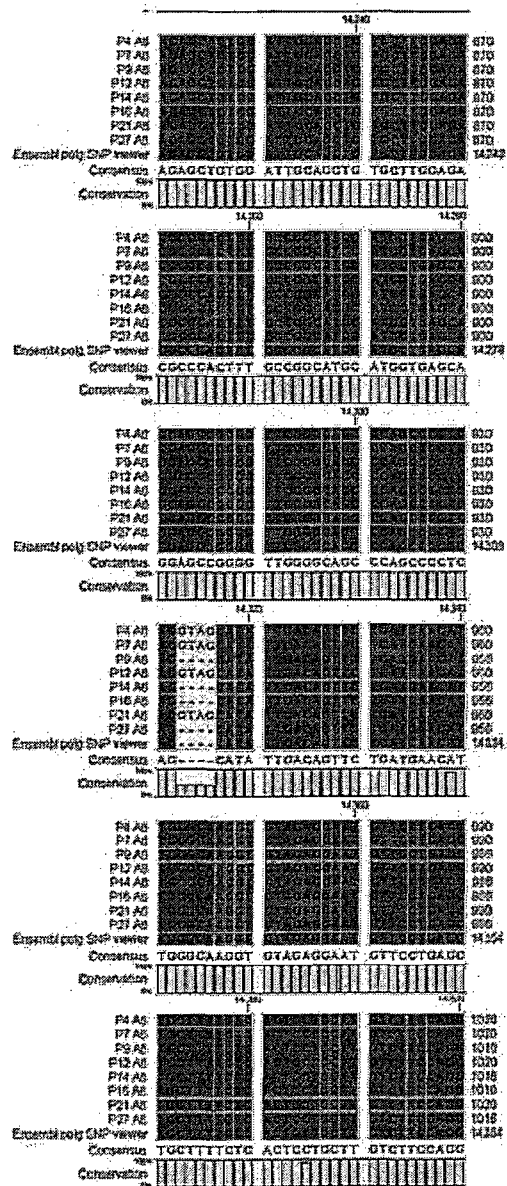
The occurrence of mitochondrial DNA polymerase gamma gene mutations in mitochondrial deficiencies in a selection of South African paediatric patients



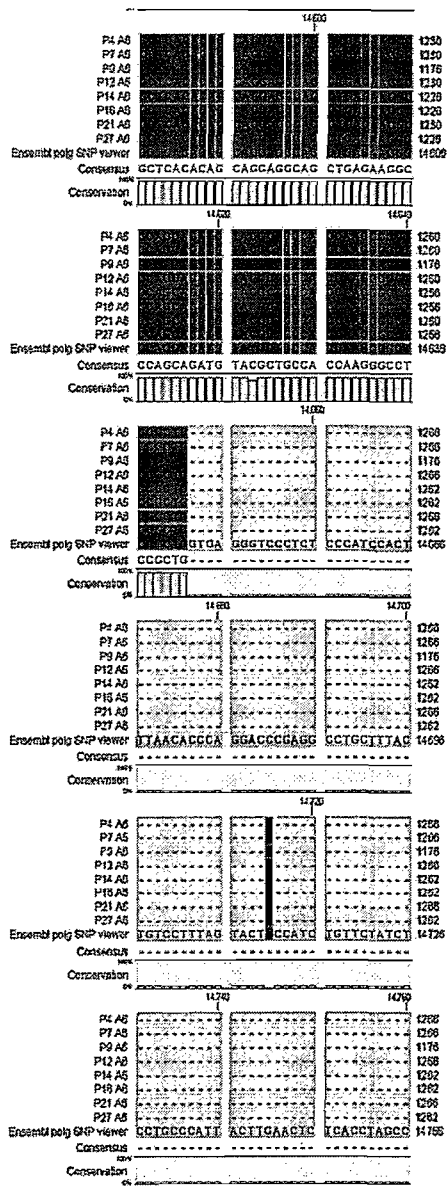
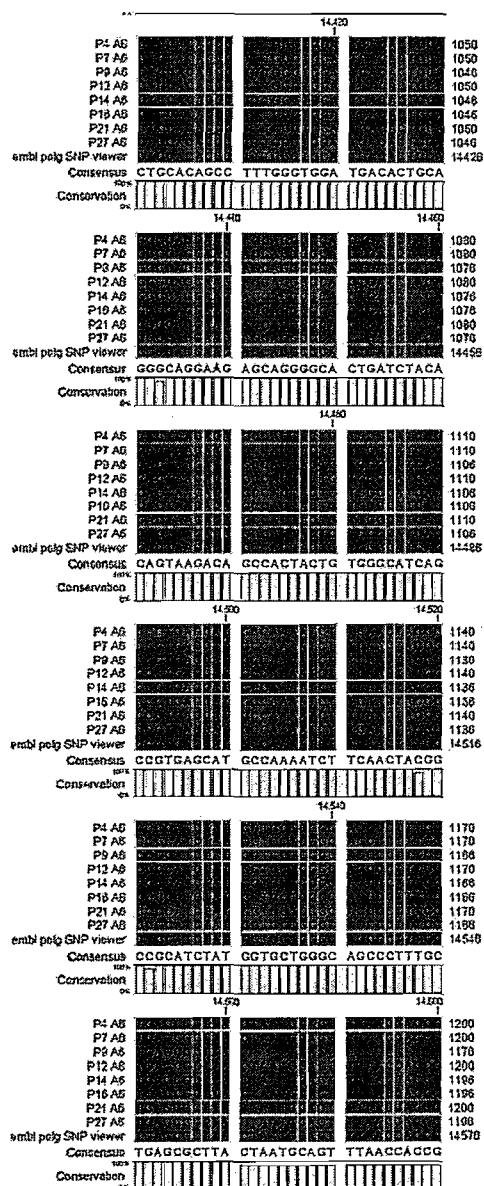
Sequence Primer: A6 (Fw2)



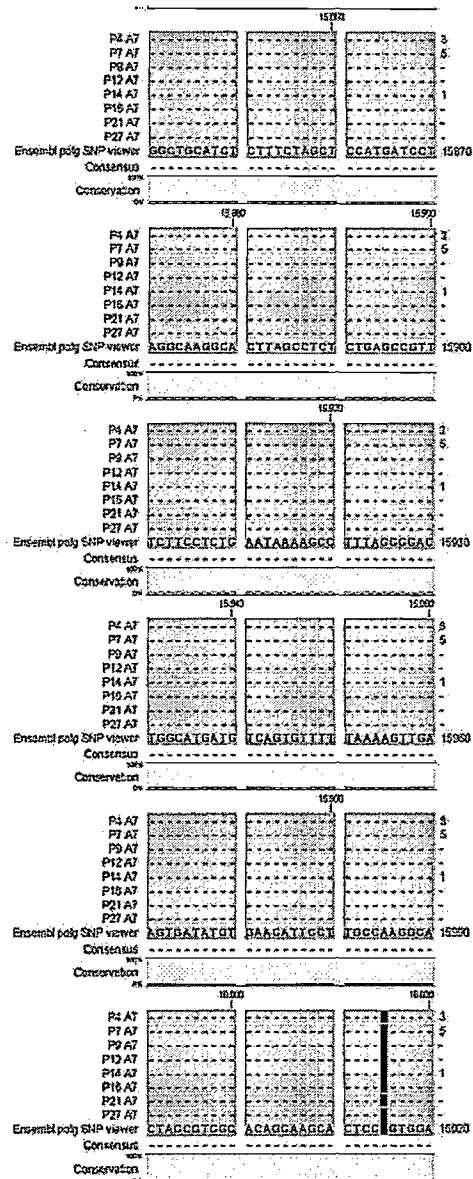
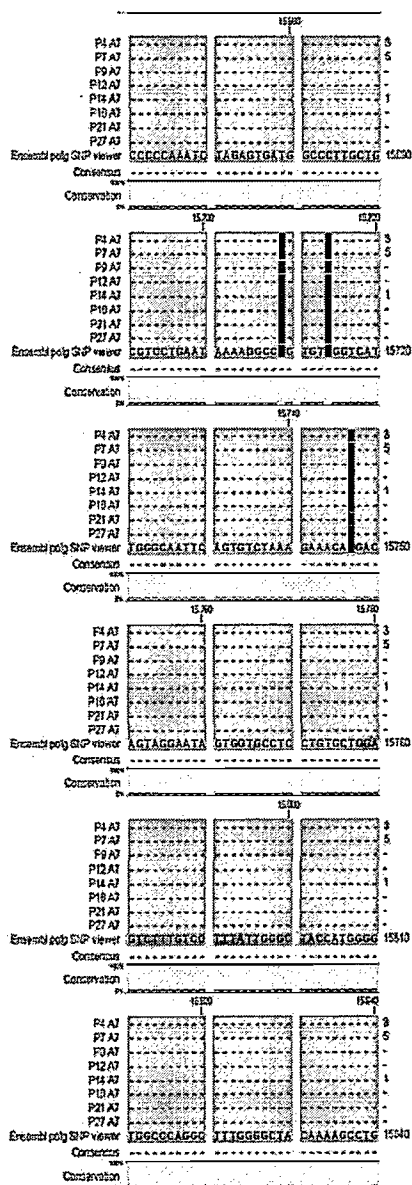
The occurrence of mitochondrial DNA polymerase gamma gene mutations in mitochondrial deficiencies in a selection of South African paediatric patients



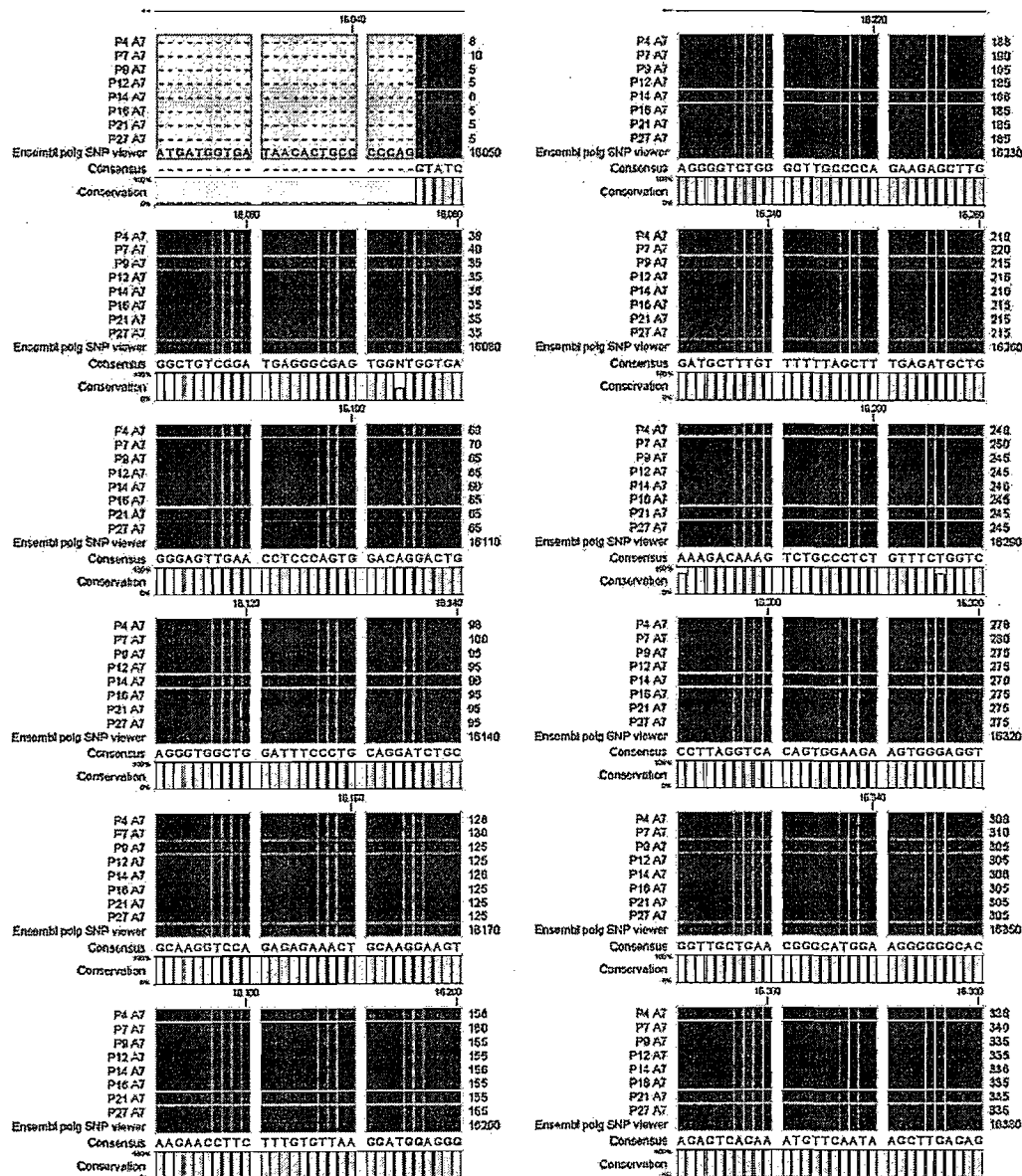
The occurrence of mitochondrial DNA polymerase gamma gene mutations in mitochondrial deficiencies in a selection of South African paediatric patients

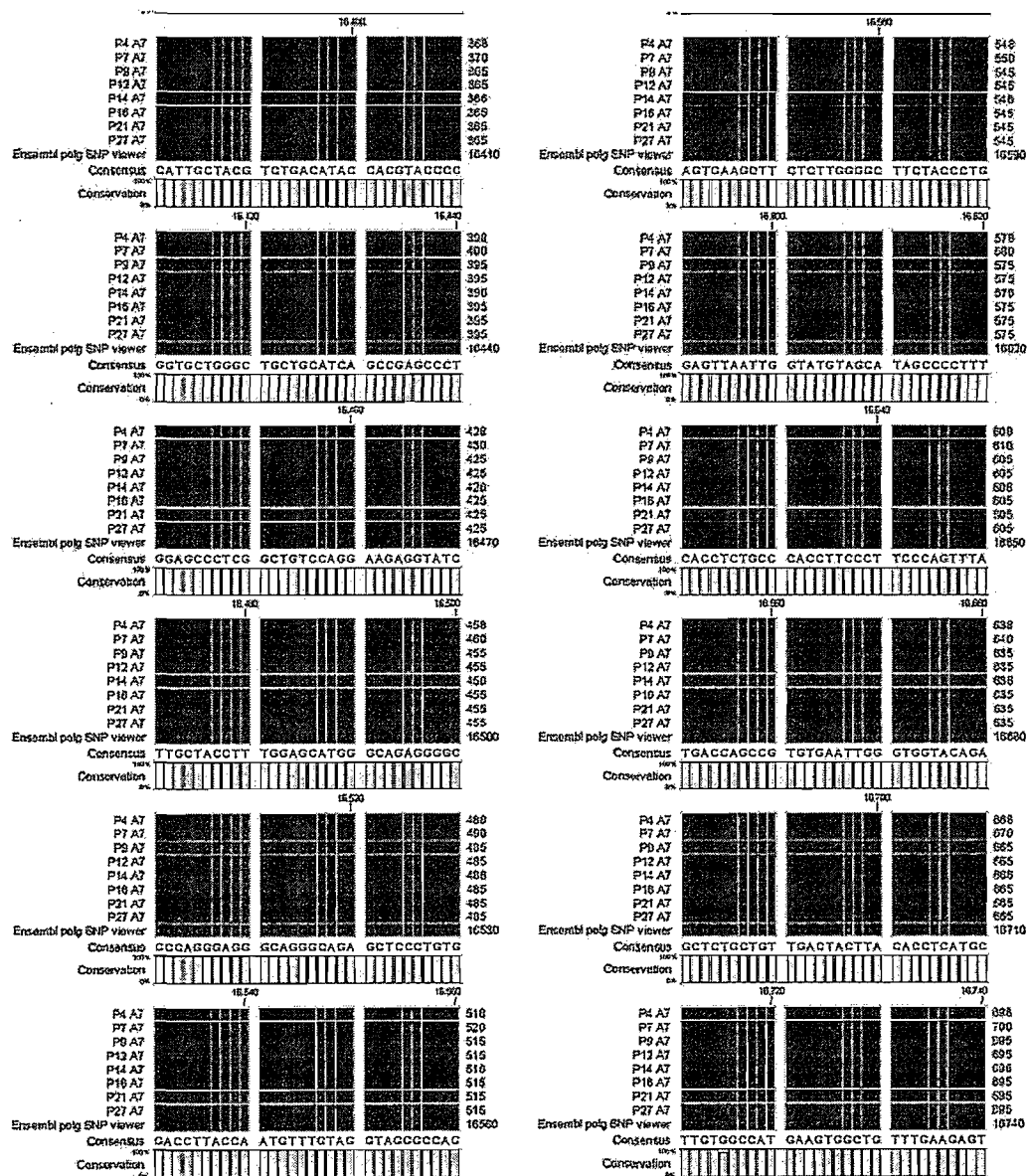


The occurrence of mitochondrial DNA polymerase gamma gene mutations in mitochondrial deficiencies in a selection of South African paediatric patients

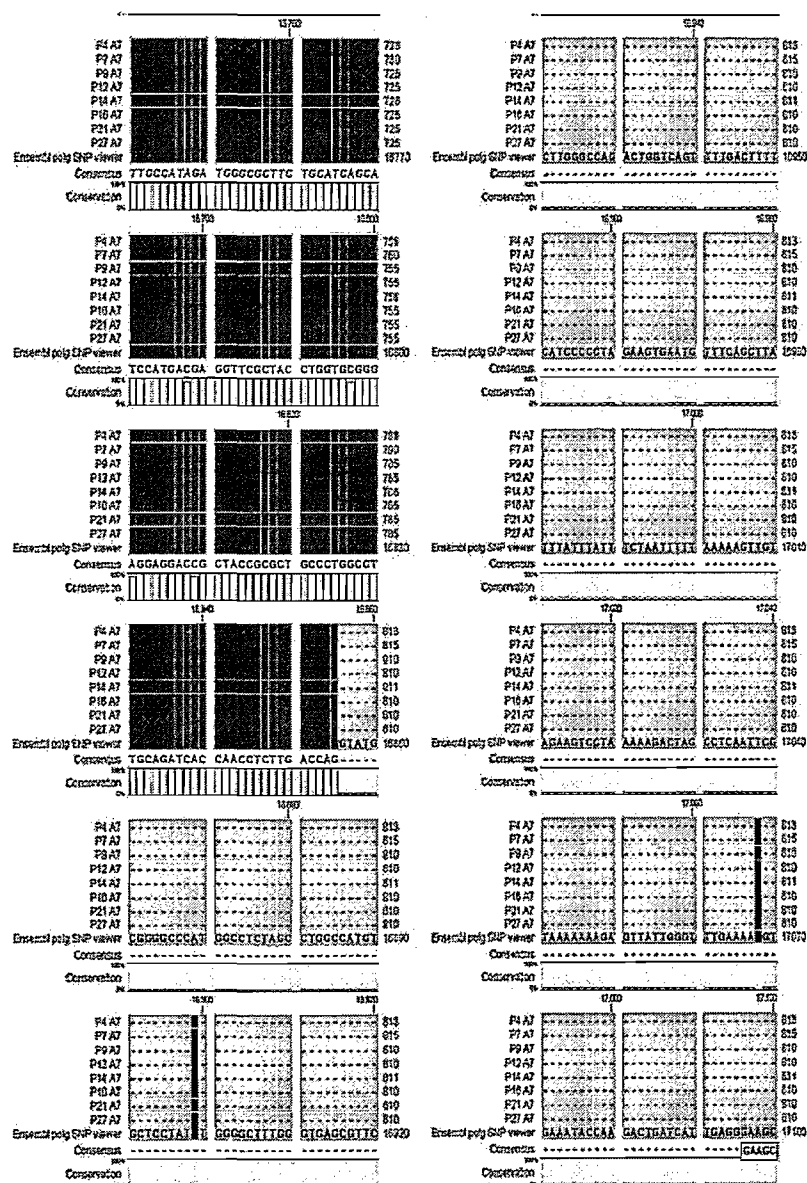


The occurrence of mitochondrial DNA polymerase gamma gene mutations in mitochondrial deficiencies in a selection of South African paediatric patients



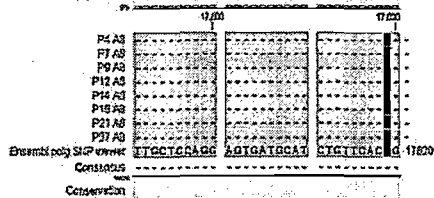


The occurrence of mitochondrial DNA polymerase gamma gene mutations in mitochondrial deficiencies in a selection of South African paediatric patients

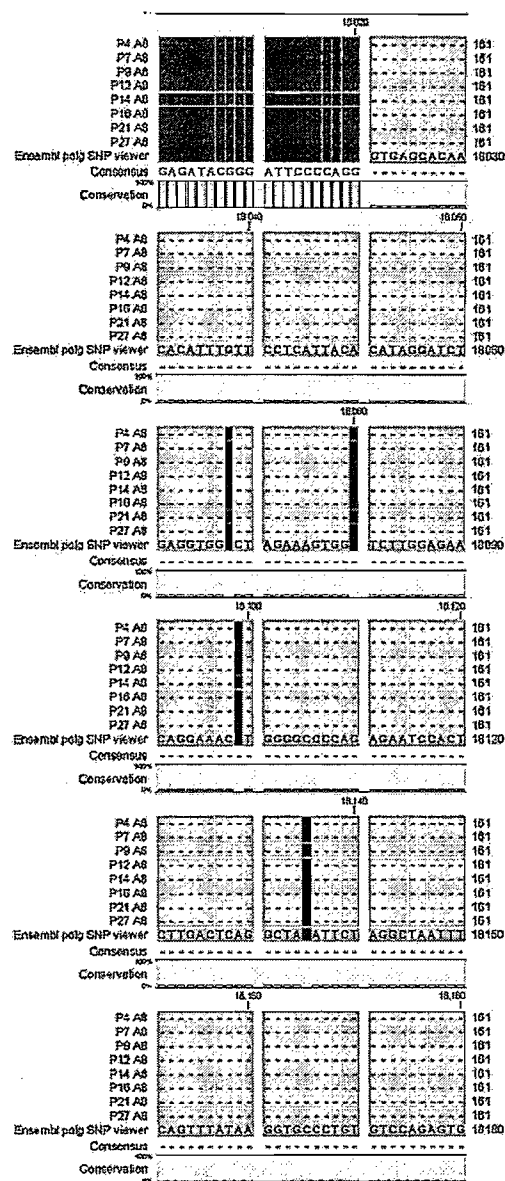
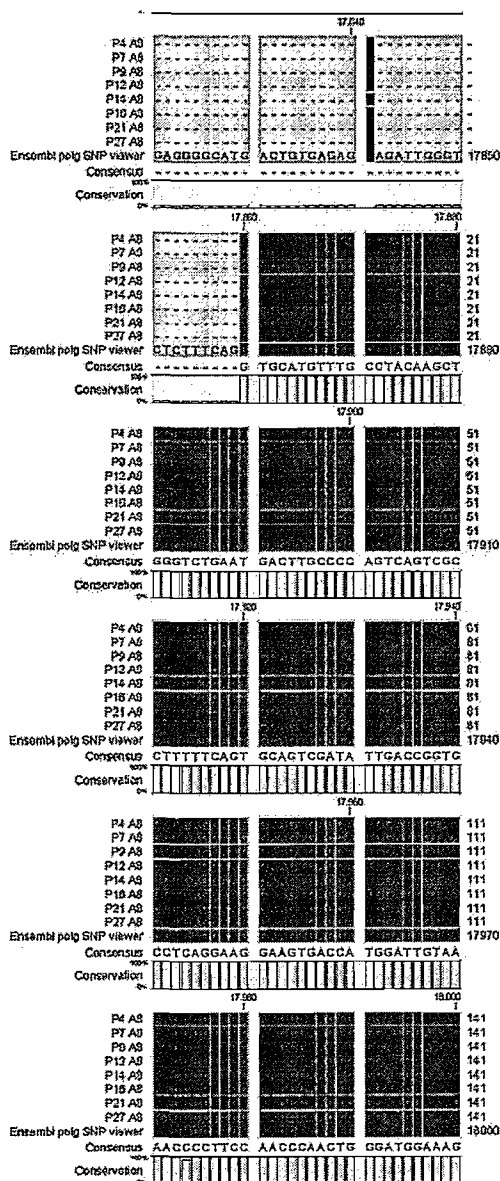


Reverse Primer: A7
Sequence Primer: A7 (Rw)

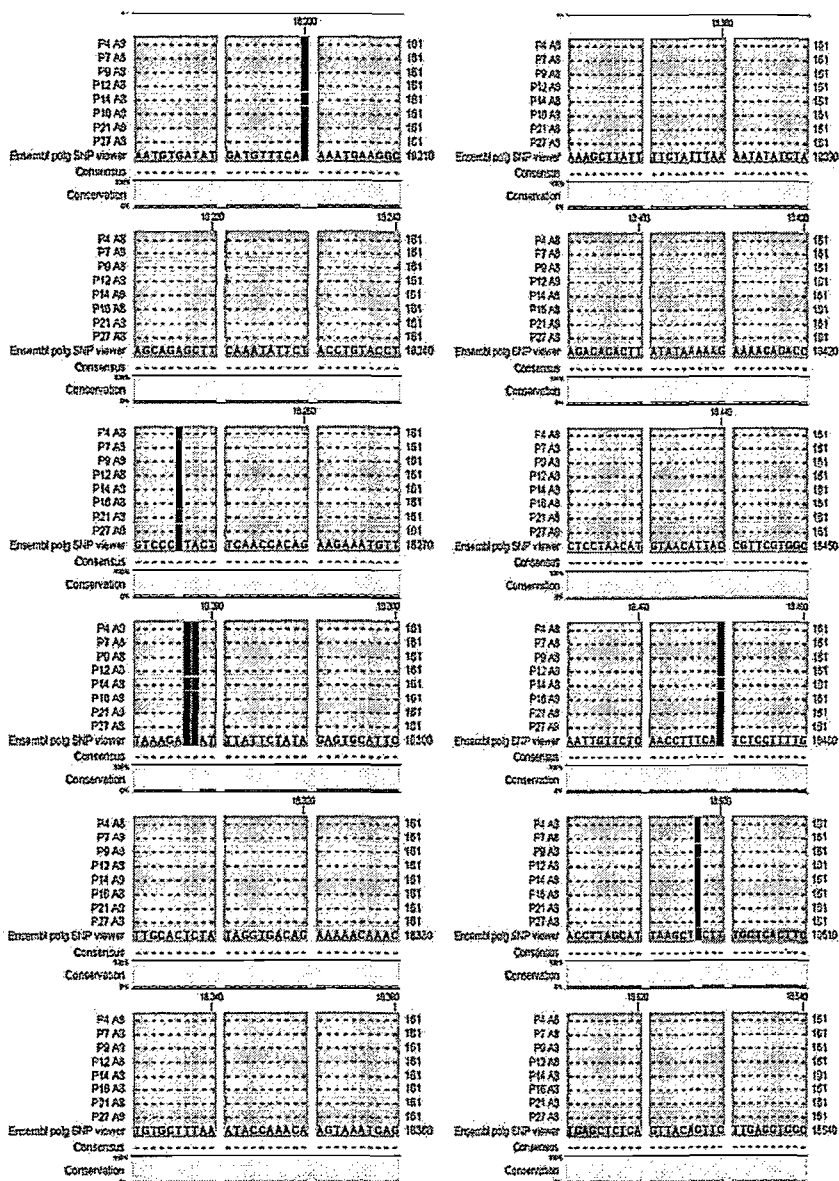
Figure 1 displays sequence logos for the 5' and 3' regions of the 17S rRNA gene. The figure is divided into two main panels, 5' and 3', each showing a grid of sequence logos for positions P1A3, P7A3, P12A3, P14A3, P16A3, P21A3, and P27A3. The logos represent the conservation of nucleotides at these positions. Below the logos, the consensus sequence is given as AAGTTTGTGA for the 5' region and TTGACIGCT for the 3' region. The 17S rRNA gene is indicated on the right.



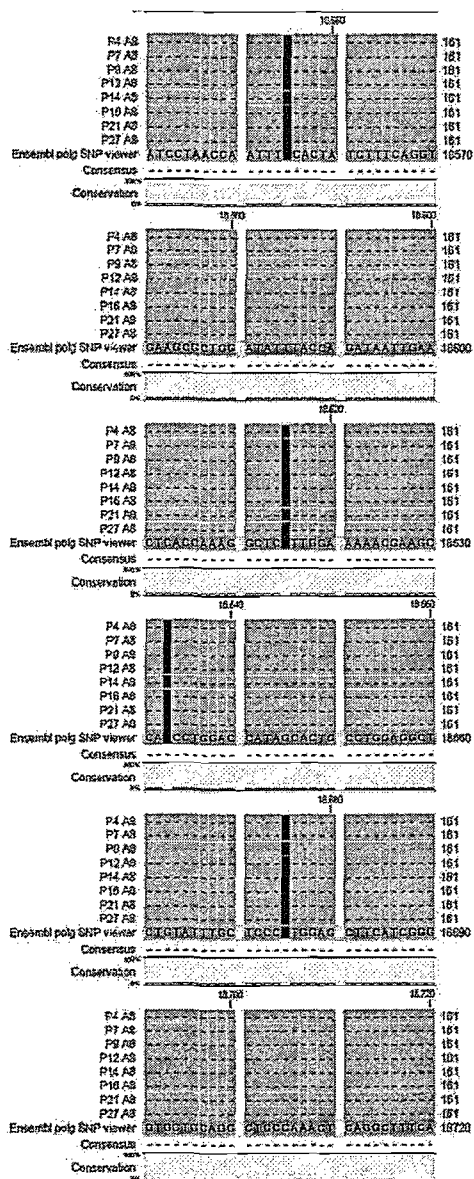
The occurrence of mitochondrial DNA polymerase gamma gene mutations in mitochondrial deficiencies in a selection of South African paediatric patients



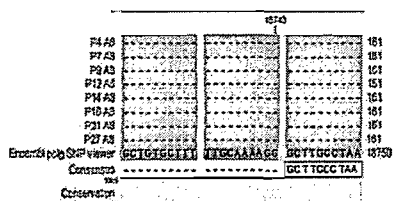
The occurrence of mitochondrial DNA polymerase gamma gene mutations in mitochondrial deficiencies in a selection of South African paediatric patients



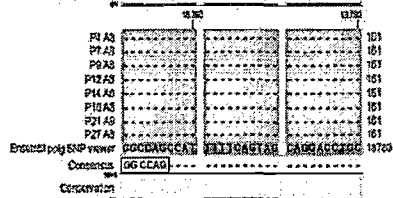
The occurrence of mitochondrial DNA polymerase gamma gene mutations in mitochondrial deficiencies in a selection of South African paediatric patients



The occurrence of mitochondrial DNA polymerase gamma gene mutations in mitochondrial deficiencies in a selection of South African paediatric patients



Sequence Primer: A8 (Rw)



Reverse Primer: A8

