DEVELOPMENT OF A RAPID DIAGNOSTIC SCREEN FOR TELOMERASE MUTATIONS ASSOCIATED WITH IMMUNOSUPPRESSIVE THERAPHY FAILURE IN PATIENTS WITH APLASTIC ANAEMIA.

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A dissertation submitted in fulfilment of the academic requirement for the degree of Master of Science in Medicine in the Discipline of Heamatology, Department of Clinical Laboratory Sciences, Faculty of Health Sciences, University of Cape Town.

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PREFACE

The scientific research undertaken and described in this dissertation was performed and at the Division of Haematology, Clinical Laboratory Sciences, Faculty of Health Sciences, University of Cape Town from April 2011 to May 2012, under the supervision of Dr Karen Shires and co-supervision of Dr Shaheen Mowla.

This study was conducted to develop a diagnostic assay which may be applicable to the molecular diagnostic laboratories. It represents the original work by the author and has not otherwise been submitted in any form for another degree or diploma at any University. In case where the work of others has been used, it is correctly acknowledged in the text.

Khethelo Richman Xulu (candidate)

Dr Karen Shires (supervisor)

Dr Shaheen Mowla (co-supervisor)

DEDICATION

I would like to dedicate this work to my grandfather Bambokudala Matalansi Xulu and my mother Thandiwe Sibonakaliso MaDludla Xulu. You have shown a great support to me despite that you never understood the journey I had undertaken. This journey for me has not been easy, consisted a lot of sacrifice, endurance and a desire to achieve something different from my family. I thank our creator, the almighty God for providing me with you to be my family.

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Finally, my almighty, creator of everything, my God for giving me the gift of life.

This is a quote that has drawn persistence in my life and my education journey:

"*Nothing in the world can take the place of persistence. Talent will not; nothing is more common than unsuccessful men with talent. Genius will not; unrewarded genius is almost a proverb. Education will not; the world is full of educated derelicts.* ***Persistence*** *and* ***determination*** *alone are omnipotent. The slogan, '****press on'*** *has solved and always will solve the problems of the human race"* John Calvin Coolidge

I thank myself for relying on nothing to get something in life.

DECLARATION – PLAGIARISM

I, Khethelo Richman Xulu declare and certify that the dissertation hereby submitted for the MSc Medicine (Haematology) degree at the University of Cape Town is my independent effort and had not previously been submitted for a degree at another university/faculty.

This dissertation does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

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Signed: 

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LIST OF SCIENTIFIC ABBREVIATIONS AND ACRONYMS

A	Adenosine
ABI	Applied Biosystems
AS-M-PCR	Allele-specific multiplex polymerase chain reaction
ATP	Adenosine triphosphate
B-cells	Bursa of Fabricus cells
bp	Base-pairs
C	Cytosine
cDNA	Complementary deoxyribonucleic acid
CHR	Complete haematological response
CML	Chronic myeloid leukaemia
CMR	Complete molecular response
DHPLC	Denaturing high performance liquid chromatography
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylenediamine tetra acetic acid
EGF	Epidermal growth factor
et al.	*et alia* (and others)
g	Gram
G	Guanine
HR	Haematological response
HRM	High-resolution melting

IL-3	Interleukin-3
JAK	Janus kinase
kDa	kiloDalton
M	Molarity
MAPK	Mitogen-activated protein kinase
mg	Milligram
$MgCl_2$	Magnesium chloride
µL	Microlitre
ml	Millilitre
mM	Millimolar
ng	Nanogram
mRNA	Messenger ribonucleic acid
PI3K	Phosphatidylinositol 3 kinase
PCR	Polymerase chain reaction
pH	Percentage hydrogen
pmol	Picomole
RNA	Ribonucleic acid
RQ-PCR	Real-time quantitative polymerase chain reaction
rpm	Revolutions per minute
SCT	Stem cell transplantation
STAT	Signal transducers and activators of transcription
T	Thymine

TAE	Tris acetate EDTA
T –cells	T-lymphocytes
Tm	Melting temperature
U	Unit
UV	Ultraviolet
V	Volts
˚C	Degree Celsius
%	Percentage
µg	Microgram
µl	Microlitre

ABSTRACT

BACKGROUND

Aplastic Anaemia (AA) is a rare haematological disease characterised by extreme depletion in the number of haematopoietic stem cells. Autoimmune attack by T-lymphocytes is the main mechanism for the depletion of stem cells. Another disease mechanism causing AA is dysfunctional telomere addition which results in shortened stem cell life span due to premature apoptosis. Mutations in both *TERC* and *TERT*, the key components of telomerase, have been detected in some patients with AA and have been shown to be responsible for the reduction in telomere length and premature stem cell death. Patients that have this alternate mechanism for AA development do not respond to immunosuppressive therapy (IST), the main chemotherapy option for patients with AA, thus, it is imperative that these mutations are detected prior to the initiation of this expensive and potentially life-threatening treatment.

AIM

The aim of the study was to develop a rapid diagnostic assay to detect clinically relevant telomerase mutations in AA patients.

METHODS

We generated plasmid based controls of *TERT* (G202A, C412T, G694A, C704T, A846G, T1015C, and G1090A) and *TERC* (del53-87, del110-113, G178A and C180T) mutations using PCR-based site-directed mutagenesis, followed by cloning of the mutated PCR product into pGEM-T-Easy vector. These plasmid-based mutants were diluted with wild-type genomic DNA and then used as templates to develop a multiplex assay system to detect seven TERT mutations and four TERC mutations using allele-specific primers in a standard PCR assay. Primers were combined to try to develop a one-tube assay to detect all four TERC mutations simultaneously and another to detect all seven TERT mutations, with the co-amplification of a control amplicon in both reactions. Assay optimisation was performed to create a single PCR cycling platform to allow amplification of all mutations simultaneously, which included cycling parameters, primer concentrations and the addition of additives such as DMSO and glycerol.

A number of assay parameters such as mutation detection sensitivity, reproducibility and accuracy were tested, as well as the determination of the assay cost and turnaround time to assess its relevance in a diagnostic environment.

RESULTS

We successfully assessed DNA samples for all eleven clinically relevant telomerase mutations in a single PCR cycling platform. Allele-specific forward primers for all four TERC mutations: TERC53, TERC110, TERC178 and TERC180 were combined with a control amplicon forward primer and a common reverse primer to allow for simultaneous assessment of all mutations and the co-amplification of a positive control amplicon. The combination of all the primers necessary for the detection of seven TERT mutations proved to be more challenging, showing non-specific amplification and other mutants not amplified when all primers were combined. We had to develop two separate reaction mixes to solve the problems. TERT master mix one (TTMM1) reaction mix contained the primers necessary for the allele-specific detection of TERT412, TERT704, TERT846, TERT1015 and TERT1090 while TERT master mix two (TTMM2) reaction mix allowed for the detection of TERT202 and TERT694, as well as a positive control amplicon. The developed *TERC*/*TERT* assay had a clinically relevant sensitivity of 2% mutant allele detection and showed good reproducibility and accuracy when known and blind samples of wild-type and mutants were assessed. The cost of the assay was found to be 2 times cheaper than the conventional direct sequencing alternative and 2.5 times faster – with an expected turnaround time of 4 hours.

CONCLUSIONS

We have successfully generated an allele-specific multiplex assay for the detection of *TERC* and *TERT* mutations that are associated with an alternate disease mechanism and IST failure in AA patients. This assay is cost-effective bearing in mind the detection of eleven mutations simultaneously and provides accurate results in a short time frame, making it suitable for use in a clinical molecular diagnostic laboratory.

1 CHAPTER ONE: LITERATURE REVIEW

1.1 DEFINING APLASTIC ANAEMIA: Clinical presentation and main disease mechanism

1.1.1 Introduction

Aplastic Anaemia (AA) is a life threatening haematological disease, which belongs to a group of disorders known as Bone Marrow Failure Syndromes (BMFS) (Young *et al*., 2000, Gordon-Smith *et al*., 2001). BMFS are characterised by the failure to generate a normal haematopoietic component within the bone marrow microenvironment, which in turn leads to the inappropriate generation of peripheral blood cellular components (*i.e*: leukocytes). These syndromes, which include AA, leukaemia (acute and chronic) and myelodysplastic syndrome (MDS), originate from the defects affecting the haematopoietic stem cells, such as increased proliferation, increased apoptosis or failure to differentiate (Gordon-Smith *et al*., 2001, Dokal and Vulliamy *et al.,* 2003). In the case of AA, there is a reduction in the number of stem cells, which results in a deficiency in the number of all three major components of the peripheral blood, namely red blood cells (erythrocytes), white blood cells (leukocytes), and platelets, which is also collectively called pancytopenia (Calado *et al*., 2009). Similarly to other bone marow diseases, acquired AA (discussed below in more detail) is potentially a clonal disease affecting a specific cell lineage at an early point in development (haemopoetic stem cell) (Calado *et al* 2009 and Cooper *et al.*, 2008)

1.1.2 Clinical presentation and causes of AA

AA has a broad spectrum of phenotypical symptoms ranging from fatigue, dizziness, pale skin, chest pains, headaches, coldness in palm of hands and feet and shortness of breath (Ball *et al*., 1998, Gordon-Smith *et al.,* 2001, Fogarty *et al*., 2003). Several of these symptoms are caused by reduced levels of the iron-rich protein haemoglobin, which is present in erythrocytes and transports oxygen in the human circulatory system (Ball *et al*., 1998, Du *et al.,* 2009). A reduction in erythrocytes and the consequential reduction in haemoglobin in AA patients cause the heart to work extremely hard to pump blood and circulate the highly needed oxygen to tissues (Dokal *et al.,* 2003). This may lead to the development of other conditions such as arrhythmias, which may in turn result in heart failure (Ball *et al*., 1998, Gordon-Smith *et al.,* 2001, Fogarty *et al*., 2003, Du *et al.*, 2009). Anaemic hypoxia may also develop due to the reduced oxygen levels (Young *et al.,* 1997).

The reduced white blood cell count in AA patients leaves patients vulnerable to bacterial and viral infections, due to a reduced immune response capacity. Many AA patients often present with multiple opportunistic infections and are more severely affected by common ailments due to the reduced immune response (Ball *et al*., 1998, Gordon-Smith *et al.,* 2001, Fogarty *et al*., 2003). The reduction in platelets impairs the ability to form clots to repair wounds, as platelets produce necessary chemicals such as platelet derived growth factor (PDGF) required for tissue repair (Kagan *et al.,* 1976; Gordon-Smith *et al.,* 2001). This will in turn lead to symptoms of uncontrolled bleeding in the case of cuts and severe bruising. The severity of the disease is determined by bone marrow cellularity and peripheral blood cell count. This dictates the classification of AA patients as moderate AA, severe AA (SAA) or very SAA (VSAA) (Marsh *et al.,* 2009).

In addition to the symptoms and problems listed above, patients with SAA and VSAA are also prone to clonal development, resulting in MDS, acute leukaemia or paroxysmal nocturnal hemoglobinuria (PNH) (Shu-ye *et al.,* 2012; Sutton *et al.,* 2012). The risk of death is very high for SAA and VSAA cases (>12 months survival), with increased age being a negative prognostic factor (Marsh *et al.,* 2009, Young *et al.,* 2010). It is essential to treat AA as the disease is inevitably fatal. Most cases of AA are treatable, with overall survival 5-year rate being as high as 70% (Young *et al*., 2000, Gordon-Smith *et al*., 2001; Spellman *et al.,* 2012; Scheinberg *et al.,* 2012). Fundamentally, AA is thought to be an autoimmune disease but the factors that trigger the development of the disease remains unclear. Unfortunately, the majority of AA cases are idiopathic, meaning that no known cause has yet been identified, which obviously makes prevention of this disease difficult. In the acquired category of this disease, there is evidence that exposure to different chemicals and drugs, including those used in chemotherapy regimens (i.e: benzene, chloramphenicol, carbamazepine, and others), though the incidences are minimal (Nguyen *et al.,* 1999) can cause the disease. Furthermore, a virus called Parvovirus B19 (Erythrovirus B19) has been reported to be linked to acquired AA. This virus attacks the haematopoietic stem cells using P antigen as a receptor to invade cells and prevents the proliferation of a broad range of haematopoietic cells (Nguyen *et al.,* 1999, Qian *et al.,* 2001, Kawakami *et al.,* 2012).

As part of an alternate disease mechanism (discussed below in detail), mutations in the telomerase complex have also been associated with both inherited and acquired forms of AA. In most cases of AA, the trigger for disease development is unresolved (Fogarty *et al*., 2003, Calado *et al*., 2009, Du *et al,* 2009).

1.1.3 Immune attack as the main pathogenic mechanism in AA

AA is characterised by extreme depletion in the number of haematopoietic stem cells. Patients with AA have an increased level of apoptosis of stem cells, which in turn results in the limited synthesis of all three cellular haematopoietic components as mentioned above (Young *et al.,* 2006). In many patients, the main cause of stem cell reduction is due to an autoimmune response, where T-lymphocytes attack stem cells that are situated in the bone marrow (Carroll *et al*., 2009, Young *et al.,* 2006). This autoimmune mechanism is mediated by the cytokines interferon-gamma (IFN-γ**)** and tumor necrosis factor-alpha (TNF-α), which play a pivotal role in the pathogenesis of AA and are produced by the T-lymphocytes. They work synergistically in the suppression of haematopoiesis by activating the P38 Map Kinase pathway in the stem cells, which in conjunction with other activated downstream pathways consequently signals the apoptosis of these haematopoietic cells (Young *et al,* 1997, Verma *et al*, 2002, Sun *et al.,* 2003).

However, it is still not clear which mechanism and pathway is used by the T-lymphocytes and how they are initially triggered (Verma *et al.,* 2002, Sun *et al.*, 2003). Experiments conducted on cultured bone marrow cells from AA patients demonstrated that this may be due to an over expression of these cytokines (Verma *et al*., 2003) and an elevated level of activated cytotoxic lymphocytes (Kagan *et al.,* 1976, Sato *et al.*, 1995; Young *et al.*, 1997). The activated T-lymphocytes attack the stem cells in the bone marrow, resulting in a hypocellular bone marrow (space, mostly replaced by fats) (Kagan *et al.*, 1976; Verma *et al*., 2003), and as a result the production of all haematopoietic progenitors is severely depleted (Passweg *et al.*, 2010; Kim *et al*., 2011). Although T-lymphocytes have been shown to attack the stems cells in AA patients (Verma *et al*., 2003), it is not clear what causes this response and if in fact it is due to an underlying stem cell defect (Cooper *et al.*, 2008, Passweg *et al.*, 2010, Kim *et al*., 2011). The success of stem cell transplants in AA patients suggests that the stem cells themselves give rise to the defective T-lymphocytes which deplete the stem cell pools from bone marrow, although the genetic defect in this pathogenic mechanism is not yet known (Young *et al*., 2010, Passweg *et al.*, 2010, Kim *et al*., 2011, Scheinberg *et al.*, 2012).

1.2 TREATMENT FOR AA

1.2.1 Transplantation as a therapy in AA

The most effective therapy in the treatment of BMFS, including AA, is stem cell transplantation (Young *et al*., 2006; Buchbinder *et al.,* 2013). Unfortunately this procedure is not only costly and potentially life-threatening, it is also complicated by the need to find a suitable human leukocyte antigen (HLA) matched donor (Spellman *et al.,* 2012). The complications of finding a suitable donor are even more difficult in a South African environment, where there are issues surrounding tissue/organ donation in some cultures and the mixed race ancestry of many South Africans. Transplantation does offer a cure and in the cases of SAA and VSAA it is viewed as the best therapeutic option (Arcese *et al.,* 2006, Viollier *et al.,* 2008, Chu *et al*., 2011, Buchbinder *et al.,* 2013). It is also recommended in children and young adults with AA, but not in the elderly, who tend to recover less efficiently after the transplant although this is presently changing with new developments in research and new therapeutic regimens (Maury *et al.,* 2009, Rocha *et al.,* 2004, Passweg *et al.,* 2010, Samarasinghe *et al.,* 2012, Spellman *et al.,* 2012).

Transplanted cells replenish the bone marrow with healthy stem cells from a suitable donor, which is usually a family member (sibling or parent); HLA-matched unrelated donors (MUD) are also used in some cases (Deeg *et al.,* 2006, Viollier *et al.,* 2008, Spellman *et al.,* 2012). Transplantation involves infusing the stem cells from a donor to the patient, which could have been harvested from the bone marrow, peripheral blood (after mobilisation of the stem cells from the bone marrow) or cord blood. The suitability of a donor is determined by a variety of parameters stipulated by The National Marrow Donor Program (NMDP) responsible for the search and identification of a suitable HLA matched donor. HLA are the group of proteins (*i.e.*: HLA-A, -B, -C, and -DRB1 loci) which play a pivotal role in the body's self-defence from different foreign agents such as bacteria and viruses.

In order for a donor to be suitable it requires that the HLA of a donor is closely related to the patient's; otherwise the immune cells of the patient will recognize the donor's cells as foreign and attack them or vice versa (Viollier *et al.,* 2008, Spellman *et al.,* 2012). Graft versus host disease (GVHD) is the attack of host cells by the donor/graft cells caused by immune incompatibility and can lead to severe complications for the patient (Deeg *et al.,* 1998, Arcese *et al.,* 2006, Chu *et al.,* 2011, Kim *et al.,* 2011, Scheinberg *et al.,* 2012).

Graft rejection however, results from the attack of donor cells by the host's immune response and is the major cause of graft failure (Maury *et al.*, 2007). To prevent graft rejection, prior to the transplantation procedure, the patient is conditioned to prevent a stem cell attack by the host's immunity (Flomenberg *et al.,* 2004, Deeg *et al.,* 2006). The conditioning is achieved through different approaches, but in most cases immunosuppressants are used, a prophylaxis therapy to deplete the host's T-lymphocytes which may attack the transplanted stem cells after recognising them as foreign cells (Marsh *et al.,* 2010, Novitzky *et al.,* 2013).

Transplantation is successful in many cases, but there are risks attached. These include the risk of infections post-transplant, due to the patients reduced immunity and the risk of developing either graft rejection or GVHD as mentioned above (Flomenberg *et al.,* 2004, Deeg *et al.,* 2006, Young *et al.,* 2006, Spellman *et al.,* 2012, Novitzky *et al.,* 2013). All of these complications can be life-threatening. Due to the toxic chemotherapy agents used in the transplantation process and the strain on the haematopoietic cells, clonal mutation evolution can occur. The development of other BMFS such as MDS and leukaemia has been associated with a subset of transplanted patients (Young *et al.,* 2006).

Developments are currently underway to improve the outcomes of transplantation, including an improvement in conditioning procedures of patients prior to transplantation with an introduction of other strategies which are less aggressive and consisting of reduced intensity conditioning characteristics compared to other conditioning regimens (Maury *et al.,* 2009, Marsh *et al.,* 2010, Novitzky *et al.,* 2013). Studies have indicated that using less aggressive regimens, such as a combination of fludarabine and cyclophosphamide and inclusion of alemtuzumab normally known as CAMPATH, improves long term survival of patients with fewer infections compared to the traditional intensive conditioning chemotherapy, which includes a combination of busulfan and cyclophosphamide and antithymocyte (ATG) (Maury *et al.,* 2009, Marsh *et al.,* 2010, Spellman *et al.,* 2012, Novitzky *et al.,* 2013).

CAMPATH is also used as part of conditioning during the preparation of donated grafts for transplantation into recipient patients. This allows for the depletion of donor T-lymphocytes from the graft, reducing the likelihood of GVHD (Novitzky *et al.,* 2009, Marsh *et al.,* 2010). The reduced intensity conditioning regimens have been shown to be associated with an increased rate of engraftment and reduced GVHD, without the intensive immunodepletion to the recipient patient (Novitzky *et al.,* 2009, Marsh *et al.,* 2010, Novitzky *et al.,* 2013).

1.2.2 Immunosuppression therapy in AA

Transplantation is not an option for every AA patient due to the reasons explained above. Thus alternative treatment options are sought, even if these control the disease rather than cure it. Since the primary pathogenesis of AA has been well described, with the autoimmune attack by T-lymphocytes on haematopoietic stem cells being the defining feature of the disease, immunosuppressive therapy (IST) is usually the main chemotherapy for all AA patients (Young *et al.,* 2006, Socie *et al.,* 2007, Young *et al.,* 2010, Spellman *et al.,* 2012). The IST is usually a combination of cyclosporine (CsA) and ATG, which is used to control, but not cure the disease.

ATG is used to treat several autoimmune disorders (Young *et al.* 2010, Affable *et al*, 2011). In AA patients, ATG exerts a range of effects on human immunology, which includes reducing the number of T-lymphocytes in the blood and peripheral tissues. It achieves this by modulating adhesion molecules and chemokine receptors on the surface of the T-lymphocytes, thus blocking their function (Affable *et al*, 2011). It further increases the uptake of T-lymphocytes by the reticuloendothelial system (Tchervenkov *et al*., 2004). This down-regulation of the immune system results in reduced apoptosis of hematopoietic stem cells, hence, better generation of stem cells in AA. CsA is also widely used in several immune-mediated diseases; it acts by preventing the signal transduction needed to activate lymphocytes, thereby inhibiting an autoimmune attack (Stepkowsk *et al*., 1997). CsA is a cyclic 11 amino acid peptide normally produced pharmaceutically and biotechnologically using fungus *Tolypocladium inflatum* (Ranger *et al*., 2000, Jin *et al*., 2002, Wang *et al.*, 2005). It has a high affinity for cyclophilin A (CypA) and forms a complex molecule, CsA-CypA, which binds to calcineurin (CnA), a calcium-calmodulin-activated serine/threonine specific protein phosphatase (Ranger *et al*., 2000, Huai *et al.,* 2002, Jin *et al*., 2002).

After binding, the complex becomes rigid and the regulatory and catalytic domains of CnA are inhibited, preventing the phosphatase function of the enzyme. The normal activity of CnA is to dephosphorylate the nuclear factor of activated T-lymphocytes (NFAT), which then activates T-lymphocytes. The CsA molecule therefore inhibits this activation process (Ranger *et al*., 2000, Jin *et al*., 2002, Wang *et al.*, 2005).

The combination of ATG and CsA allows a synergistic approach to reduce the attack of haematopoietic progenitors by activated T-lymphocytes. However, despite the successful utilization of IST in some patients, only 60% of AA patients respond to therapy (Yamaguchi *et al*., 2005, Vulliamy *et al.*, 2005, Young *et al*., 2006, Calado *et al*., 2008, Young *et al.* 2010). This suggested that alternative and as yet undescribed disease mechanisms exist in AA and research in the past few years has embarked on trying to unravel the disease mechanism(s) of these unresponsive AA patients. One such mechanism involves proteins that form part of the telomerase complex and is the basis for the research undertaken in this thesis.

1.3 TELOMERASE COMPLEX AND DISEASE PATHOGENESIS

1.3.1 Telomerase complex and AA disease pathogenesis

1.3.1.A: Alternate disease mechanism for AA

Apart from an autoimmune attack by T-lymphocytes, a second disease mechanism for AA development involves inefficient telomerase complex in the haematopoietic stem cells. The telomerase complex includes a reverse transcriptase (*TERT*), an RNA template (*TERC*) and associated proteins (dyskerin, NOP10, NHP2 and GAR) and is responsible for genomic stability by capping the chromosome with telomeres (Yamaguchi *et al*., 2005, Vulliamy *et al.*, 2005, Young *et al*., 2006). Reduced functioning of this complex results in incomplete chromosome capping, activation of DNA repair mechanisms and then signalling of cellular apoptosis (Verma *et al*, 2002, Young *et al*., 2006). In AA, reduced telomerase complex activity results in a reduced stem cell pool due to premature cell death (Yamaguchi *et al*., 2005, Du *et al.*, 2009)

This mechanism was initially shown to occur in a number of AA patients who did not respond to IST therapy (Kagan *et al.*, 1976, Verma *et al*, 2002). Patients were found to have haematopoietic stem cells and leucocytes that demonstrated shortened telomeres in comparison to cells from age-matched controls (Ball *et al.*, 1998, Oshima *et al.*, 2003). Further investigation revealed that these patients had reduced telomerase activity (Greenwood *et al.*, 2003, Yamaguchi *et al*., 2005, Marrone *et al.*, 2007) and telomerase complex mutations (Vulliamy *et al*., 2007, Calado *et al*., 2008, DU *et al.*, 2009).

Studies using plasmid constructs of either WT or mutated *TERC* or *TERT* genes showed that cultures transfected with mutated genes showed reduced telomerase activity (Yamaguchi *et al*., 2005, Marrone *et al.,* 2007). Researchers also showed that cell death was more prominent in cultures of haematopoietic cells from some AA patients (Young *et al*, 1997, Verma *et al*, 2002). This indicated that mutations harboured within *TERT* and *TERC* genes were part of AA disease pathogenesis, especially in patients who did not respond to IST.

1.3.1.B: Telomerase complex and telomere formation

1.3.1.B.i: Telomeres

Telomeres are tandem repeat DNA sequences (i.e. repeats of $TTAGGG_n$) situated at the end of eukaryotic chromosomes (Figure 1.1) and also on some prokaryotic chromosomes (Cong *et al*., 2002, Mitchell *et al*., 2010). Eukaryotic organisms normally have a long linear chromosomal DNA which is prone to DNA loss during each cell replication cycle. To prevent the loss of important coding sequences, the chromosomes are completed by the addition of protective telomeres (Young *et al.,* 2006; Calado *et al*., 2012). This process is important in cells capable of cell division, and is therefore normally limited to stem cells, including haematopoietic stem cells and activated lymphocytes (Calado *et al.,* 2008, Du *et al.,* 2009).

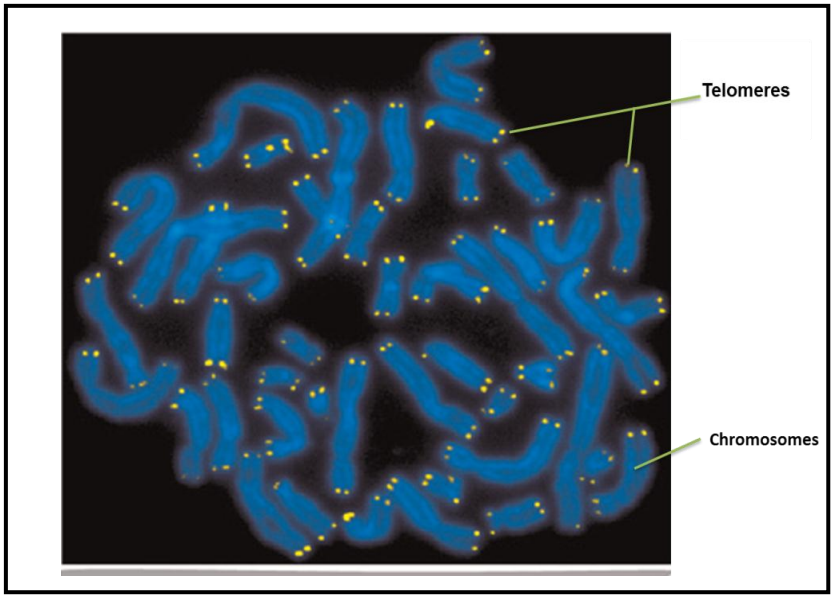

Figure 1. 1: Telomeres at the end of chromosomes

Telomeres (yellow) were microscopically visualized at the ends of chromosomes (four yellow signals of telomeres either side of chromosome) via fluorescence in situ hybridization (FISH).Telomeres are capping the chromosomal ends to protect the loss of genomic information after each replication during cell division (Young *et al*., 2010).

1.3.1.B.ii: Telomerase complex

The formation of telomeres requires a coordinated effort from a collection of proteins that form part of the telomerase complex (Figure 1.2). These include *TERT* and *TERC* (forming telomerase), the ribonucleoprotein subunit 1 (GAR1; 25 kDa), the ribonucleoprotein subunit 2 (NHP2; 22 kDa), the ribonucleoprotein subunit 3 (NOP10; 10kDa) and the ribonucleoprotein subunit 4 (dyskerin or DKC1; 57 kDa) (Calado *et al.*, 2008; Mitchell, *et al*., 2010). While *TERT* and *TERC* are the main components, the accessory proteins play a key role in processing *TERC* molecules and stabilising the complex. DKC1 binds to *TERC* for stabilization within the complex, GAR1 modifies small nuclear RNA for the pre-processing of *TERC*, while NHP2 is required for biogenesis of ribosomes for protein assembly within the complex and NOP10 provides the correct intramolecular processing and trafficking (moving within the cell) of *TERC* within the telomerase complex. The proper coordination of these different components within the telomerase complex ensures that the telomeres are synthesised correctly for capping of the chromosomes (Ly *et al*., 2005; Calado *et al.*, 2008).

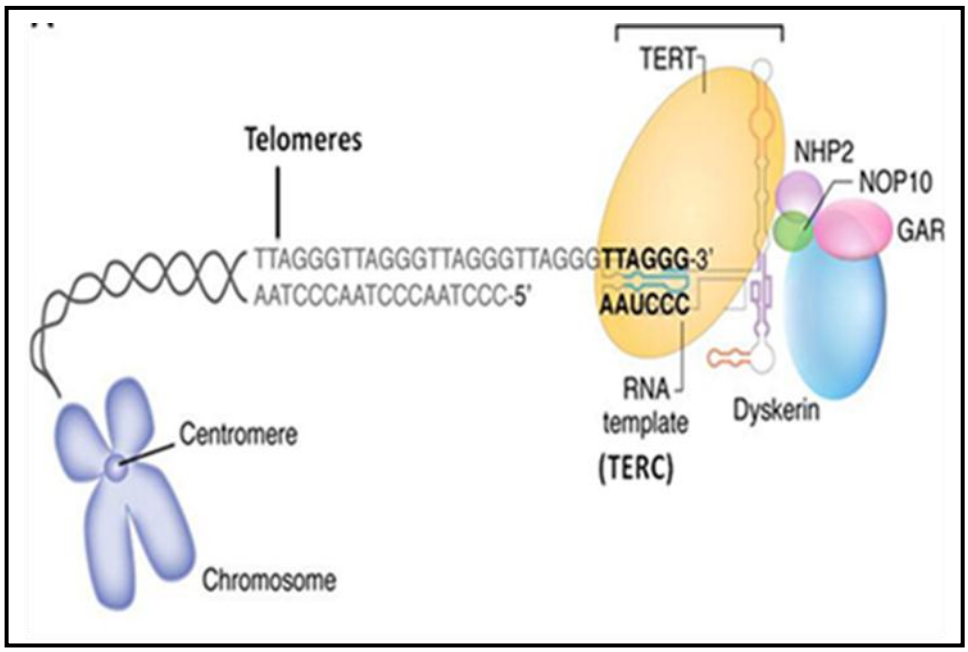

Figure 1.2: Schematic diagram of the telomerase complex.

The picture above shows the synthesis of telomeres via the telomerase complex indicating the *TERT* and *TERC* including associated proteins (dyskerin, NOP10, NHP2 and GAR1) that play a pivotal role for RNA stability in the complex. Telomeres are capping the end points of chromosomes after DNA replication. The picture was adapted from Calado and Young (2008) and modified.

TERC RNA molecule contains various core domains: CR2/CR3 (together called core-pseudoknot), CR4-CR5, box H/ACA (CR6/CR8), and CR7, as well as the hypervariable paired region, which can have flexible length and sequence (Figure 1.3) (Chen *et al*., 2002). Each of these domains plays a significant role during the interaction of *TERC* and *TERT* to ensure that the RNA substrate is processed appropriately for the synthesis of telomeres, thus the telomerase activity is dependent upon specific domains of *TERC* for normal functioning (Ly *et al*., 2005; Chen *et al.,* 2008). The 5'region (core-pseudoknot domain) contains the telomere-encoding domain and forms a specific secondary structure to expose this template encoding region of the RNA molecule.

It also plays a vital role during telomerase assembly by facilitating dimerization of *TERC* (Ly *et al*., 2005; Mitchell, *et al*., 2010). The box H/ACA domain and CR7 (3'region) allow direct binding of DKC1 and other accessory proteins to ensure accurate processing of *TERC* and correct exposure of the *TERC* encoding core-pseudoknot domain (5'region) (Ly *et al*., 2005; Mitchell, *et al*., 2010). They also play an important role in the localisation of *TERC* within the nuclear compartment of the cell via the nucleolar-targeting signal (NTS) mechanism facilitated by the C-domain of *TERT* (Mitchell, *et al*. 1999; Pogacic *et al.*, 2000).

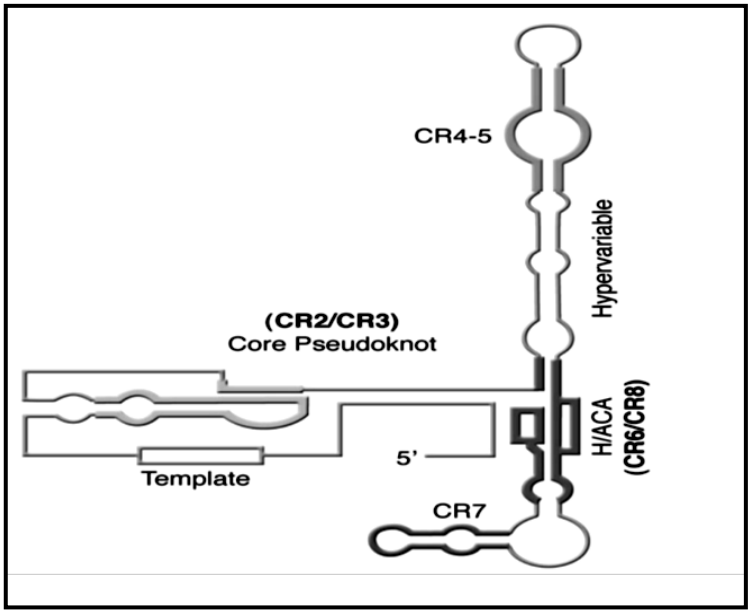

Figure 1. 3: Schematic diagram showing the structure of *TERC*

The image showing *TERC* with its core domains: CR2/CR3 (Core Pseudoknot) the 5' encoding region, CR4-CR5, box H/ACA, and CR7 domains (both located at the 3'region), and the hypervariable paired region. The picture was adapted from Ly *et al*., (2005) and modified to indicate certain regions.

TERT consists of C-terminal region, reverse transcription region and N-region (Figure 1.4), which are crucial for the proper interaction with the *TERC* template. The C-terminal region comprises of highly conserved domains (E-I, E-II, E-III, and E-IV) that provide direct intramolecular interaction between *TERC* and *TERT* during the complex formation and are vital for enzymatic function (Pogacic *et al.*, 2000; Chen *et al*., 2002; Ly *et al.*, 2005, Calado *et al*, 2008). The reverse transcription region is the catalytic region of the protein and contains seven domains (1, 2, A, B, C, D and E), all of which are important for transcription of *TERC.*

The N-terminal region comprises of the N-terminal domain (TEN), the CP domain, and the QFP domain, which play a crucial role for RNA (*TERC*) interaction (Calado *et al,* 2008). It also comprises of an anchor site that binds primer nucleotides for the determination of nucleotides repeats that would be processed and added during telomeres synthesis (Pogacic *et al.,* 2000; Lin *et al.,* 2008; Calado *et al,* 2008).

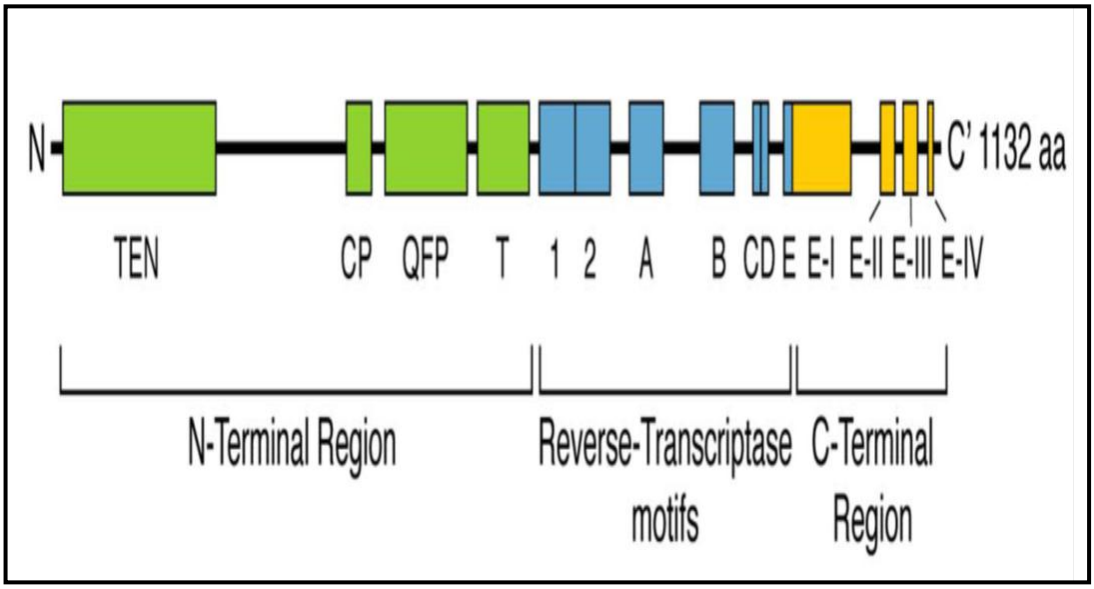

Figure 1. 4: Schematic diagram showing the structure of *TERT*

Schematic diagram of *TERT* showing the various regions and domains in the protein, N-terminal with its domains (TEN, CP, QFP and T), reverse transcription region comprises of domains 1, 2, A, B, C, D and E, and C-terminal region containing domains E-I, E-II, E-III, and E-IV (Calado *et al*., 2008).

In summary, telomere synthesis is achieved through properly coordinated interactions within the telomerase complex, where the 5' region of *TERC* and the reverse transcription region of *TERT* are responsible for the direct formation of the telomeres (Lin *et al*., 2008; Calado *et al.,* 2008; Mitchell *et al.,* 2010). The 3'region of *TERC* interacts with accessory proteins to provide stability and proper processing of RNA (*TERC*) within the complex (Pogacic *et al.,* 2000; Lin *et al*., 2008).

1.3.1.B.iii: Shelterin and telomerase complex interactions

In addition to the telomerase complex, the shelterin complex is involved in the synthesis of telomeres. The shelterin complex comprises of the telomeric repeat binding factor 1 (TRF1), TRF2, the TRF1-interacting protein 2 (TIN2), protection of telomeres 1 (POT1), the POT1-TIN2 organizing protein (TPP1, also known as TINT1, PTOP or PIP1) and the repressor or activator protein 1 (RAP1) (Ye *et al.*, 2004, de Lange *et al*., 2005, Chen *et al*., 2008).

As telomeres are synthesised to cap chromosome, they form a structural loop called the "telomeres loop", normally known as the T-loop (Figure 1.5). The T-loop is generated through the coiling of the 3' end of the telomeric overhang onto the double stranded telomeres and this is facilitated by proteins of the shelterin complex (Griffith *et al*., 1999; de Lange *et al*., 2005, Calado *et al*., 2012). Towards the end of the T-loop, a single-stranded telomere DNA that has a complementary sequence to either strand of DNA at the chromosomal end is formed (de Lange *et al*., 2005). It binds to one of two strands and forms a triple-stranded DNA displacement loop (D-loop)that prevents base pairing to one of the two strands, thus, maintaining the appropriate chromosomes (Ye *et al*., 2004; de Lange *et al*., 2005). Failure in the shelterin complex results in the inability to protect chromosomes properly, leading to DNA repair system to be triggered to repair chromosomal ends (Griffith *et al*., 1999; Ye *et al*., 2004; de Lange *et al*., 2005).

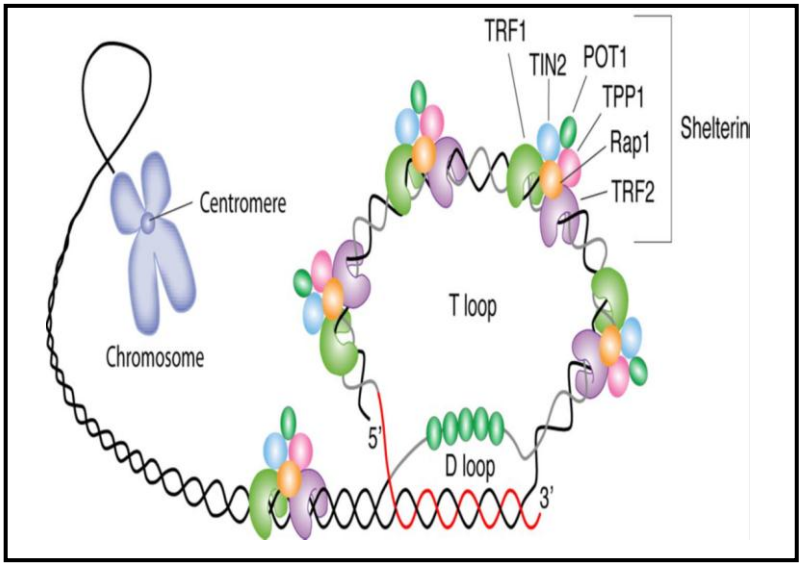

Figure 1.5: A diagram illustrating the shelterin complex during chromosome capping

The picture above shows the shelterin complex interacting with telomeres during the capping of chromosomes after DNA replication. Proteins of shelterin that are involved in the formation of T-loop and D-loop, responsible for chromosomal ends protection are shown binding to the telomeres and collectively provide a shielding mechanism for the chromosomal ends (Calado *et al*., 2012)

1.3.1.B.iv: Consequences of incomplete telomere capping

The proper capping of the ends of chromosomes ensures that the 3` end of telomeric DNA is not recognised by the cell's DNA damage machinery as a double strand break (DBS) and that the DNA damage response (DDR) is not triggered. However, if the synthesis of telomeres is impaired and the capping of chromosomes is incomplete, a potent DDR is elicited (Deng *et al.,* 2008). This leads to the expression of DDR markers such as phosphorylated γ-H2AX, 53BP1, NBS1, MDC1 and CHK2, amongst others. These proteins localize to the telomeres forming dysfunctional telomere-induced foci (TIFs), which have been described as a feature of senescent cells. Alternatively, dysfunctional telomeres have also been shown to activate cell cycle checkpoints such as the PI3 kinase ATM (ataxia-telangiectasia mutated) and ATR (ataxia-telangiectasia and Rad3 related). These kinases phosphorylate downstream CHKI and CHK2 kinases, which in turn phosphorylate p53. The phosphorylation of p53 results in the displacement of its sequester, murine double minute 2 protein (MDM2), thus, preventing its targeting for ubiquitin-dependent degradation. Therefore, the stabilized p53 proceeds to stimulate the expression of the cyclin-dependent kinase inhibitor p21, promoting cell cycle arrest or apoptosis (Young *et al.,* 2005, Calado *et al.*, 2012).

1.3.1.C: Telomerase complex mutations associated with AA pathogenesis

As mentioned earlier, studies have shown that AA patients have shortened telomere lengths on their chromosomes (Yamaguchi *et al*., 2005; Vulliamy *et al.,* 2007; Calado *et al*., 2008; Calado *et al.,* 2012). This led researchers to investigate the corresponding telomerase activity and take a closer look at the genetics of both the *TERT* and *TERC* components to try to identify possible disease causing mutations (Yamaguchi *et al*., 2005; Young *et al.,* 2006; Calado *et al*., 2008; DU *et al*., 2009). This was stimulated by the finding that dyskeratosis congenita was actually caused by the inheritance of telomerase mutations (Vulliamy *et al.,* 2002). Comparative studies have shown that haematopoietic stem cells and leucocytes from AA patients harbour mutations and have altered telomere length compared to cells from age-matched healthy individuals. Table 1.1 shows a list of mutations that are associated with AA. Although many mutations have been discovered, these particular ones listed were found to be associated with reduced telomerase enzyme activity in cells harvested from patients harbouring these mutations. In most studies, the frequency of mutations in either of these genes was found to be about 5-10% (Yamaguchi *et al*., 2005; Young *et al.,* 2006; Vulliamy *et al.,* 2007; Calado *et al*., 2008; Du *et al*., 2008).

All these mutations have been found in AA patients that have failed to respond to IST therapy or have only a transient response. It is therefore postulated that the presence of these mutations serves as a diagnostic feature of this alternate disease mechanism and detecting these mutations when diagnosing AA is an important tool for determining treatment strategy.

Table 1.1: *TERT* and *TERC* mutations associated with AA

Mutation*	Telomerase activity#	IST therapy response$	ReferenceΩ
c.5766G>A; p.Ala202Thr (*TERT*202)	Reduced	Transient	Yamaguchi *et al*., 2005
c.6396C>T; p.His412Tyr (*TERT*412)	Reduced	Failure	Yamaguchi *et al*., 2005
c.20707G>A; p.Val694Met (*TERT*694)	Reduced	Failure	Yamaguchi *et al*., 2005
c.20737C>T; p.Pro704Ser (*TERT*704)	Reduced	Failure	Du *et al*., 2009
c.31483A>G; p.Tyr772Cys (*TERT*772)	Reduced	No profile	Yamaguchi *et al*., 2005
c.31483A>G; p.Tyr846Cys (*TERT*846)	Reduced	Failure	Yamaguchi *et al.,* 2005
c.44647T>C; p.Cys1015Arg (*TERT*1015)	Reduced	Failure	Calado *et al*., 2008
c.45653G>A; p.Val1090Met (*TERT*1090)	Reduced	Failure	Yamaguchi et al., 2005
r.52_86del35 (*TERC*53)	Reduced	Failure	DU *et al., 2009*
r.35C>U (*TERC*35)	Reduced	No profile	DU *et al.,* 2009
r.37A>G (*TERC*37)	Reduced	No profile	Ly *et al*, 2005
r.72C>G (*TERC*72)	Reduced	Partialy	Young *et al.,* 2006
r.110_113del GACU (*TERC*110)	Reduced	Failure	Vulliamy *et al*., 2007
r.116C>U (*TERC*116)	No activity	No profile	Vulliamy *et al.,* 2007
r.117A>C (*TERC*117)	No activity	No profile	Yamaguchi *et al*., 2005
r.178G>A (*TERC*178)	Reduced	Failure	Vulliamy *et al.,* 2007
r.180C>U (*TERC*180)	Reduced	Failure	Vulliamy *et al*., 2007
r.204C>G (*TERC*204)	No activity	No profile	Du et *al*., 2009
r.305G>A (*TERC*305)	Reduced	No profile	Du *et al*., 2009

*****: Mutations of *TERT* and *TERC* associated with AA, depicted according to their exons, the base changes in the DNA sequence and the amino acids before base change and after mutation, mutants names are shown in brackets for conveniently used throughout the manuscript.

#: Profile of telomerase activity - as measured by telomeric repeat amplification analysis of cell lysates and average telomere length at chromosomal ends by flow fluorescence in situ hybridization from AA patients peripheral blood cells compared to aged match healthy individuals (Yamaguchi *et al*., 2005; Du *et al*., 2009; Calado *et al*., 2008).

$: Response to IST response as measured by blood cell count and increased in cell count showing response (Yamaguchi *et al*., 2005; Young *et al.*, 2006; Vulliamy *et al*., 2007; Du *et al*., 2009).

Ω: References for the studies investigated mutations, telomerase activity and patient' response from IST.

The interaction between *TERC* and *TERT* is essential for telomerase activity and telomere formation and is dependent upon the structure of both. Mutations within these two telomerase complex components affect the normal functioning of the system leading to inappropriate interactions between *TERC* and *TERT* and associated proteins, resulting in reduced activity of the telomerase and ineffective telomere formation (Yamaguchi *et al*., 2005, Young *et al.,* 2006, Du *et al*., 2008, Calado *et al*., 2008, Du *et al*., 2009). Some mutations result in no telomerase activity at all while others reduced the enzyme activity significantly (Young *et al.,* 2006, Du *et al*., 2008, Calado *et al*., 2008).

The mutations affect telomerase activity in different ways depending on the location of these mutations within the various structures and the role that these structures play in the interactions between different components. Mutations of *TERT* gene (listed in Table 1.1) result in reduced enzyme activity and are found in different domains of the enzyme. *TERT*202 and *TERT*412 are found in the N-terminal region, a domain crucial for substrate (*TERC*) interaction. *TERT*694, *TERT*704 and *TERT*846 occur in the reverse transcription region, the catalytic region, while *TERT*1015 and *TERT*1090 are found in the C-terminal region which is very important for the intramolecular interactions of *TERC* and *TERT* as the complex assembles (Pogacic *et al.,* 2000, Chen *et al*., 2002, Ly *et al.,* 2005, Calado *et al,* 2008).

Mutations of *TERC* which are directly associated with IST failure are also linked with reduced telomerase activity, as opposed to total inhibition. All four *TERC* mutations: *TERC*53, *TERC*110, *TERC*178 and *TERC*180 occur in the 5' core-pseudoknot domain on a template region shown in Figure 1.6 (Pogacic *et al.,* 2000, Vulliamy *et al*, 2007). This leads to decreased substrate to enzyme interaction, resulting in low enzyme activity, and also incorrect coding of telomeres that cause an incorrect sequence product and also cause less interaction to the enzyme for proper telomeres sequence synthesis (Pogacic *et al.,* 2000, Calado *et al.,* 2008; Du *et al.,* 2009).

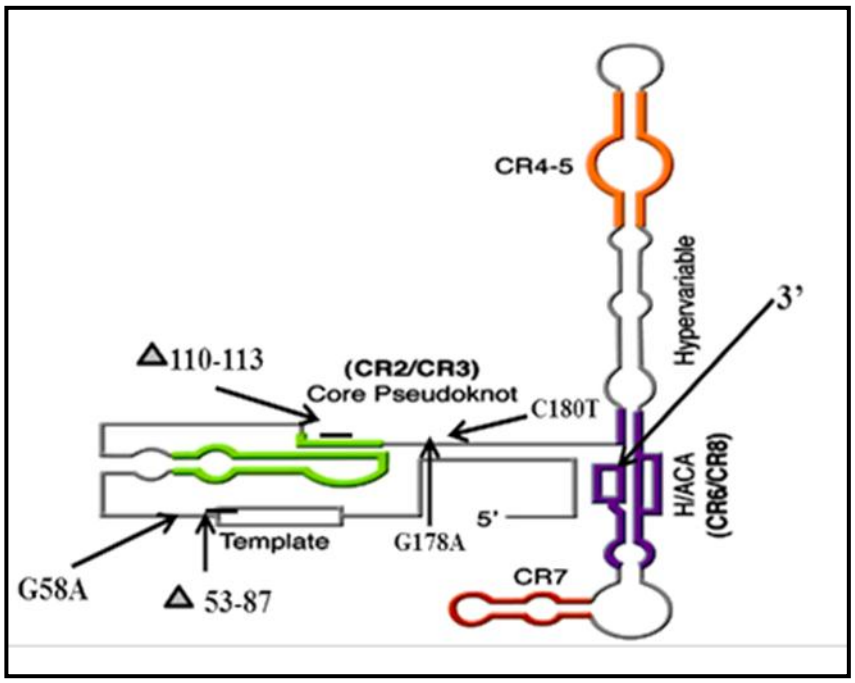

Figure 1. 6: Schematic diagram *TERC* showing the location of mutations

Schematic structure of *TERC* depicting the positions of mutations spanning across the structure of *TERC* and showing the base changes and deletions. The picture was adapted from Ly *et al*., (2005) and modified to indicate certain regions and mutations.

As mentioned earlier, IST is the main chemotherapy for AA patients. However if a patient harbours the specific mutations shown in Table 1.1 they are very likely to have the alternate disease mechanism for AA pathogenesis and therefore would not respond to this type of therapy. If given IST therapy their condition could deteriorate and the patient be exposed to unnecessary secondary infections due to the therapy and even die because of the on-going disease (Young *et al.*, 2006, Calado *et al.*, 2008). Therefore, for survival of these patients, an urgent transplant would be necessary (Gordon-Smith *et al*., 2001, Young *et al.*, 2006, Calado *et al*., 2009). Some of these mutations have an inheritable link (Young *et al.*, 2006; Calado *et al.*, 2008); therefore, it is essential to screen the family members for mutations prior to transplantation to screen out these donors (Yamaguchi *et al.*, 2005, Du *et al.*, 2009). Thus, detecting and knowing the *TERC/TERT* mutation status prior to initiating any therapeutic strategy for AA patients is a crucial step for the decision making about therapy. In most cases 5%-10% of AA patients have both TERC or TERT mutations and likely 15-20% of those mutations are investigated in this work.

1.3.2 Mutations of telomerase complex associated with other diseases

1.3.2.A: Idiopathic Pulmonary fibrosis and liver cirrhosis

Studies investigating the involvement of telomere length in the pathogenesis of idiopathic pulmonary fibrosis (IPF) and liver cirrhosis have found that telomerase complex dysfunction contributes towards these two diseases (Rudolph *et al.,* 2000, Tsakiri *et al.,* 2007, Chaiteerakij *et al*., 2011, Calado *et al*., 2011, Hartmann *et al.,* 2011). Studies investigating telomere length and telomerase activity in the hepatic stem cells (liver cells) and pulmonary stem cells (lung cells) extracted from either IPF or liver cirrhosis patients showed reduced telomerase activity and significantly shortened telomeres compared to cells from age matched healthy individuals (Tsakiri *et al.,* 2007, Calado *et al*., 2011, Hartmann *et al.,* 2011). Furthermore, studies conducted using mouse models deficient of *TERC* genes showed failure to regenerate liver cells after a partial hepatectomy compared to WT mice that had sufficient regeneration of the liver and exhibited reduced cirrhosis (Rudolph *et al*., 2000).

Mutations linked to IPF have been shown in the *TERC* gene are A37G (Ly *et al,* 2005), G182A (Kirwan *et al.,* 2011) and two AA associated deletions [*TERC*53 and *TERC*110 (Table 1.1)]. Four *TERT* mutations [*TERT*202, *TERT*412, *TERT*694, *TERT*772, and *TERT*1090 (Table 1.1)] were found to be associated with AA pathogenesis. Other mutations associated with IPF and liver cirrhosis are G33524A (Arg>His), G45569A (Ala>Thr) (Yamaguchi *et al.,* 2005), as well as C5155T (Pro>Ser) (Tsakiri *et al.,* 2007), T5222A (Leu>Gln) (Armanios *et al*., 2005), G5592A (Val>Met), C6618T (Arg>Cys) (Tsakiri *et al.,* 2007), C21268T (Ala>Val) (Du *et al*., 2009), G27852A (Val>Ile), and C33558G (His>Gln) (Du *et al*., 2008). Although detecting these mutations in IPF and liver cirrhosis patients does not have a direct effect on therapeutic decisions, screening their family members could be beneficial as a precautionary measure to prevent disease development. The failure of pulmonary cells and hepatic cells to regenerate due to mutations of *TERC* and *TERT* shows that the telomerase complex plays a pivotal role during regeneration of cells.

1.3.2.B: Dyskeratosis congenita

Dyskeratosis congenita (DKC) is another disease linked to the malfunctioning of the telomerase complex due to the mutations found within the genes of the various role-players. It is a rare inheritable disorder characterised by skin pigmentation problems, nail dystrophy and bone marrow failure (Vulliamy *et al*., 2006; Du *et al.,* 2009).Mutations of *TERC*, *TERT* and DKC1 genes affects haematopoietic stem cells and activate leucocytes leading to premature stem cell ageing and death. Studies have shown that these mutations play a major role in DKC disease progression (Vulliamy *et al.,* 2001, Vulliamy *et al*., 2006; Du *et al.,* 2009). Mutations in the *TERC* gene that are associated with AA have been found in DKC and these mutations are *TERC*53, *TERC*110, *TERC*178 and *TERC*180 (Table 1.1). Specific mutations associated directly with DKC are C408G and GC107AG (Vulliamy *et al.,* 2001).

Two mutations found in *TERT* gene (*TERT*412, *TERT*704) from patient suffering from AA were also found in DKC patients .Studies have delineated other *TERT* mutations that are only associated with DKC; A27796G (Tyr>Cys) (Yamaguchi *et al.,* 2005), C28892T (Arg>Cys), C35502T (Arg>Trp) (Marrone *et al.,* 2007) and G35507C (Lys>Asn) (Armanios *et al.,* 2005). Since DKC is part of the BMFS and in most cases transplantation is the main cure/treatment for DKC patients, screening the donor for these mutations is vital.

1.3.3 Null mutations in the telomerase complex

Apart from reported pathogenic telomerase complex mutations, there is evidence describing point mutations within *TERC* and *TERT* that are not associated with disease. These include several mutations that seem to be race-specific, including *TERC* mutations G58A (Vulliamy *et al.,* 2002, Yamaguchi *et al.,* 2003) and G228A (Yamaguchi *et al,* 2003, Marrone *et al*., 2004). G58A has been shown to occur at a frequency of 4-10% and G228A occurs at a frequency of 82% in African Americans. G58A is located in the telomere coding region (core-pseudoknot domain), a highly conserved region, thus, impacts negatively on *TERC* and *TERT* interaction for proper synthesis of telomeres and it was assumed that it may reduce or stop telomerase activity. However, investigators found that this mutation had no significant effect on the telomerase activity giving similar activity as the WT in *in vitro* studies and is thus regarded as a null mutation (Fu *et al.,* 2003, Ly *et al*., 2003, Marrone *et al*., 2004). Investigations on the G228A have shown that this mutation does not confer any susceptibility to diseases pathogenesis (Yamaguchi *et al*., 2003, Marrone *et al*., 2004).

These findings show that it is imperative to look for specific mutations when assessing the patient for mutations associated with specific disease. Sequencing either the whole *TERC* gene or relevant *TERT* exons may detect point mutations that are not related to disease.

1.4 VARIUOS TECHNIQUES AND METHODS TO DETECT MUTATIONS

There are many techniques that are used to detect mutations in human genomic DNA. These include allele-specific PCR, standard Sanger sequencing, next generation sequencing (NGS), and real-time PCR using hydrolysis or hybridisation probes. Below we discuss other possible techniques which can be used to detect mutations too.

1.4.1 Sequencing for mutation detection

While the sequence analysis of *TERC* would be very productive, allowing the detection of all 4 mutations in the same PCR and sequencing reaction, the analysis of *TERT* is more involved, requiring at least 4 reactions. Both strands of the amplicon would also need to be sequenced, to limit sequencing artifacts and impurities thus analysis in duplicate would be preferential to be certain that the mutations were not introduced in error by Taq polymerase. Standard sequencing also has a detection sensitivity of 20- 30% (Kollner *et al.*, 1993). While this is sensitive enough for inherited mutations, clonal mutation development would have to be fairly advanced to detect the mutations. As sequence analysis is a post-PCR technique, which requires purified amplicon for the reaction, extra time is also required for this analysis. This is compounded by the need to analyse all the sequencing data and compare it to WT sequence. However this methodology still remains the method of choice in most diagnostic laboratories.

1.4.2 NGS for mutation detection

NGS is a new sequencing technology that is used primarily to sequence large tracts of DNA (Izawa *et al.,* 2013). During NGS, DNA is fragmented into small fragments which are then sequenced in different parallel reactions and the results are assembled for analysis (Dong *et al*., 2012; Sule *et al.,* 2013). NGS is highly sensitive to detect point mutations thus giving an advantage over other techniques (Sule *et al.,* 2013). Unfortunately, although NGS offers a sensitivity advantage over standard sequencing, the cost is currently quite prohibitive and the requirement of novel hardware for the analysis may limit its use in developing countries that do not have requisite skills and funds.

1.4.3. Real-time PCR

Real-time PCR is another powerful molecular technique which allows the PCR reaction to be monitored during cycling, through the activation of a fluorescent molecule, which can be attached to a sequence-specific probe or incorporated into the double-stranded product (i.e: syber green) (VanGuilder, *et al.,* 2008). Through the use of hydrolysis or hybridisation probes, it is possible to detect amplification of a mutated product only. This approach is highly sensitive and very specific (Reynisson *et al.,* 2006, VanGuilder, *et al.,* 2008). However, each mutation requires a specific probe and current multiplex technology on this platform does not allow the simultaneous detection of more than 3 products simultaneously (Luo *et al.,* 2011). Although real-time PCR has advantages in detection of these mutations (specificity and sensitivity), the drawback is the expensive reagents required, the inability to effectively multiplex all the mutations and the need for specialised molecular equipment, which is an issue particularly in developing countries that lack resources.

1.4.5 Allele-specific multiplex PCR

Allele-specific multiplex PCR is a rapid molecular technique which is applicable in many areas of DNA analysis. It allows a combination of reactions using different pairs of primers that are used in one tube to amplify two or more target sequences (Chamberlain *et al*., 1988, Newton *et al.,* 1989, Edkins *et al.,* 1996). Allele-specific primers are 100% homologous to the mutant sequence and have base differences located specifically at the terminal 3' end of a forward primer to allow for amplification to occur only in the presence of mutations. This technique ensures that the mutated sequences are preferentially amplified over the WT DNA sequence. Allele-specific primers are preferable because they bring this discriminatory factor compared to standard PCR primers.

Although allele-specific primers brings the advantage of detecting mutations by amplifying only in the presence of a mutation, sometimes the WT DNA is amplified since the difference between allele-specific primer and WT DNA sequence is only one base at 3'end of primer. Chemically modified primers can enhance the discrimination factor of allele-specific primers. These modifications can be achieved by using locked-Nucleic Acid (LNA) primers, Zip-Nucleic Acid (ZNA) primers or peptide nucleotide acid (PNA) primers (Maertens *et al.,* 2006, Moreau *et al.,* 2009, Terahara *et al.,* 2011).

Chemically modified primers based on either LNA or ZNA provides a thermal stability to the primers by increasing the annealing temperature which aids to increase specific amplification as the primers would not be able to bind to the WT DNA but would only bind to their targets (Moreau *et al.,* 2009, Paris *et al.,* 2010, Ivanova *et al*., 2011). PNA based primers also increase specificity by allowing the PNA to bind to the WT DNA sequence and leaving allele-specific primers to bind only to their targets (Terahara *et al.,* 2011). Using PNA primers clamping system requires that two sets of oligonucleotides are designed for the PCR reaction, one is PNA and another is allele-specific primers and both sets are used in one reaction. Therefore chemically modified primer could be potentially used as an option to improve specificity of the allele-specific multiplex PCR if 3'end based primers bind to the WT DNA. However chemically modified primers may increase costing since it's a new technology.

Some of the phenomenal applications for the allele-specific PCR which have been well described are detection of gene deletions, analysis of polymorphisms and mutations and DNA quantitative analysis (Newton *et al.,* 1989, Edkins *et al.,* 1996, Markoulatos *et al.,* 2002, Hindiyeh *et al.,* 2005). Multiplex PCR as a concept solves many issues, but it also comes with its own challenges due to the introduction of multiple primer sets and different sized amplicons being produced. In a singleplex reaction, where there is only a single pair of primers, the primers can be designed to prevent non-specific binding and primer dimerization. However, in a multiplex environment, even well-designed primers start to behave differently and require a lot of optimization to ensure that the reaction is successful (Chamberlain *et al*., 1988, Markoulatos *et al.,* 2002, Schoske *et al.,* 2003). These difficulties include favouring of a certain target to be amplified against others, poor specificity and sensitivity (Markoulatos *et al.,* 2002).

This occurs because a PCR reaction requires a certain primer to template ratio, and once another primer pair is introduced in the reaction, the ratio changes and the PCR reaction is compromised (Markoulatos *et al.,* 2002, Schoske *et al.,* 2003). In most cases in a multiplex PCR, non-specific PCR products are formed, primer dimers are also synthesized, and this impairs the efficiency of PCR reaction (Henegariu *et al.,* 1997, Loffert *et al.,* 1999). The non-specific products consume reaction reagents, which in turn compromises the annealing and extension to the target regions. As indicated above, designing specific primers that would not cause primer-dimer formation is crucial for any PCR, but with a multiplex PCR it is challenging since multiple primers are used in one reaction. Thus finding a common annealing temperature is not easy as these primers are binding in different DNA sequences, some with high GC content which raises the Tm, while others with high AT content causing low Tm.

Generally, all the primers in the multiplex PCR should have a similar predicted melting temperature (Tm) to avoid non-specific binding but the practicality is determined by a region where the target gene to be amplified is located (Chou *et al*., 1992, Wagner *et al*., 1994, Loffert *et al.,* 1999, Markoulatos *et al.,* 2002). Furthermore, the reagents interactions fluctuate in the multiplex PCR that causes an amplification of certain products of interest while others are not amplified. This favouring of certain amplification in a multiplex PCR is called PCR drift (Walsh *et al.,* 1992). Optimization is required to improve amplification in the multiplex PCR.

During optimization of the multiplex PCR, it is important to perform singleplex PCR reactions, using each primer pair separately. Furthermore, it is important to optimize the concentration of PCR reagents (*i.e.* dNTP's, primers and enzymes) since in a multiplex PCR different amplicons are usually amplified concurrently (Henegariu *et al.,* 1997, Markoulatos *et al.,* 2002 Walsh *et al.,* 1992, Loffert *et al.,* 1999, Markoulatos *et al.,* 2002). The optimum magnesium chloride concentration is critical in the PCR reaction since Taq DNA polymerase is a magnesium-dependent enzyme (Markoulatos *et al.,* 2002). Magnesium chloride binds also to the double stranded DNA, hence, if it is in excess it could stabilize the template and affect completion of denaturation, which could decreases the PCR product.

Furthermore, if magnesium chloride concentration is in excess, it could increases the non-specific primer binding which would increase non-specific products and decreases sensitivity. However, inadequate magnesium chloride concentration may also decrease the amplicon production or lead to no amplification at all (Loffert *et al.,* 1999, Markoulatos *et al.,* 2002). Other than the optimisation of PCR reagents, the cycling conditions may also change during a multiplex scenario as compared to the single-plex reactions. Considering decrease/increase annealing temperature, increase extension time, could also be checked if it has an impact in the multiplex PCR to produce a target amplicon and minimizes the non-specific products, this helps to find suitable common cycling conditions (Loffert *et al.,* 1999, Markoulatos *et al.,* 2002).

The additives in the multiplex PCR have also been indicated to improve the efficiency of PCR, especially in a multiplex setting. Adding dimethyl sulfoxide (DMSO), glycerol, formamide and bovine serum albumin (BSA) in multiplex PCR reactions have been shown to improve the efficacy of the reaction (Masoud, *et al.,* 1992, Henegariu *et al.,* 1997, Markoulatos *et al.,* 2002). DMSO is suggested to enhance the PCR efficiency by using various mechanisms such as DMSO decreasing the Tm of the primers, affecting the thermal activity profile of Taq DNA polymerase, and enhances the effective denaturation of the double stranded template (Masoud, *et al.,* 1992, Walsh *et al.,* 1992). Glycerol relaxes DNA, helping the primers to bind easily to their specific target sequences in a multiplex PCR setting (Markoulatos *et al.,* 2002). While BSA is suggested to improve a multiplex PCR efficiency by affecting the reaction in different ways, but on how it works is not clearly described.

As each unique primer pair is added to the multiplex reaction, the reaction becomes susceptible to primer dimerization. To improve the success of multiplex PCR, hot-start applications have been introduced to ensure that the key components of PCR remain inactive until high temperature of thermo cycling (Henegariu *et al.,* 1997). This can either be achieved by keeping all reactions at a very low temperature before cycling, by adding the Taq polymerase only once the cycling temperature is above the primer annealing temperature or by using chemically modified polymerases that have been modified to be only activated at high temperatures, such as Go Taq hot start polymerase (Promega, USA), One Taq hot start DNA polymerase (NEB, England), Maxima hot start Taq DNA polymerase (Thermo Scientific, SA). These enzymes require a period at a high temperature (~95°C) to destabilise the protective structure and release the polymerase (Henegariu *et al.,* 1997, Le *et al*., 2009).

Similarly, some work has been done to elucidate the effect of modifying the dNTP's structure to only work once heat- activated. The hot start dNTP's such as CleanAmp dNTP's (TriLink, USA) are also chemically modified with a thermolabile 3′-tetrahydrofuranyl (THF) which ensures that dNTP's remain inactive until high temperature of thermo cycling, then is released to begin the PCR (Koukhareva *et al.,* 2009).

This prevents the interaction of primers at ambient temperature that could results in primer dimerization, particularly in multiplex PCR where there are many different primers. Therefore, using the hot start dNTP's is also suggested to improve multiplex PCR efficacy without many alterations which would be required when optimizing (Koukhareva *et al.*, 2009, Le *et al*., 2009). Allele-specific multiplex PCR is a good technique which allows amplification of different target sequences of DNA. This allows simultaneous analysis of multiple DNA sequences in a single PCR reaction, crucial for detecting genetic mutations and deletions. This technique requires extensive optimisation to ensure that all primers involved in multiplex PCR are able to work under one PCR condition and cycling.

1.5 AIMS AND OBJECTIVES

The aim of the study was to develop a novel diagnostic screening assay for rapid and cost-effective detection of 7 *TERT* and 4 *TERC* mutations that are specifically associated with IST failure in AA patients.

The first objective of this study was to create a range of positive controls by site-directed mutagenesis PCR to generate separate amplicons containing a single mutation that would be cloned into a plasmid vector to develop the assay.

The second objective was to develop a multiplex assay, preferably in a single PCR reaction mix for the detection of the 7 *TERT* mutations and 4 *TERC* mutations allele-specific primers.

CHAPTER TWO: GENERATION OF *TERT* AND *TERC* MUTATIONS FOR ASSAY DEVELOPMENT

2.1 INTRODUCTION

As described in Chapter 1, AA patients fail to produce sufficient haematopoietic cells due to a depletion of stem cells (Young *et al*., 2000; Gordon-Smith *et al*., 2001; Dokal and Vulliamy *et al.*, 2003). One of the disease mechanisms responsible for this depletion is a defective telomerase enzyme complex, which results in premature apoptosis of the affected stem cells (Young *et al*., 2000). Mutations in both *TERC* and *TERT*, the key components of the telomerase complex have been shown to be responsible for this defect in many AA patients (Yamaguchi *et al*., 2005; Vulliamy *et al*., 2007; Calado *et al*., 2008; DU *et al.*, 2009). These mutations include: deletions *TERC*53 and *TERC*110, and single polymorphisms *TERC*178 and *TERC*180 in the intron-less *TERC* gene and single/duplex base polymorphisms of *TERT* at positions GC763TA (*TERT*202) and C1393T (*TERT*412) in exon 2, G1570A (*TERT*694) and C15735T (*TERT*704) in exon 5, A26461G (*TERT*846) in exon 9, T39644C (*TERT*1015) and G40650A (*TERT*1090) in exon 14 and exon 15, respectively (details given in Table 1.1) (Greenwood *et al.*, 2003; Yamaguchi *et al*., 2005; Marrone *et al.*, 2007; Vulliamy *et al*., 2007; Calado *et al*., 2008; DU *et al.*, 2009). Patients with these specific mutations do not respond to the standard IST administered to AA patients, due to this alternative disease mechanism (Young *et al.*, 2006, Marrone *et al.*, 2007; Calado *et al*., 2008) and thus it is essential to detect these mutations early in the diagnostic process.

In order to develop a diagnostic assay, it is necessary to have positive mutant controls. AA is a rare disease and less than 40% of patients have a single mutation in one of these genes (Young *et al*., 2000; Calado *et al*., 2009). The positive mutant controls must therefore be artificially generated for use in the assay, since the likelihood of finding patients harbouring mutations is low. Hence, the first aim of our study was to generate *in vitro* positive controls of the relevant *TERC* and *TERT* mutations, which would then be used to develop the diagnostic assay (described in Chapter 3). The strategy was to generate these *TERC* and *TERT* mutations using site-directed mutagenesis.

PCR primers were designed which contained each of the desired mutations separately. These were used to amplify the desired amplicon from human wild type (WT) genomic DNA. In this way individual mutations were introduced into the amplicons. To ensure pure mutant populations and the ability to generate large quantities as positive controls, amplicons were cloned into the pGEMT-Easy plasmid vector. In order to demonstrate the process of creating TERC and TERT mutants,

2.2 MATERIALS AND METHODS

2.2.1 Site-directed mutagenesis (SDM) primers

The primers for *TERC* and *TERT* mutants were designed using Primer3 software, ensuring minimal self-complimentary and hairpin formation. These mutagenic primers were longer (25-30 bases) than standard PCR primers to ensure a reasonable 3' clamp after the mutated bases and sufficient 5' sequence to allow development of primers for the diagnostic assay (Chapter 3). Primers were checked for specificity against the human genome using NCBI blast, to ensure no non-specific binding. All primers were generated at the University of Cape Town (Department of Molecular and Cell Biology) and are shown in Table 2.1 below.

Table 2.1: SDM primers to produce *TERC* and *TERT* mutations and deletions

Primer[$]	Sequences (5' to 3')
TC53 (Forward Primer)	TTTGTCTAACCC_*CCCGCGCGTG
TC110 (Forward Primer)	CTGTTTTCTCGCT_TTCAGCGG
TC178 (Forward Primer)	TTCATTCTAGAGCAAACAAAAAATA#TCAGC
TC180 (Forward Primer)	TTCATTCTAGAGCAAACAAAAAAGTTAGC
TCR (Reverse Primer)	CACGTCTCCTGCCAATTTGC
TT202, Exon2 (Forward Primer)	GTCGGGATGCGAACGGTACTGG
TT412, Exon2 (Forward Primer)	CGGGCTCCTCAAGACGTACTGC
TT694, Exon5 (Forward Primer)	GGGCCTGGCGCACCTTCATGCT
TT704, Exon5 (Forward Primer)	GCCCAGGACCCGCCGTCTGAGC
TT848, Exon9 (Forward Primer)	CTGCTCTGCAGCCTGTGCG
TT1015, Exon14 (Forward Primer)	GCCATCCTCTCAGGTTTCACGCACGTGT
TT1090, Exon15 (Forward Primer)	GCTGACTCGACACCGTGTCACCTATGC
TT-exon2R (Reverse Primer)	CCTCACCTGGGCTCCTGC
TT-exon5R (Reverse Primer)	CCTGGTGGCTGAGCCGTTG
TT-exon9R (Reverse Primer)	AGTCCTCAGGCTGTGCAAC
TT-exon14-15R (Reverse Primer)	GACACCAGCGTTTAATCACATAG

[$]: Primers used to generate mutants of *TERC* and *TERT* are named according to their mutations.

[*]: Positions of deleted bases are underlined in red.

[#]: Base substitutions are indicated in red.

2.2.2 Genomic DNA preparation

Peripheral blood (EDTA, 5ml) was collected from a healthy individual with informed consent (approved by the Faculty of Health Sciences Research Ethics Committee, University of Cape Town -HREC REF: 131/2011). A concentrated white cell buffy layer was obtained by centrifugation of the sample at 2500 rpm for 10 minutes and the DNA from this layer was extracted using the QIAmp® DNA blood mini kit (Qiagen, USA). Briefly, 200µl of the buffy layer was mixed with proteinase K (4U/mg) to degrade proteins, RNAse A (10mg/ml) to degrade RNA and 200µl lysis buffer (contained 5% SDS and low pH). This solution was incubated at 56^0C for 10 minutes to aid cell lysis.

Thereafter 200µl of ethanol was added to the sample to dehydrate the DNA (aids in silica binding) before loading onto the silica QIAamp® mini spin column, via centrifugation (8000 rpm for 1 minute). The bound DNA was washed sequentially with two buffers (AW1 and AW2) to eliminate contaminants (proteins and salts) (500µl each buffer, 8000 rpm for 1 minute). Residual wash buffer was removed by a final centrifugation at 14 000 rpm for 1 minute. The DNA was finally eluted from the silica column by incubation with 200µl nuclease-free water at room temperature for 1 minute followed by centrifugation at 8000 rpm for 1 minute. The concentration of DNA was quantified using a UV-Vis Spectrophotometer Nano-Drop 2000 (Thermo Scientific, USA) and stored at -20^0C.

2.2.3 SDM PCR for generation of *TERC* and *TERT* mutants

PCR reactions for the generation of each mutant consisted of the following in a final reaction volume of 25µl: 1X Green GoTaq® Flexi Buffer (Promega, USA), 2U GoTaq® Hot Start Polymerase (Promega, USA), 0.8mM dNTPs, 2mM magnesium chloride (Promega, USA), 25pmol of each forward and reverse primer (Table 2.1), and 40ng human genomic DNA. The PCR cycling conditions were as follows: polymerase activation and initial genomic DNA denaturation at 95°C for 4 minutes, followed by 35 cycles of template denaturation at 94°C for 30 seconds, primer annealing at a range of 55°C- 65°C for 30 seconds, and extension at 72°C for 90 seconds. A final extension step of 72°C for 8 minutes was included to ensure amplicon completion. PCR products were resolved by agarose gel electrophoresis, using a 2% TAE agarose gel and electrophoresis in 1X TAE buffer (40mM Tris, 20mM acetic acid, 1mM EDTA) at 75 volts for ±1.5 hours. The DNA amplicons were stained with ethidium bromide (0.5 µg/ml) (Sigma, USA) and visualized under UV light using the UV doc system (UVItec, UK).

2.2.4 Purification of PCR products

Following successful amplification, each relevant amplicon band was excised from the gel and purified using the QIAquick® PCR purification kit (Qiagen, USA) following the manufacturer's instructions. Briefly, the excised gel fragment was dissolved in 3 gel volumes of buffer QG, by incubation at 50°C for 10 minutes. To aid in binding of the DNA to the silica column, one gel volume of isopropanol (Sigma, USA) was added and the solution loaded onto the QIAquick® Minielute column followed by centrifugation at 13000rpm for 1 minute.

The bound DNA was washed with 500µl of buffer QG (13000 rpm, 1 min), followed by a second wash with 750µl of buffer PE (to remove salts) (13000rpm, 1 min). Residual ethanol from the PE buffer was removed via a second centrifugation (13000rpm, 1 minute). Finally the amplicon DNA was eluted with 10µl nuclease-free water and quantified using a UV-Vis Spectrophotometer Nano-Drop 2000 (Thermo Scientific, USA) and stored at -20°C.

2.2.5 Cloning of PCR products into pGEMT-Easy

2.2.5.A: Ligation

Purified PCR products (section 2.2.4) containing the relevant mutations were cloned into the pGEMT-Easy bacterial vector using T4 DNA ligase (Promega, USA). A vector: insert ratio of 1:3 was used for all ligation reactions, with 50ng of pGEM®T-Easy vector being used in each reaction. To calculate the relevant amount of insert (purified PCR product) the following formula was used: [(50ng of vector × kb size of insert) ÷ (kb size of vector) × ratio of 3 = Xng of insert required for the reaction] (Promega Bio Math online, http://www.promega.com/techserv/tools/biomath/calc06.htm). The ligation reaction was performed according to the manufacturer's recommendations (Promega, USA), with a 10µl reaction volume and incubation at 4°C overnight.

2.2.5.B: Transformation and small scale DNA preparation

An aliquot of the ligation reaction (2µl) was mixed with 100µl of calcium-chloride prepared competent *Escherichia coli (E. coli)* (*DH5 Alpha* strain) (Dagert *et al.,* 1979) and incubated on ice for 25 minutes. Thereafter the samples were subjected to heat shock by incubating the cells at 45^0C for 2 minutes and chilled on ice for a further 5 minutes to allow DNA uptake. This was followed by the addition of 900µl Luria Broth (LB) bacterial medium and incubation for 1 hour at 37^0C, to allow expression of the enzyme beta-lactamase, responsible for resistance to ampicillin in the transformed bacteria, before the selection pressure was applied. An aliquot of the transformation mix (100µl) was plated onto LB-Agar plates containing 50µg/ml ampicillin (Sigma-Aldrich), 40µg/ml 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) (Sigma-Aldrich) and, 0.1mM isopropyl beta-D-thiogalactopyranoside (IPTG) (Sigma-Aldrich) and incubated overnight (16 hours) at 37^0C. White bacterial colonies, indicating successful transformation were selected and cultured in 5ml LB media containing ampicillin 50µg/ml overnight at 37^0C.

Plasmid DNA was extracted from these O/N cultures using the Nucleobond® Xtra maxi-prep kit (Macherey-Nagel, USA), following manufacturer's instructions with modification. Bacteria from 2ml of O/N culture were harvested by centrifugation (6000rpm, 15 minutes, 4^0C) and the supernatant discarded. Bacterial cell pellets were resuspended in 300µl buffer N1 containing RNase A, followed by an addition of 300µl buffer N2 and incubation at room temperature for 5 minutes to allow cell lysis. Pre-cooled buffer N3 (300µl) was added to precipitate the proteins (5 minutes, on ice), and then the sample was centrifuged (14 000rpm, 30 minutes, 4^0C) and the clear supernatant transferred to a new tube. Isopropanol (0.7 volumes) was added to precipitate the DNA, followed by immediate centrifugation (14 000rpm, 10 minutes, 4^0C) and washing of the DNA pellet with 1ml 70% ethanol to remove salts (14 000rpm, 5 minutes, 4^0C). The plasmid DNA was resuspended in 50µl nuclease-free water (50µl) and then digested with restriction enzymes to verify successful cloning (see below).

2.2.5.C: Analysis of clones using Restriction Enzyme digestion

To confirm the cloning of amplicons into the pGEM®T-Easy vector, the purified plasmids (section 2.2.5.B) were digested with *EcoR1* (Promega, USA) to release the amplicon insert from the vector. Each restriction enzyme digestion reaction (20µl) contained the following, 1X restriction enzyme buffer (Promega, USA), 1µg plasmid DNA, 10U *EcoR1* (Promega, USA), and nuclease-free water (Qiagen, USA). The restriction enzyme digestion mix was incubated at 37^0C for 2 hours and the digested DNA was resolved on a 2% agarose TAE gel (75V, 1.5 hours). The restricted products were stained with ethidium bromide (0.5µg/ml) and visualized under UV light using the UV doc system (UVItec, UK).

2.2.5.D: Large scale plasmid preparation

After the restriction enzyme digests confirmed successful cloning of inserts (section 2.2.5.C), corresponding bacterial cultures were inoculated in 50ml of LB media containing 50µg/ml ampicillin and grown O/N at 37˚C, with vigorous shaking. Large-scale, high quality plasmid DNA was extracted from the cultures using the Nucleobond® Xtra Maxi-prep kit (Macherey-Nagel, USA), following manufacturer's instructions. Briefly, overnight cultures (40ml) were collected using centrifugation (6000rpm, 15 minutes, 4^0C) and the supernatant removed. Bacterial cell pellets were resuspended in 12ml buffer S1 (RNase A added), followed by an addition of 12ml buffer S2 and incubation at room temperature for 5 minutes to allow cell lysis.

Pre-cooled buffer S3 (12ml) was added to precipitate the proteins (5 minutes, on ice), followed by filtering to remove cell debris and proteins through a filter paper flow-through system. The cleared lysate was loaded onto a hydrated column and the bound DNA was washed twice with buffer N3 (2X 32ml) to remove residual salts. The DNA was finally eluted in 15ml buffer N5 and precipitated using 0.7 volume isopropanol. The precipitated DNA was collected via centrifugation (14000rpm, 30 minutes, 4^0C) and washed with 70% ethanol to remove residual salts (14000rpm, 10 minutes, 20^0C).. The plasmid DNA was reconstituted by dissolving the pellet in nuclease-free water (Qiagen, USA). Purity and quantity of DNA was determined by UV-Vis Spectrophotometer Nano-Drop 2000 (Thermo Scientific, USA) and stored at -20°C.

2.2.6 Sequence analysis of DNA fragments

The *TERC* and *TERT* purified PCR products and plasmid constructs were sequenced using the ABI Prism BigDye® Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, USA). Each sequencing reaction consisted of BigDye® mix (0.25X), sequencing buffer (0.3X), 3.2pmol of the relevant reverse primer (Table 2.1), 10ng of each individual mutant PCR product or 10ng plasmid construct and nuclease-free water to a final volume of 10µl. The cycling conditions for the sequencing reaction were as follows: 96°C for 15 seconds, 30 cycles at 96°C for 15 seconds, 50°C for 15 seconds and 60°C for 4 minutes. Fragments were analysed on an ABI3120 genetic analyser (Applied Biosystems, USA) at the DNA Sequencer Unit, Stellenbosch University. The sequences were analysed using the Chromas Lite software (Technelysium, Australia) and BIOEdit software (BIOEdit, UK).

2.3 RESULTS

2.3.1 Experimental design

The mutations that were selected for inclusion in the proposed diagnostic assay were based on their association with a telomerase malfunction and confirmed association with IST failure in AA patients (Yamaguchi *et al*., 2005, Vulliamy et al., 2007, DU *et al.*, 2009). Table 2.2 shows the list of clinically relevant mutations chosen for this study.

Table 2.2: Summary of *TERC* and *TERT* mutations used in this study

Mutation*	DNA base change Ω	Region€	Amino Acid Changeβ	Reference
*TERC*53	Del53-87	*TERC* gene	N/A	Du *et al.*,2009
*TERC*110	Del110-113	*TERC* gene	N/A	Vulliamy *et al*.,2007
*TERC*178	G178A	*TERC* gene	N/A	Vulliamy *et al*.,2007
*TERC*180	C180T	*TERC* gene	N/A	Vulliamy *et al*.,2007
*TERT*202	G763T, C764A	Exon 2	A202Y	Yamaguchi *et al.*, 2005
*TERT*412	C139T	Exon 2	H412Y	Yamaguchi *et al*., 2005
*TERT*694	G15704A	Exon 5	V694M	Yamaguchi *et al.*, 2005
*TERT*704	C15753T	Exon 5	P704S	Du *et al*.,2009
*TERT*846	A26461G	Exon 9	Y846C	Yamaguchi *et al*., 2005
*TERT*1015	T39644C	Exon14	C1015R	Calado *et al*.,2008
*TERT*1090	G40650A	Exon15	V1090M	Yamaguchi *et al.*,2005

***** **:** The *TERC* (light red shaded) and *TERT* mutants

Ω: DNA based change refers to specific DNA sequence on the NCBI database with accession number NC 000005 for *TERT* and NR 001566 for *TERC.*

€: Region represents the part where mutations occur on the *TERC* gene and *TERT* gene and exon depicts the position a mutation is found in the DNA sequence of *TERT* gene and N/A refers to not applicable.

β: Amino acid change shows the modification in the protein synthesized due to DNA base changes in the sequence indicated by the corresponding single-letters

To create the desired controls for detection of these specific mutations, point mutations and deletions were introduced into amplicons using forward primers containing the various mutations (SDM). *TERC* is a short intron-less gene, encoding the RNA template (451bp) for telomerase (Yamaguchi *et al.* 2003), making it possible to use a common reverse primer (TCR) with four mutated forward primers (TC53, TC110, TC178 and TC180) to generate the desired amplicons. The specific SDM primers are listed in Table 2.1 (section 2.2.1 of the Material and Methods), while Figure 2.1 shows the binding positions of these primers.

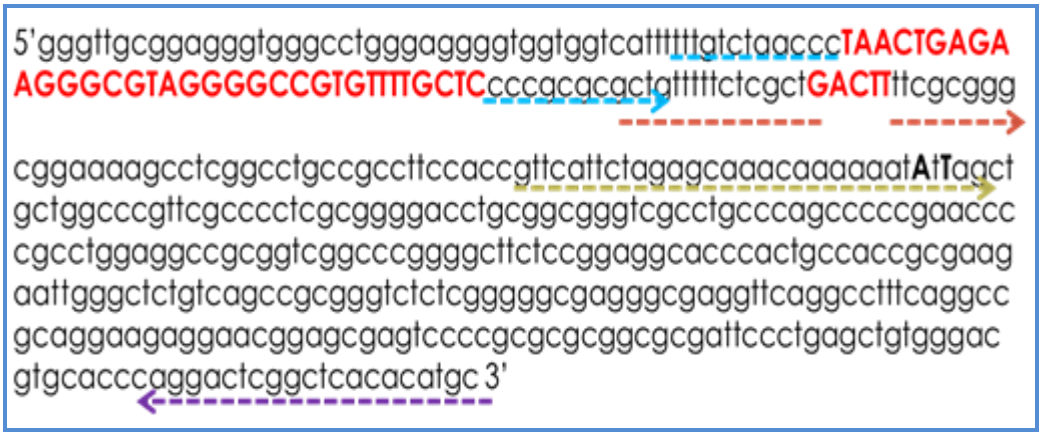

Figure 2.1: Binding positions of the SDM primers used to generate *TERC* mutants.

Nucleotide sequence of *TERC* (from position 1-421), NCBI database NR 001566, used to generate mutants. The colour-coded arrows indicate the location (5' - 3' orientation) of the SDM primer sets (Table 2.1), used for generation of mutants *TERC*53, *TERC*110, *TERC*178 and *TERC*180**.** The red bold nucleotides show an area where a deletion is generated and black bold indicates where a single base change is introduced. The purple line indicates the common reverse primer for all forward primers.

TERT is a large 45kb gene, comprising 16 exons. The clinically relevant mutations chosen for the study (Table 2.2) were spread over 5 exons, requiring multiple forward/reverse primer sets to amplify the relevant areas. Figures 2.2 and 2.3 show the binding positions of the various primer sets, which included the specific SDM forward primer and an exon-specific reverse primer (details given in Table 2.1) to allow the creation and amplification of all the mutant products. Some exons contained more than one relevant mutation, which then allowed a common reverse primer for the generation of those mutants (i.e. exons 2 and 5). Due to their close proximity, amplification of the exon 14 and 15 mutations was also achieved with a common reverse primer.

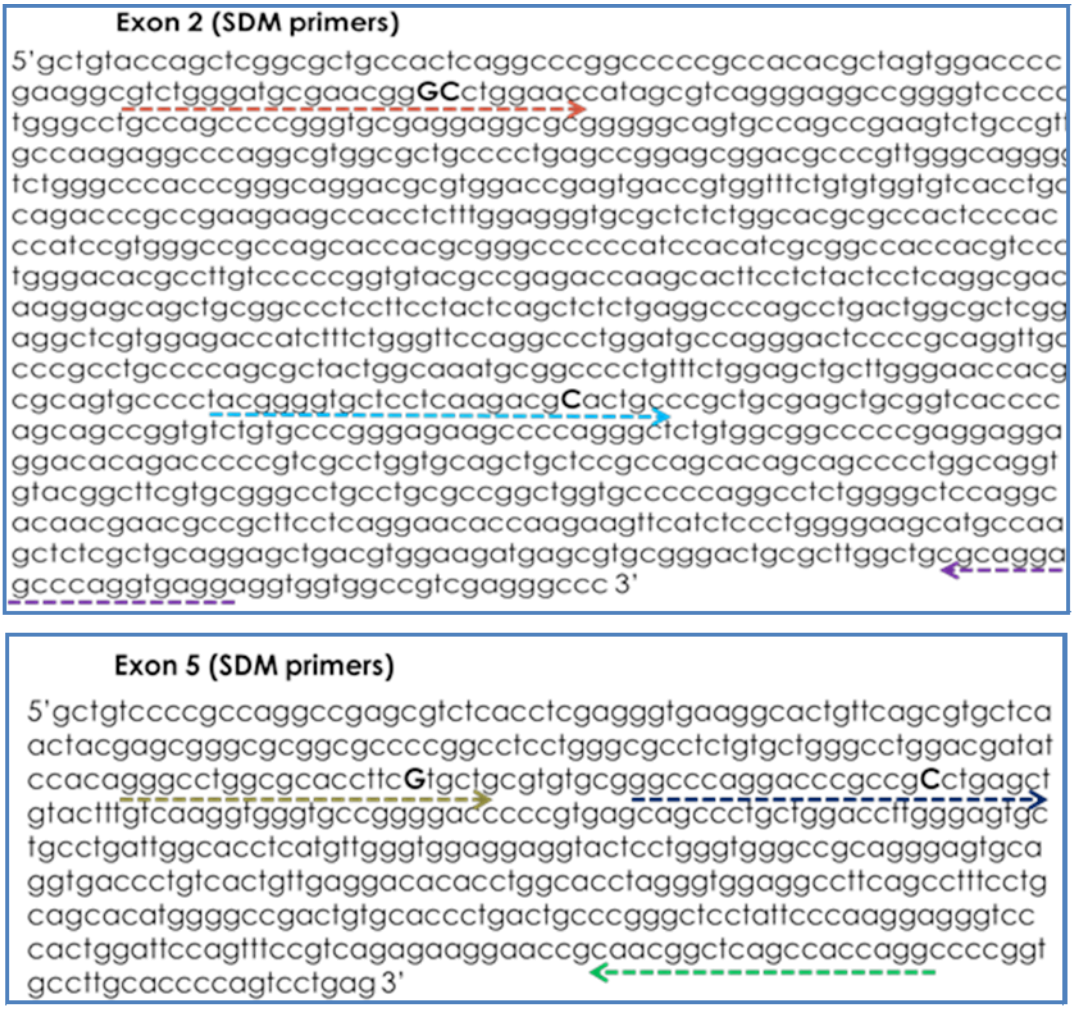

Figure 2.2: Binding positions of SDM primers for the amplification of *TERT* mutations in exons 2 and 5

Nucleotide sequences of exon 2 (682- 1760) and exon 5 (15575- 16042) of the human *TERT* gene (NCBI database with accession number NC 000005), which was used to generate *TERT*202, *TERT*412, *TERT*694 and *TERT*704 mutants. The colour-coded arrows indicate the binding positions (5' - 3' orientation) of the primers that were used to generate each corresponding mutation (sequences given in Table 2.1). The bold nucleotides show an area in the WT sequence where a mutation was introduced to generate the desired mutant. The purple line indicates the common reverse primer on the exon 2, while the green line shows the common reverse primer on the exon 5.

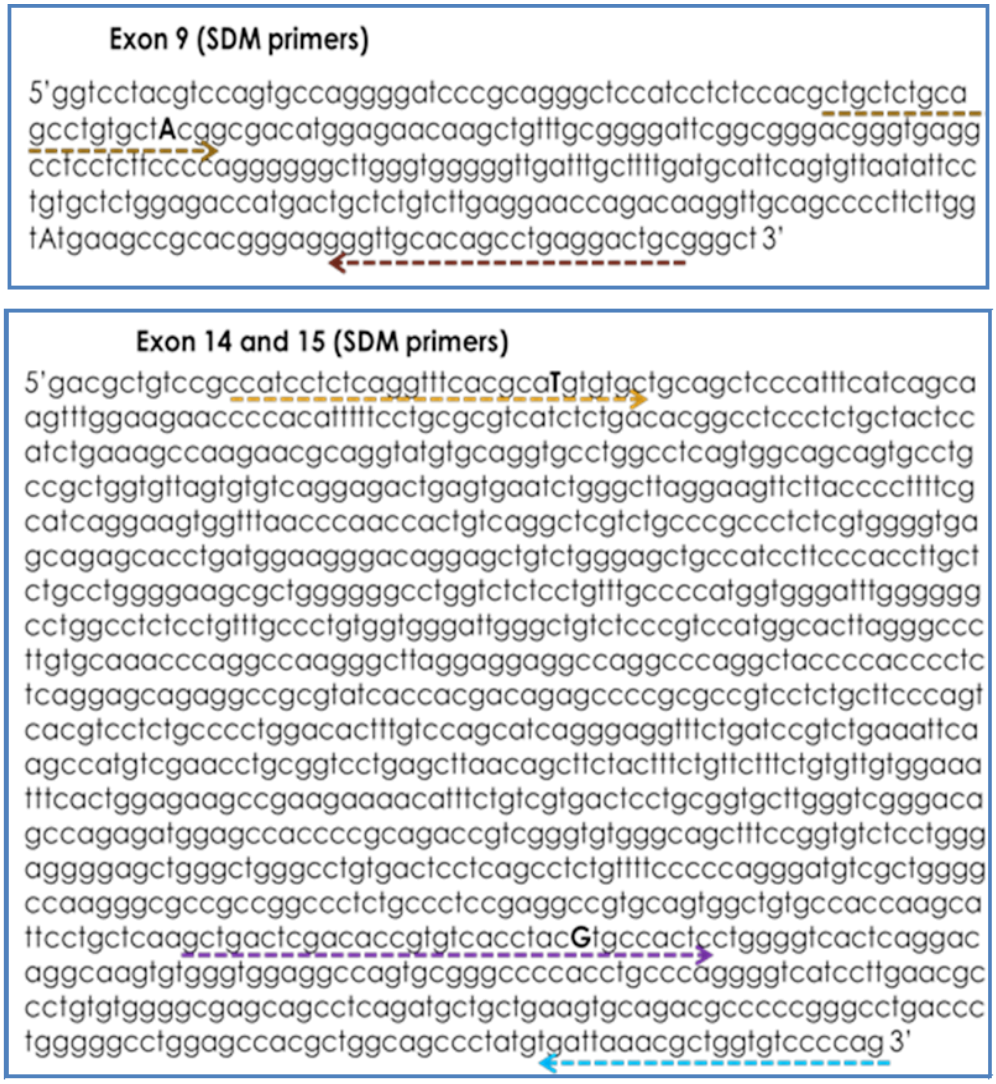

Figure 2.3: Binding positions of SDM primers for the amplification of *TERT* mutations in exons 9, 14 and 15

Nucleotide sequences of exon 9 (26412- 26710), exon 14 and 15 (39611- 40850) of the human *TERT* gene (NCBI database with accession number NC 000005), which was used to generate *TERT*846, *TERT*1015 and *TERT*1090 mutants. The colour-coded arrows indicate the binding position (5' - 3' orientation) of the primers that were used to generate each corresponding mutation (sequences given in Table 2.1). The bold nucleotides show an area in the WT sequence where a mutation was introduced to generate the desired mutant. The brown line indicates the reverse primer on the exon 9 and the line blue line shows the common reverse primer on the exon 14 and 15 respectively.

2.3.2 Creation of SDM *TERC* mutant amplicons

DNA from the blood of a healthy individual was isolated (described in section 2.2.2) and used as a template with the specific SDM primers listed in Table 2.1 to amplify fragments containing the desired mutations. For the generation of all *TERC* mutant amplicons, a temperature gradient of 55^0C- 64^0C was used to identify the most appropriate annealing temperature to generate a specific, single and well-amplified product (where possible) that could be excised and used for subsequent sequence analysis and cloning into the pGEMT-Easy vector. PCR reactions were performed as described in section 2.2.3, with *TERC*53 SDM amplicon created with primers TC53 and TCR (Table 2.1) (544bp), *TERC*110 SDM with primers TC110 and TCR (470bp), *TERC*178 SDM amplicon with primers TC178 and TCR (445bp) and *TERC*180 SDM amplicon with primers TC180 and TCR (450bp). A reagent blank, negative control (NC) was also included in each PCR experiment to monitor contamination. Figure 2.4 shows the results analysis of all 4 mutants: *TERC*53 SDM amplicon, *TERC*110 SDM amplicon, *TERC*178 SDM amplicon, and *TERC*180 SDM amplicon.

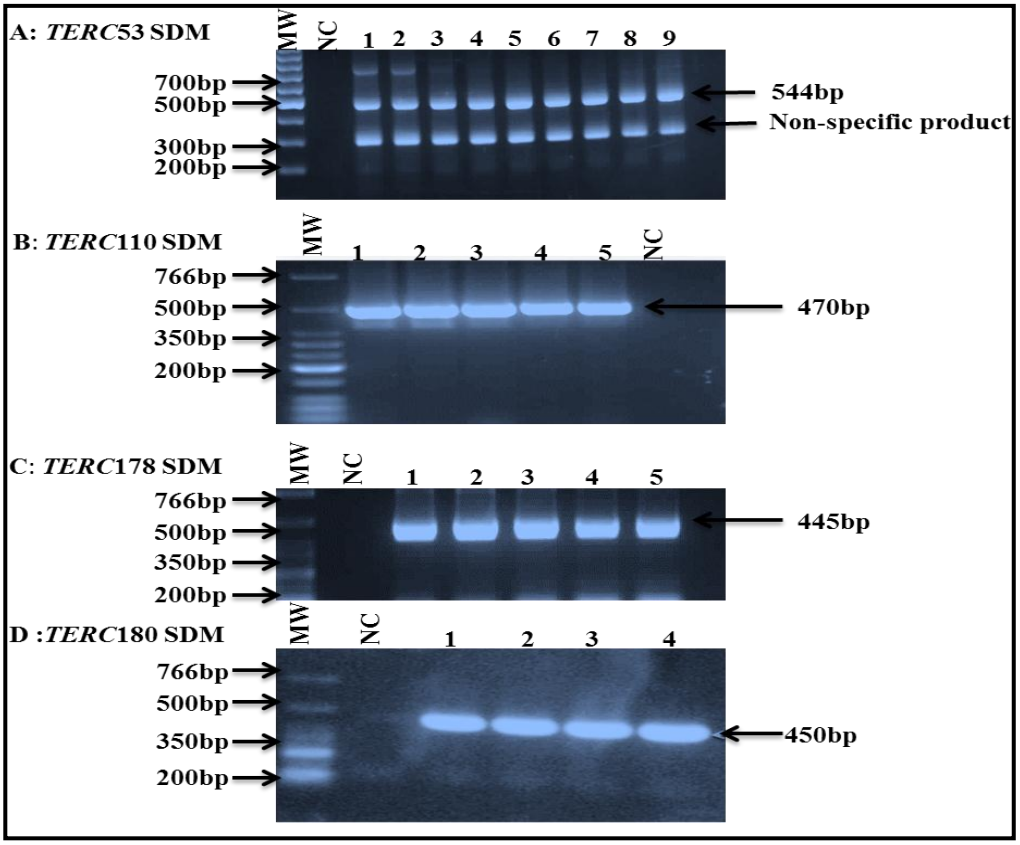

Figure 2.4: Electrophoretic analyses of annealing temperature gradient PCR for *TERC* mutant amplifications using SDM primers

SDM *TERC* amplicons generated using specific SDM primers. A temperature gradient was used for the annealing temperature to determine the most appropriate amplification conditions. This is a photograph showing the amplicons resolved a 2% agarose TAE gels, stained with ethidium bromide and visualized with UV light. Lane MW: low molecular weight ladder (NEB), lane NC: negative control and lane numbers refer to the annealing temperature used. Panel A: *TERC*53 amplicons, lane 1: 58°C, 2: 60°C, 3: 62°C, 4: 63°C, 5: 64°C; B: *TERC*110, lane 1: 58°C, 2: 60°C, 3: 61°C, 4: 63°C, 5: 64°C; C: *TERC*178, lane 1: 55°C, 2: 56°C, 3: 57°C, 4: 58°C, and 5: 60°C; and D: *TERC*180, lane 1: 59 °C, 2: 61°C, 3: 63°C, and 4: 64°C.

For *TERC*110, *TERC*178 and *TERC*180, single amplicons of the expected sizes were clearly observed with the following annealing temperatures yielding optimal results: 64^0C for *TERC*110 (Figure 2.4.B), 56^0C for *TERC*178 (Figure 2.4.C), and 63^0C for mutant *TERC*180 (Figure 2.4.D). However, the amplification of *TERC*53 consistently produced a non-specific band over this temperature gradient (Figure 2.4.A).

To eliminate non-specific banding, various approaches were undertaken including increasing the annealing temperature range, magnesium chloride titration (1.5mM- 3mM) and decreasing the PCR extension time. Unfortunately the amplification of *TERC*53 persisted in yielding non-specific products (results not shown). Products were again created and amplified using the most appropriate annealing temperatures as indicated, with *TERC*53 being amplified at 64^0C. The expected sized amplicons were excised and purified as described in section 2.2.3 and 2.2.4, and analysed for the presence of mutations via sequence analysis using the TCR primer (section 2.2.6). Sequences were aligned to the WT human *TERC* gene (NCBI database, accession number: NR 001566) and two examples of this analysis are shown in Figure 2.5. This figure shows the sequence alignment of the *TERC*53 SDM amplicon (and the creation of the desired deletion of bases 53-87) with its corresponding WT human *TERC* sequence (panel A), as well as SNP *TERC*178 (G178A) (panel B). All the *TERC* mutations as described in Table 2.2 (section 2.3.1) were successfully generated using this approach.

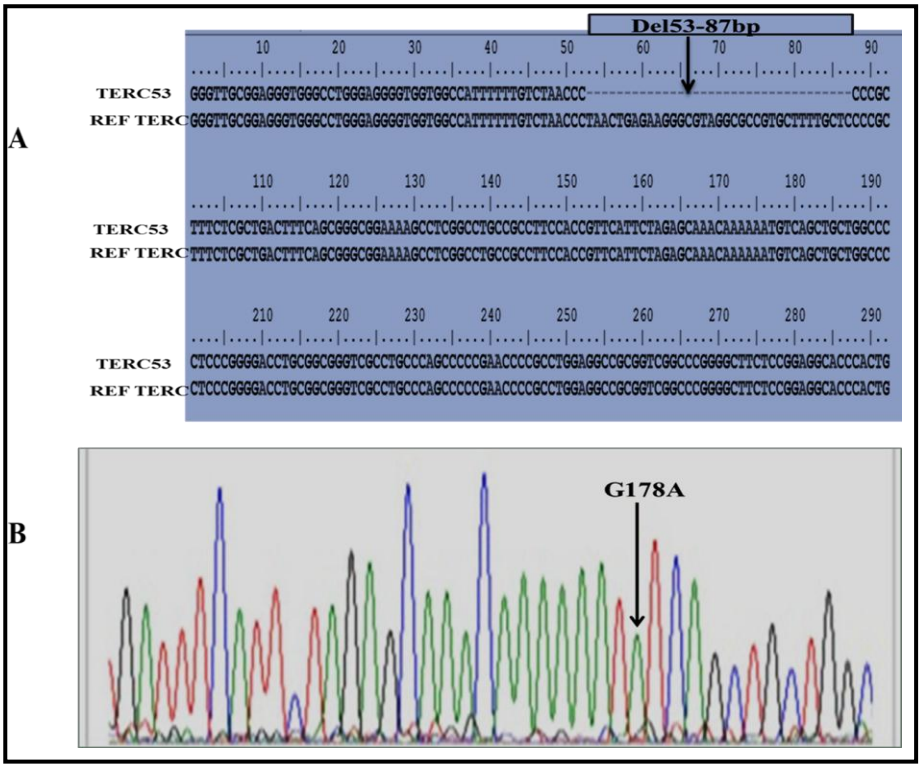

Figure 2.5: Sequence analysis of *TERC*53 and *TERC*178 SDM products

Panel A**:** Alignment of *TERC*53 SDM amplicon (reverse complement sequence) and WT *TERC* gene (REF *TERC*) (NCBI NR 001566) from position (1- 292) indicating the deleted region 53- 87 in the *TERC*53 sequence. Panel B: Sequencing chromatogram of *TERC*178 SDM amplicon (reverse complement sequence) of PCR product (128-187bp) showing the mutated region (G178A).

2.3.3 Creation of mutant *TERT* amplicons

The DNA from a healthy individual, isolated as described in section 2.2.2, was used as a template with the specific SDM primers listed in table 2.1 to produce PCR amplicons containing the desired *TERT* mutations. As was done for the *TERC* mutants, an annealing temperature gradient was performed to identify optimal conditions for the generation of a single specific fragment, without non-specific amplicons. PCR reactions were performed as described in section 2.2.3 (using a temperature gradient of 55ºC- 64ºC for the annealing step) with mutant *TERT*202 amplified with primers TT202 and TT-exon2R (Table 2.1) (900bp), *TERT*412 amplified with primers TT412 and TT-exon2R (378bb), *TERT*694 amplified with primers TT694 and TT-exon5R (356bp), mutant *TERT*704 amplified by primers TT704 and TT-exon5R (323bp), *TERT*846 amplified with primer TT846 and TT-exon9R (243bp), *TERT*1015 amplified with primers TT1015 and TT-exon14-15R (1224bp) and mutant *TERT*1090 amplified with primers TT1090 and TT-exon14-15R (221bp). To control for DNA contamination in the PCR reagents, a reagent blank/negative control (NC) was also performed in each experiment.

Single specific bands, of the correct size were amplified for mutant fragments *TERT*412, *TERT*694, *TERT*704, *TERT*1015 and *TERT*1090 at all annealing temperatures used. Figure 2.5 below shows some of these results.

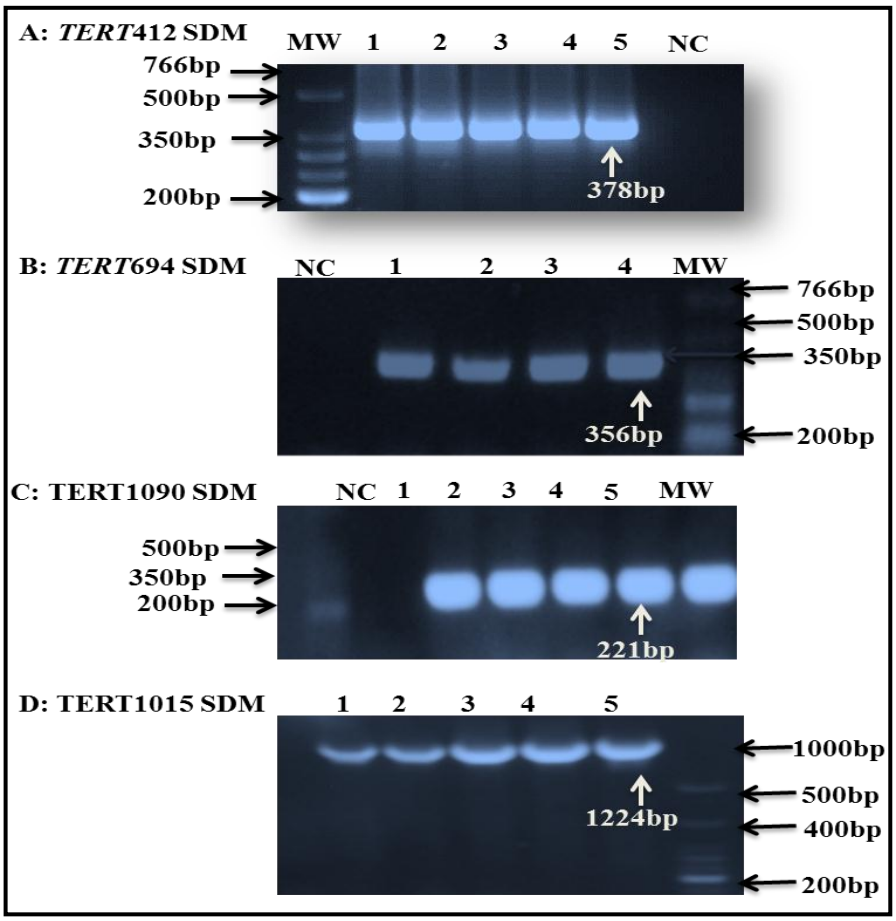

Figure 2.6: Electrophoretic analyses of annealing temperature gradient PCR for *TERT* mutant amplifications using SDM primers.

SDM *TERT* amplicons generated using specific SDM primers. The photograph shows the amplicons resolved on 2% agarose TAE gels, stained with ethidium bromide and visualized with UV light. Lane MW: low molecular weight ladder (NEB), lane NC: negative control. Lane numbers in each panel refer to the annealing temperature used. Panel A: *TERT*412 SDM amplicons, lane 1: 56°C, 2: 58°C, 3: 60°C, 4: 62°C, 5: 64°C; B: *TERT*694, lane 1: 58°C, 2: 60°C, 3: 64°C, 4: 64°C; C: *TERT*1090, lane 1: 56°C, 2: 58°C, 3: 60°C, 4: 64°C; and D: *TERT*1015, lane 1: 59 °C, 2: 61°C, 3: 63°C, and 4: 64°C.

In the case of mutants *TERT*202 and *TERT*846, multiple bands were generated at all annealing temperatures used, and although there was an increase in amplification of the band of the correct size as the annealing temperature was gradually increased, non-specific fragments were also consistently amplified. Further optimization was carried out to try and improve specific amplification of these two mutants by performing a magnesium chloride titration ranging from 1- 3mM. However, this did not improve the result significantly (results not shown).

Figure 2.6 above shows examples of troubleshooting the non-specificity experienced when amplifying mutants of *TERT*202 and *TERT*846. In addition to temperature gradient and magnesium chloride titration, a decrease of PCR extension time was also performed but the non-specific products were consistently amplified.

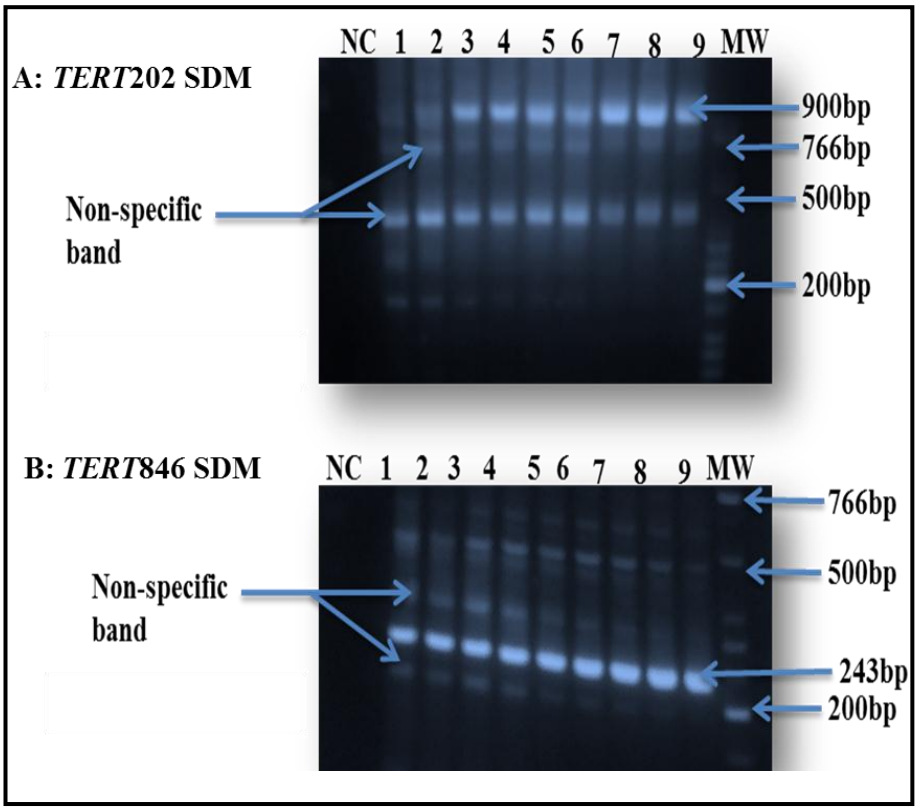

Figure 2.7: Electrophoretic analyses of annealing temperature gradient PCR for *TERT202* and *TERT*846 mutant amplifications using SDM primers.

SDM PCR products were created using specific SDM primers. The above photograph shows the amplicons resolved on 2% agarose TAE gels, stained with ethidium bromide and visualized with UV light. A temperature gradient was used for the annealing temperature to identify the most suitable conditions for amplification. Lane MW: low molecular weight ladder (NEB), lane NC: negative control. Lane numbers refer to the annealing temperature used. Panel A: *TERT*202, Panel B: *TERT* 846. Lanes showing annealing temperatures used. Panel A: *TERT*202 SDM amplicons, lane 1: 55°C, 2: 56°C, 3: 58°C, 4: 59°C, 5: 60°C, 6: 61°C, 7: 62°C, 8: 63°C; 9: 64°C, B: *TERT*694, lane 1: 55°C, 2: 56°C, 3: 58°C, 4: 59°C, 5: 60°C, 6: 61°C, 7: 62°C, 8: 63°C; 9: 64°C.

The SDM PCR reactions were repeated using the following specific annealing temperatures: 60°C for *TERT*412 and *TERT*1015, 62°C for *TERT*1090 and *TERT*704, and 64°C for *TERT*694. For *TERT*202 and *TERT*846, SDM PCR reactions were repeated using an annealing temperature of 65°C, which displayed the least non-specific amplification. All SDM amplicons were excised and purified as described in section 2.2.4 and sequenced as described in section 2.2.6 to check for the creation of the specific mutations via sequencing analysis. The *TERT*202 and 412 amplicons were sequenced using the reverse primer TT-exon2R (Table 2.1), *TERT*694 and 704 with primer TT-exon5R (Table 2.1), *TERT*846 with primer TT-exon9R (Table 2.1) and *TERT*1015 and 1090 with TT-exon14-15R (Table 2.1).

To verify and confirm the presence of the desired mutations before cloning into pGEM®T-Easy, the sequencing products were aligned using BIOEdit to the reference human *TERT* gene (NCBI database with accession number: NC 000005). Figure 2.8 below shows sequencing analysis of *TERT*202 SDM product and *TERT*846 SDM product as example of other sequencing analyses.

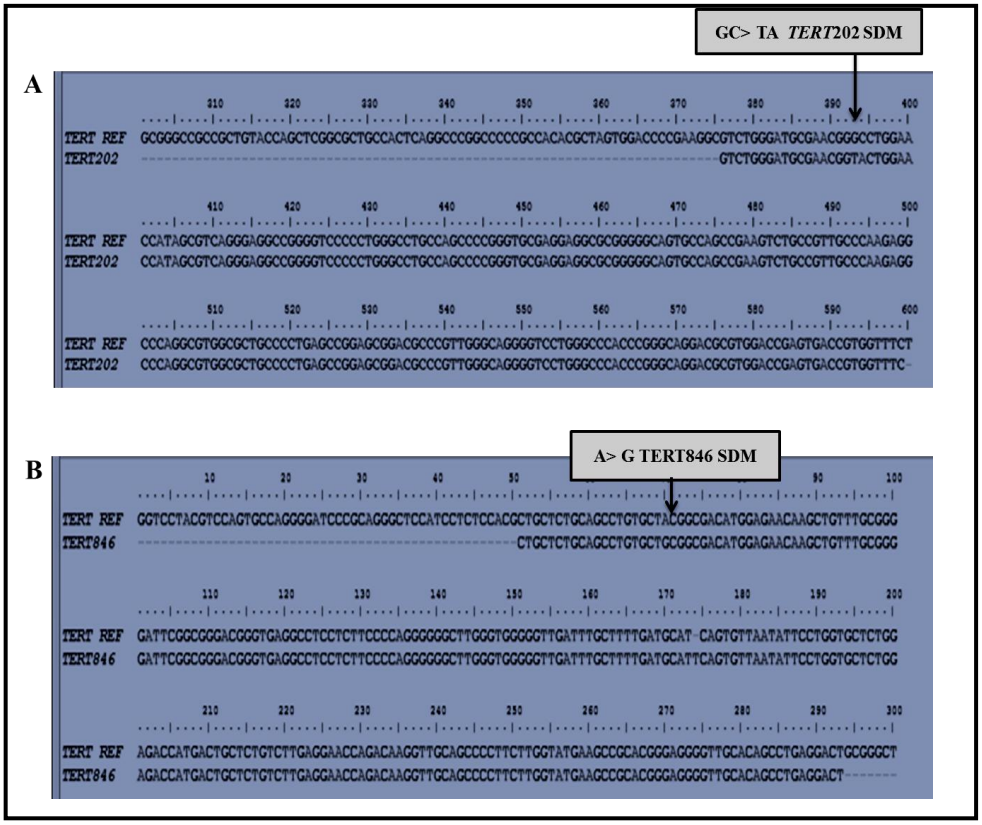

Figure 2.8: Sequence analysis of *TERT*202 and *TERT*846 SDM products

Panel A**:** Alignment of *TERT*202 SDM amplicon (reverse complement sequence) and WT *TERT* gene (NCBI database with accession number: NC 000005) from position (301- 600) indicating the mutations GC>TA in the *TERT*202 sequence. Panel B: Alignment of *TERT*846 SDM amplicon (reverse complement sequence) and WT *TERT* (exon 9) gene (NCBI database with accession number: NC 000005) from position (1- 300) indicating the base change A>G in the *TERT*846 sequence compared to the reference gene.

2.3.5 Cloning of SDM PCR amplicons *TERC* and *TERT* into the pGEMT-Easy plasmid

Following sequence confirmation, the SDM PCR products described above (for all *TERT* and *TERC* mutations) were cloned into pGEM®T-Easy as described in section 2.2.5. Amplicons were ligated at a ratio of 3:1 to pGEM®T-Easy and transformed into competent *E. coli*. White colonies (3-5) were selected on X-gal/IPTG plates; small scale cultures were established and plasmid DNA was prepared via small-scale plasmid preparations (section 2.2.5.B). To confirm the cloning of the SDM amplicon into the vector, restriction enzyme digests were performed on all of the selected clones (*TERC* and *TERT*), using *EcoR1* (section 2.2.5.C).

The multiple cloning site of the pGEM®T-Easy plasmid is flanked by recognition sites for *EcoRI*, which can be used to release the cloned amplicon (with 122bp of added vector sequence). Figure 2.9.A shows the results of several digests. Figure 2.9.A shows the successful digestion of plasmid DNA from a single *TERT*202-pGEMT-Easy prospective clone, showing the production of two fragments (lane 2) one representing the *TERT*202 SDM PCR product together with a small piece of plasmid (1022bp) another for pGEMT (2893bp). The plasmid from a single clone potentially containing *TERC*180 SDM product also gave the expected fragment sizes of 567bp for *TERC*180 SDM PCR product together with a small piece of pGEM®T) and 2893bp for pGEMT-Easy plasmid.

Figure 2.9.B represents the restriction enzyme digestions of plasmid DNA from clones of *TERT*846 SDM product and *TERT*1090 SDM. From the *TERT*846 SDM clone two fragments (lane 1 and 2) were produced, one indicating the *TERT*846 SDM product (365bp) with flanking pGEMT-Easy sequence and another corresponding to linearized pGEMT-Easy plasmid (2893bp), while *TERT*1090 SDM clone also produced two fragments, one representing *TERT*1090 SDM product (343bp) with flanking pGEMT-Easy sequence and the other corresponding to linearized pGEMT-Easy plasmid (2893bp).

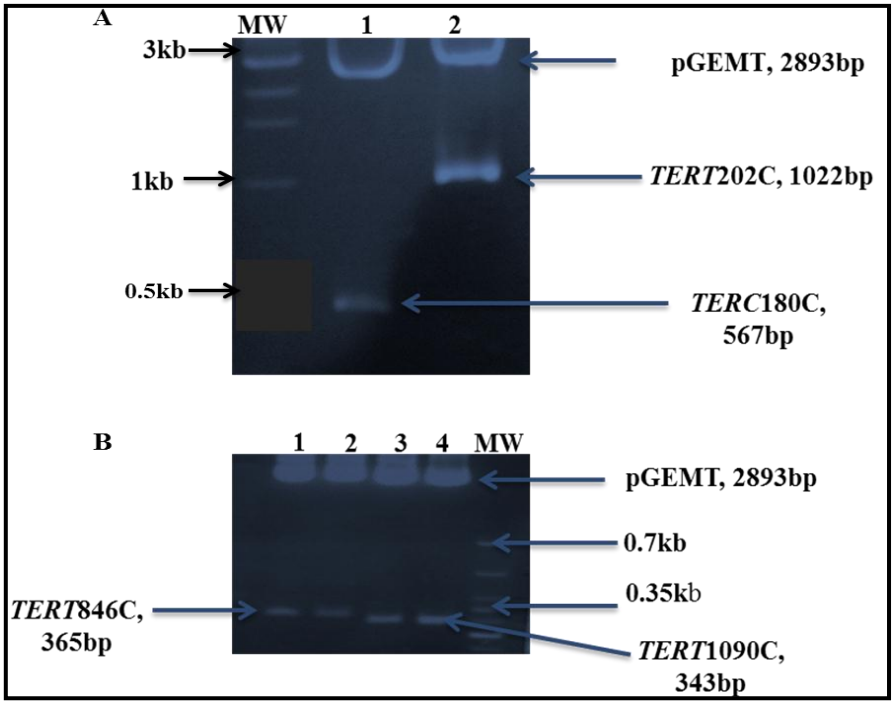

Figure 2.9: Electrophoretic analyses of restriction enzyme digests using *EcoRI* of *TERC* and *TERT* clones.

Restriction enzyme digests of *TERC* and *TERT* clones with *EcoR1* to release the cloned SDM products from the pGEMT-Easy. Digested fragments were resolved on 2% agarose TAE gels, stained with ethidium bromide and visualized over UV light. Lane MW: molecular weight ladder (NEB). Panel A: lane 1: *TERC*180C with flanking vector sequence (567bp) and linearized pGEMT backbone (2893bp), lane 2: *TERT*202C (900bp) with flanking vector sequence (1022bp) and pGEMT-Easy linearized backbone (2893bp). Panel B: lane 1 and 2: *TERT*846C with flanking vector sequence (365bp), and lane 3 and 4: *TERT*1090C with flanking vector sequence (343bp), and all four planes also contain the linearized pGEMT backbone (2893bp).

Selected clones of *TERC* and *TERT* from each cloning experiment were used to perform a large scale DNA preparation as described in section 2.2.5.D. Although the SDM PCR products were sequenced before cloning, there remained a small possibility that non-mutated or non-specific amplicons were present within the purified products. While restriction enzyme digestion confirmed the cloning of the correct sized amplicon, it did not reveal the mutational status of the cloned amplicons. Since cloning involves the expansion of a clone containing a single amplified product, it was essential to confirm that the correct mutated product had been cloned before it could be used as a positive control for the assay development.

Therefore, purified DNA from the plasmid constructs was sequenced according to section 2.2.6 and the sequences aligned using BIOEdit to either the human *TERC* DNA reference sequence (NCBI database with accession number: NR 001566) or the human *TERT* DNA reference sequence (NCBI database with accession number: NC 000005). Figure 2.10.A shows the result of an alignment of the cloned *TERC*110 SDM sequence with the WT human *TERC* gene. The presence of the relevant mutation is clearly seen.

Figure 2.10.B shows the sequence chromatogram of *TERC*180 cloned into pGEM®T-Easy, and again, the relevant mutation is present. These results are representations of how all of the cloned *TERC* mutants were verified. Similarly, Figure 2.11.A shows the results of an alignment of the cloned *TERT*412 SDM sequence with the WT human *TERT* gene. The presence of the mutation of interest is confirmed. Figure 2.11.B shows the sequence chromatogram of *TERT*704 cloned into PGEM®T-Easy vector, once more, the desired mutation is present. These results are representations of all the cloned *TERT* mutants were confirmed.

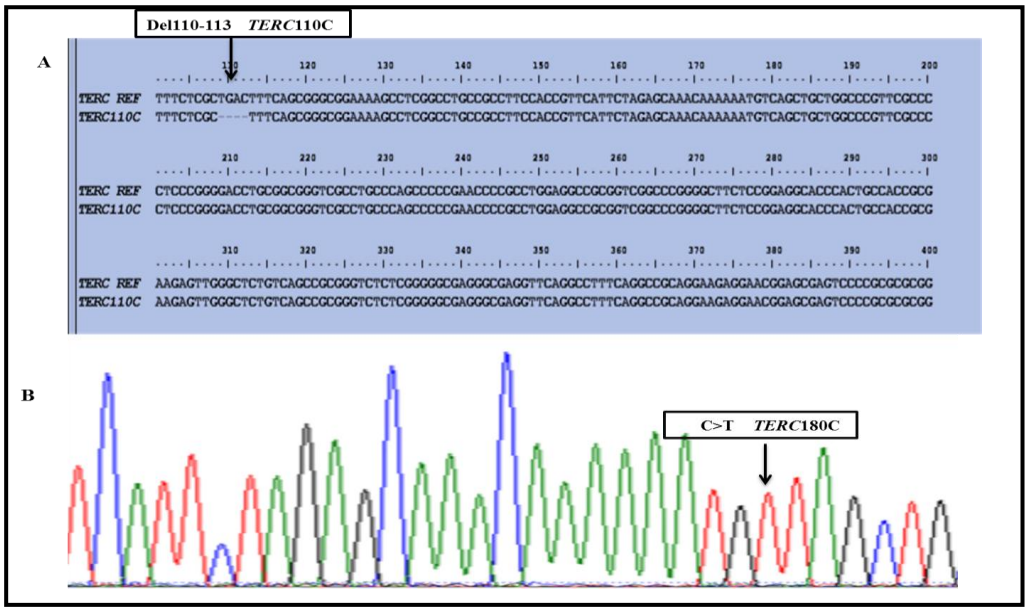

Figure 2.10: Sequence analysis of *TERC*110C and *TERC*180C cloned SDM products.

Panel A**:** Alignment of *TERC*110C SDM amplicon sequence (reverse complement sequence) cloned into pGEMT-Easy and WT human *TERC* gene (NCBI database with accession number: NR 001566) from position (91- 400bp) indicating the deletion (del110-113, TGAC) in the *TERC*110C sequence. Panel B: sequence chromatogram of *TERC*180C (reverse complement sequence) compared to the WT human *TERC* gene (NCBI database with accession number: NR 001566) showing the mutations (SNP, C>T) in the *TERC*180C sequence.

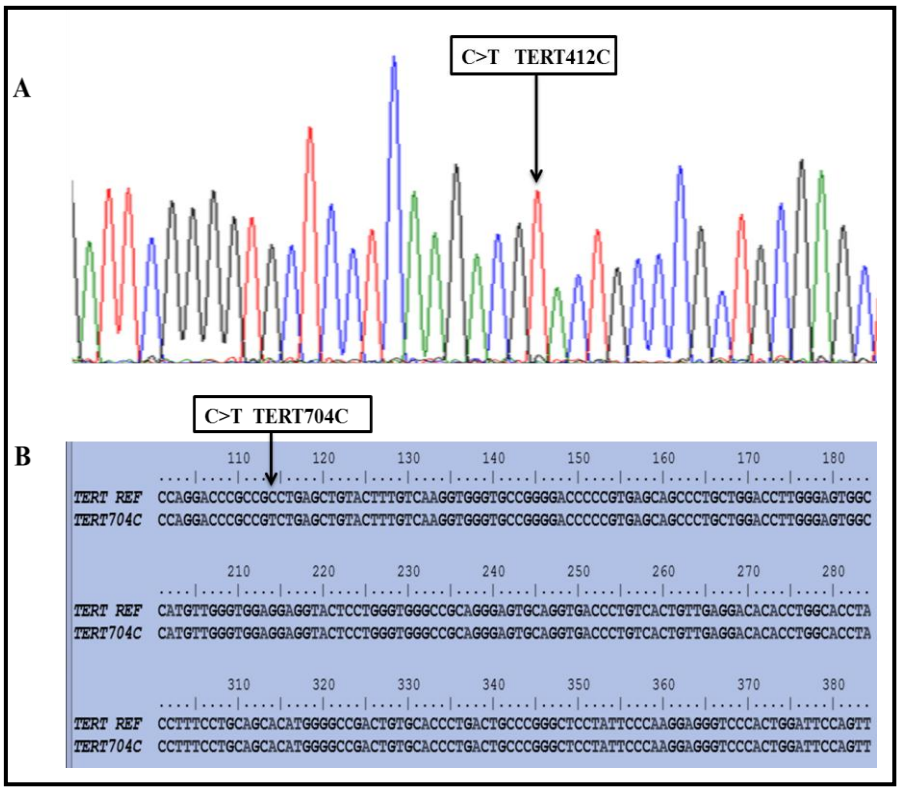

Figure 2.11: Sequence analysis of *TERT*412C and *TERT*704C cloned SDM products.

Panel A: Sequence chromatogram of *TERT*412C (reverse complement) compared to the WT human *TERT* gene (NCBI database with accession number: NC 000005) showing the based change (C>T) in the sequence of *TERT*412C. Panel B: sequence alignment of *TERT*704C cloned into pGEMT-Easy (reverse complement sequence) and WT human *TERT* gene (NCBI database with accession number: NC 000005) from position (101-384bp) indicating a mutation (SNP, C>T) in the *TERT*704C sequence.

Following this sequence confirmation the clones were then used for all further experiments and were named according to either the position of the DNA base change (compared to the wild type gene), as used for the *TERC* clones or the resulting amino acids substitution position in the *TERT* clones (compared to the wild type protein sequence). Hence these plasmid mutants were referred to as *TERC*53C, *TERC*110C, *TERC*178C and *TERC*180C for *TERC* gene mutations and *TERT*202C, *TERT*412C, *TERT*694C, *TERT*704C, *TERT*846C, *TERT*1015C and *TERT*1090C for *TERT* gene mutations. Table 2.3 gives details on the contents of each plasmid clone.

Table 2.3: List of plasmids containing *TERC* and *TERT* mutants

Mutant€	Forward and Reverse Primer£	PCR amplicon size¥	pGEM+Mutantβ
*TERC*53C	TC53 and TCR	544bp	3559bp
*TERC*110C	TC110 and TCR	470bp	3485bp
*TERC*178C	TC178 and TCR	445bp	3460bp
*TERC*180C	TC180 and TCR	450bp	3465bp
*TERT*202C	TT202 and TT-exon2R	900bp	3915bp
*TERT*412C	TT412 and TT-exon2R	378bp	3393bp
*TERT*694C	TT694 and TT-exon5R	356bp	3371bp
*TERT*704C	TT704 and TT-exon5R	323bp	3338bp
*TERT*846C	TT846 and TT-exon9R	243bp	3258bp
*TERT*1015C	TT1015 and TT-exon14-15R	122bp	4239bp
*TERT*109C	TT1090 and TT-exon14-15R	221bp	3236bp

€: Cloned SDM products of *TERC* and *TERT* into pGEMT-Easy

£: Primers used to generate SDM products

¥: SDM PCR product sizes.

β: Cloned SDM products of *TERC* and *TERT* (SDM product + pGEMT-Easy)

2.4 DISCUSIONS AND CONCLUSIONS

AA is a life threatening disease which may cause early deaths to the affected patients (Young *et al*., 2000, Gordon-Smith *et al*., 2001). Detecting the cause of this disease and the mechanism involved in the disease development is important to be determined before patients are directed to the therapy. This is because some AA patients (approximately 40%) do not respond to the main chemotherapy, IST therapy (Young *et al.*, 2005, Spellman *et al.*, 2012). The primary disease mechanism of AA is an attack of haematopoietic stem cells by T-leucocytes with an approximate of 60% of patients who respond to IST, while 40% of AA patients remain non-responsive (Young *et al*., 2006; Affable *et al.*, 2011). Mutations in the telomerase complex, specifically from two key components of the complex, *TERC* and *TERT*, have been indicated as the main cause of the secondary disease mechanism of AA. This secondary disease mechanism is caused by ineffective of the telomerase complex to synthesize telomeres, which signal the cell to apoptosis to avoid genetic instability after chromosomes not being capped properly. Patients who harbor the mutations on the telomerase complex do not respond to IST therapy and thus it is essential to screen the patients prior to treatment decisions (Calado *et al*., 2009, Du *et al*, 2009).

Not all mutations of the telomerase complex associated with AA have been directly linked to IST failure (Yamaguchi *et al.*, 2005; Vulliamy *et al*., 2007; Calado *et al*., 2008; DU *et al.*, 2009). This study was designed to develop an assay allowing for the rapid detection of those mutations which have been specifically linked to IST failure amongst AA patients, so that the assay has a direct clinical impact. Currently, telomerase (*TERC* and *TERT*) mutations are detected via sequencing which involves intensive laboratory work, including the amplification and sequence analysis of numerous exons of the *TERT* gene and the whole *TERC* genes. Therefore an assay involving multiplex PCR would allow rapid and specific detection of these mutations with results generated in one day. This will allow early development of a therapeutic strategy for the patients.

Usually, diagnostic assays are developed using positive controls that have been taken from the patient population. These controls allow for the validation of the assay, including the determination of reproducibility, sensitivity and accuracy. Due to the fact that AA is a rare disease, these positive controls are not readily available and presently no commercial vendor supplies them. Thus, in order to develop an assay to detect these important clinically relevant mutations it was necessary to artificially create them. The approach was to design primers with mutation (SDM primers) that would produce a PCR product with containing a desired mutant.

Designing of SDM primers proved to be problematic in some instances as they had to be long enough to allow homologous annealing on either side of the mutation, but also to allow for a second round of specific amplification during the actual diagnostic assay. Furthermore, due to the nature of the DNA sequence having certain bases dominating on the sequence, positioning of the primers was limited as some sequences dominated either AT or GC contents. Therefore in most cases the primers failed the standard criteria for use in a successful PCR reaction. These parameters are 60% GC content, 18-22bp primer, an ideal annealing temperature (Tm) between 55ºC - 65ºC, hairpin formation and primer-dimer formation with the reverse primer.

Despite the challenges that these primers potentially posed, successful, single SDM amplification products were created for the *TERC* mutants: *TERC*110 SDM amplicon, *TERC*178 SDM amplicon, *TERC*180 SDM amplicon using human WT genomic DNA and the *TERT* mutants: *TERT*412 SDM, *TERT*694 SDM, *TERT*704 SDM, *TERT*1015 SDM and *TERT*1090 SDM. Using a Go-Taq® hot start DNA polymerase (Promega, USA) minimized difficulties which could have been experienced with the PCR setup. It allowed the amplification of targets because of its robust activity. Although primer dimers and non-specific products were experienced on other PCR reactions for mutants production, but most of them were created successfully. Despite that when using GoTaq® hot start DNA Polymerase (Promega, USA) the PCR reaction could be setup at room temperature; we took precautions to setup all reactions on ice to ensure that all reagents are inactive even if the protective antibody on the DNA polymerase would have been disturb and the enzyme was released to be readily active. This further allowed the specific amplification of PCR amplicons.

However, the production of a single specific product for *TERC*53 SDM amplicon, *TERT*202 SDM amplicon and *TERT*846 SDM amplicon could not be achieved. This could be due to the fact that the SDM primer for *TERT*202 SDM product had two mismatched to create two mutations, and therefore could not anneal with strong specificity to a single region in the template compared to just a single mismatch for the others. In the case of *TERT*846 SDM, the non-specific products were potentially caused by various reasons, including the fact that the primer is long and the presence of primer dimers. It could be possible that synthesized primer-dimers became a secondary template in the reaction, allowing primers to further bind and produce another product. This could be the possibilities as multiple products were experienced with the production of these mutants.

Non-specific amplification using the *TERC*53 SDM primer was expected, as it contains a large region that is not homologous to the target region. To improve specificity, various approaches were conducted, starting with performing magnesium chloride titration ranging from (1.5- 3mM), decreasing the number of PCR cycles from 35 to 30 cycles, increasing extension time from 90 seconds to 2 minutes. Changing the magnesium chloride concentration could potentially improve the specific amplification. Since magnesium is a critical co-factor for the Taq DNA polymerase requires being optimal in the reaction, therefore low or too high magnesium concentration may lead to ineffective enzymatic activity of Taq DNA polymerase.

The magnesium concentration could affect annealing of primers, template and PCR product since this may affect the interaction of the enzyme and lowers the activity (Innis *et al.*, 1988). In most cases, the extension time and number of cycles during PCR amplification depends upon the initial concentration of the template but 90 seconds for extension and 35 cycles are usually enough for amplification. Nevertheless, in some cases where at late cycles when product concentration exceeds enzyme concentration extended extension time (2 minutes) may be helpful to finish product which may be not synthesized completely. Also decreasing cycles may potentially help to reduce non-specific products which may be formed at later stages of cycling (Saiki *et al.*, 1989).

But all these attempts were unsuccessful to eliminate non-specific PCR amplification. However, despite non-specific products, the correct amplicons were extracted for purification and confirmed the desired mutations. Eventually, sequencing of all cleaned SDM products confirmed the introduction of the required mutations (SNPs and deletions) into the *TERC* and *TERT* amplicons. Since all clinically relevant mutations of *TERC* and *TERT* were created, to ensure pure mutants and the ability to generate large quantities of positive controls, mutations amplicons were cloned into the pGEM®T-Easy plasmid vector. This significantly increased the storage capacity of the mutants and avoids DNA degradation for long term usage.

As the cloned products sizes were already confirmed to be correct during the cloning process using restriction digestion. There was still a possibility that during the process the DNA sample could be tampered and get degraded after handling with different reagents, and freeze and taw. Therefore to ensure that stored cloned SDM PCR products of *TERC* and *TERT* were still intact and correct, and that only the desired mutations were available in the products. Cloned SDM products were sequenced and this proved that all stored mutants were correct and can be used on further experiments to develop a diagnostic assay.

In conclusion the first aim of the study was successfully achieved which was to generate the clinically relevant mutations in vitro and to clone them in into pGEM®T-Easy. This ensured that these positive controls can be produced in large quantities, have increased stability and be readily available for use in a diagnostic assay.

CHAPTER THREE: DEVELOPMENT OF AN ALLELE-SPECIFIC MULTIPLEX PCR ASSAY TO DETECT *TERC* AND *TERT* MUTATIONS

3.1 INTRODUCTION

The strategy to detect specific *TERC* and *TERT* mutations in the DNA of AA patients was to use an allele-specific multiplex PCR, where the presence of a mutation would result in the amplification of a PCR product. The aim was to develop a single tube allele-specific multiplex PCR assay to detect all 4 *TERC* mutations and another single tube allele-specific multiplex PCR assay to detect all 7 relevant *TERT* mutations. Each single-tube reaction also needed to incorporate a control amplification to account for possible problems with DNA quality and PCR reagents.

To achieve this ambitious aim, the approach was to first determine a common PCR program and reaction mix to allow the amplification of the *TERC* mutations, but not the WT equivalents, using single-plex PCR reactions (i.e: one set of primers and one heterozygous mutant template). To this end, the cloned *TERC* and *TERT* mutations described in Chapter 2 were diluted into WT genomic DNA to create more realistic, naturally occurring controls (20% mutated allele) and used as positive controls for the assay, while genomic only DNA served as the negative control. The primers were then combined to assess the efficiency and accuracy of amplification of each of the mutants in a multiplex environment and optimisation was performed where necessary. Once the *TERC* PCR program had been established, single-plex reactions for the *TERT* mutations were tested using these conditions and again the reactions were optimised, however now limited to reaction components (such as glycerol, magnesium chloride). Finally, the primers were combined to try to optimise the amplification of each mutant in this multi-primer environment. It must be noted that it is not expected for an individual to harbour multiple mutations of the same gene, thus amplification of single mutations within this multi-primer environment was primarily tested. Once an assay was established, the final objective was to test various aspects of the assay so that it could be effectively used in a routine diagnostic environment. This validation required the sensitivity of the assay to be established, as well as the accuracy in reporting a result and the reproducibility aspect.

3.2 MATERIALS AND METHODS

3.2.1 Templates for assay development

3.2.1.A: WT human genomic DNA control (negative control)

WT human genomic DNA was prepared as described in section 2.2.2 (Chapter 2) to be used as a mutation-free negative control. For all PCR assays, a concentration of 40ng/µl of DNA was used.

3.2.1.B: Plasmid controls (positive controls)

Plasmid controls prepared in section 2.3.5 (Chapter 2) to be used as templates in PCR reactions to mimic the naturally occurring mutations of *TERC*/*TERT* were used during the assay development. Since the assay had to mimic a real sample from a patient, each plasmid control (1×10^{-4} ng/µl) had to be diluted into 40ng/µl of a normal genomic DNA (prepared in section 2.2.2, Chapter 2) to create a heterozygous mix of a mutant allele and normal WT DNA. Mutations of *TERC*/*TERT* are usually heterozygous in patients, thus it was imperative to ensure that all the samples to be used in the development of the assay are closely similar to a real patient sample. All plasmid controls were diluted with WT genomic DNA up to 20% allele content within the mix. The principle used to generate heterozygous mixes was that a human genomic DNA has two copies of alleles which either of them could harbour a mutation of *TERC*/*TERT*, while a plasmid has one copy. Both plasmid and human genomic DNA has a number of base sequences and the length. This information was used to determine a number of plasmid copies required to make a 20% mutation allele with a dilution by a normal human genomic DNA to make a heterozygous sample.

Using the information that one human cell yields approximately 10 picomoles of DNA indicated that 40ng/µl of human genomic DNA has 8000 alleles. The number of copies of plasmid required to make 20% allele mutation and 80% WT genomic DNA were calculated. These formulas: [number of copies of plasmid (N) = number of moles (n) × Avogadro's Constant (NA= 6.022045×10^{23})], [n = Molecular weight of a double stranded DNA (dsDNA) × length of based paired plasmid DNA] were used to determine a relevant concentration of plasmid (1×10^{-4} ng/µl) needed to make a 20% allele mix with a dilution by 40ng/µl WT genomic DNA. A ratio of plasmid to WT genomic DNA of 1:19 alleles was used to create a heterozygous mix. Finally, a heterozygous mix of each mutant and a WT genomic DNA mix (1×10^{-4}ng/µl: 40ng/µl) was created to be used for assay development.

3.2.1.C: Multiple Heterozygous positive controls

Multiple heterozygous positive controls were prepared to be used as positive controls for the finalised assay. To prepare the *TERC* positive control (TCC), the heterozygous mutant positive controls (see section 3.2.1.B) for all four mutants: *TERC*53C, *TERC*110C, *TERC*178C and *TERC*180C were mixed in equal quantities, making a cocktail with each mutant allele being present at ±5%. Two *TERT* multiple positive controls were made, TTC1 containing *TERT*412C, *TERT*704C, *TERT*846C, *TERT*1015C and *TERT*1090C, resulting in a mutant allele of ± 4% after addition of equal volumes of 5 heterozygous mixes of plasmid positive controls, sitting at ±20%, and TTC2 comprising of *TERT*202C and *TERT*694C, making an allele cocktail of each mutant being available of ±10%. Each of these controls would be used when the assay is performed.

3.2.1.D: Saliva DNA samples

Saliva (2ml) was collected using the Oragene® DNA (OG-500) kit (DNA Genotek Inc, Canada) from healthy individuals with informed consent (approved by the Faculty of Health Sciences Research Ethics Committee, University of Cape Town -HREC REF: 131/2011). The saliva sample was then used to extract DNA using the QIAmp® DNA blood mini kit (Qiagen, USA). Briefly, 200µl of saliva with Oragene® DNA buffer was mixed with 20µl proteinase K (4U/mg) to degrade proteins, 5µl RNAse A (10mg/ml) to degrade RNA and 200µl lysis buffer (contained 5% SDS and low pH).

This was followed by incubation at 56^0C for 10 minutes to help cell lysis. Subsequently 200µl of ethanol was added to the sample to dehydrate the DNA (aids in silica binding) before loading onto the silica QIAamp® mini spin column, and the sample was passed through the column via centrifugation (8000 rpm for 1 minute). The bound DNA was washed sequentially with two buffers (AW1 and AW2) to remove impurities (proteins and salts) (500µl each buffer, 8000 rpm for 1 minute). Remaining wash buffer was removed by a final centrifugation at 14000 rpm for 1 minute. The DNA was then eluted from the silica column by incubation with 50µl nuclease-free water at room temperature for 1 minute followed by centrifugation at 8000 rpm for 1 minute. The concentration of DNA was determined using a UV-Vis Spectrophotometer Nano-Drop 2000 (Thermo Scientific, USA) and stored at -20^0C. This DNA sample was used during the validation process.

3.2.2 Allele-specific mutant *TERC* and *TERT* primers

Mutant-specific forward primers were designed by ensuring that the 3' base was specific to the mutation (Dieffenbach *et al*., 1993, Hirotsu *et al*., 2010, Liu *et al.* 2012). This limited the placement of the primers, but GC content, primer-dimer formation and hair-pin structures were controlled to some extent with the primer length. Common reverse primers were also designed and all the primers were checked for primer-dimer formation with one another using Primer3 software, ensuring minimal hairpin formation and self-complimentary. Theses primers were tested for specificity against the human genome using NCBI blast, to ensure no non-specific binding. Table 3.1 lists the primers that were selected for the multiplex diagnostic assay for *TERC/TERT* mutation detection. Primers were also designed to amplify a *TERC*-control region of *TERC* (using *M-TCTRL* and *M-TCR* primers), as well as *TERT* control region in exon 2, *TERT*-control (using *M-TTCTRL* and *M-TT202R* primers). All primers were supplied by the Molecular and Cell Biology Department of the University of Cape Town.

Table 3.1: Primers to detect *TERC/TERT* mutants in an allele-specific PCR

Primer	Sequences (5' to 3')
M-TC53 (Forward Primer)	TTTGTCTCTAACCCCCC
M-TC110 (Forward Primer)	CTGTTTTTCTCTCGCTTTC
M-TC178 (Forward Primer)	CTAGAGCAAACAAAAAATA
M-TC180 (Forward Primer)	GAGCAAACAAAAAATGTT
M-TCR (Reverse Primer)	CACGTCTCCTGCCAATTTGC
M-TCTRL (Forward Primer)	GCCGCGAGAGTCAGCTT
M-TT202 (Forward Primer)	CTGGGATGCGAACGGTA
M-TT412 (Forward Primer)	GGGTGCTCCTCAAGACGT
M-TT694 (Forward Primer)	GGCCTGGCGCACCGGCA
M-TT704 (Forward Primer)	GGCCCAGGACCCGCCGT
M-TT846 (Forward Primer)	GCTCTGCAGCCTGTGCTG
M-TT1015 (Forward Primer)	CTCTCAGGTTTCACGCAC
M-TT1090 (Forward Primer)	CGACACCGTGTCACCTACA
M-TT202R (Reverse Primer)	TGACACCACACAGAAACCAC
M-TT412R (Reverse Primer)	CGCACGAAGCCGTACAC
TT-exon5R (Reverse Primer)	CCTGGTGGCTGAGCCGTTG
M-TT846R (Reverse Primer)	AGTCCTCAGGCTGTGCAAC
M-TT1015R (Reverse Primer)	CCTAAGTGCCATGGACG
TT-exon14-15R (Reverse Primer)	GACACCAGCGTTTAATCACATAG
M-TTCTRL (Forward Primer)	CAGCTACCTGCCCAACACG

$: Forward and reverse primers to amplify *TERC* mutants and *TERC* control fragment and *TERT* mutants.

Allele-specific primers were named using either their DNA location or amino acid base change while reverse primers were named using the same principle. All primers designed specifically for the multiplex their names started with a letter M, and for *TERC* primers with TC after letter M while *TERT* primers with a TT. Primers for internal controls were also named based on either representing *TERC* or *TERT*.

3.2.3 Allele-specific single-plex PCR of *TERC* and *TERT* mutants

Each primer pair was initially tested in individual single-plex reactions against the specific heterogeneous mutant mix as follows: 1X Green GoTaq® Flexi Buffer (Promega, USA), 2U GoTaq Hot Start Polymerase (Promega, USA), 0.8mM dNTPs, a range of 0.5mM- 1.5mM magnesium chloride (Promega, USA), a range of 3.2pmol- 37.5pmol forward primer and 25pmol reverse primer (Table 3.1) and either 40ng genomic DNA (negative control) or heterozygous positive control (40ng genomic DNA/1 × 10^{-4}ng plasmid) or 1 × 10^{-4}ng plasmid (positive control).

The PCR cycling conditions were as follows: Taq DNA polymerase activation and initial template DNA denaturation at 95°C for 4 minutes, followed by a range of 30- 35 cycles of template denaturation at 94°C for 30 seconds, primer annealing at a range of 40°C- 65°C for 30 seconds, and extension at 72°C for 90 seconds. For amplicon completion, a final extension step of 72°C for 8 minutes was added. PCR products were resolved by agarose gel electrophoresis, using a 2% TAE-agarose gel and electrophoresis in 1X TAE buffer (40mM Tris, 20mM acetic acid, 1mM EDTA) at 70 volts for 1.5 hours. The DNA amplicons were stained with ethidium bromide (0.5 μg/ml) (Sigma, USA) and visualized under UV light using the UV doc system (UVItec, UK).

3.2.4 Allele-specific multiplex PCR

3.2.4.A: Multiplex PCR of TERC mutants

For the generation of a single-tube amplification system, all four allele-specific forward primers, the *M-TCTRL* forward primer and a common reverse primer (listed in Table 3.1) were combined in a single tube (TCMM – *TERC* master mix). PCR reactions for detecting each mutant in this multiprimer setting were as follows: 1X Green GoTaq® Flexi Buffer (Promega, USA), 0.2U GoTaq® Hot Start Polymerase (Promega, USA), 0.8mM dNTPs, 1.5mM magnesium chloride (Promega, USA), 25pmol forward primer for each *TERC* allele-specific forward primer, 3.2 pmol control primer (*M-TCTRL*) and a common reverse primer (*M-TCR*) and 3% glycerol (in H_2O) – plus either 40ng genomic DNA (negative control) or heterozygous positive control (40ng genomic DNA/1 ×10^{-4}ng plasmid) prepared from section 3.2.1.B, or multiple heterozygous positive controls prepared from section 3.2.1.C as a template.

The PCR cycling conditions were as follows: initial denaturation at 95°C for 4 minutes, followed by 30 cycles of template denaturation at 94°C for 30 seconds, primer annealing at 55°C for 30 seconds, and extension at 72°C for 90 seconds, and the addition of a final extension step of 72°C for 8 minutes. The PCR amplicons were resolved via agarose gel electrophoresis, using a 2% TAE-agarose gel and electrophoresis in 1X TAE (40mM Tris, 20mM acetic acid, 1mM EDTA) at 70 volts for 1.5 hours. This was followed by staining DNA fragments with ethidium bromide (0.5 µg/ml) (Sigma, USA) and visualized under UV light in the UV doc system (UVItec, UK).

3.2.4.B: Multiplex PCR for TERT mutants

Two different PCR reactions were prepared to detect *TERT* mutants. TTMM1 (*TERT* master mix 1) was designed to amplify mutants *TERT*202 and *TERT*694, as well as a *TERT* exon 2 control fragment. TTMM2 (*TERT* master mix 2) was designed to amplify mutants *TERT*412, *TERT*704, *TERT*846, *TERT*1015 and *TERT*1090 from plasmid constructs (Table 2.3, Chapter 2). The multiplex PCR reactions containing the allele-specific mutant forward primers contained the following in a final reaction volume of 25µl: 1X Green GoTaq® Flexi Buffer (Promega, USA), 0.2U GoTaq® Hot Start Polymerase (Promega, USA), 0.8mM dNTPs, 0.5mM magnesium chloride (Promega, USA), 5% DMSO, TTMM1 contained 25pmol *M-TT412, M-TT704, M-TT846, M-TT1015,* and *M-TT1090* forward primers and 25pmol *M-TT412R, TT-exon5R, M-TT846R, M-TT1015-R* and *TT-exon14-15R* reverse primers, TTMM2 contained 25pmol *M-TT202* and *M-TTCTRL,* and 75pmol *M-TT694* (Table 3.1). The DNA template in each reaction was either 40ng WT genomic DNA (negative control) or 40ng heterozygous positive control (section 3.2.1.B) or multiple heterozygous positive controls prepared from section 3.2.1C as a template. The PCR cycling conditions and product analysis were the same as those for *TERC* (section 3.2.4.A).

3.2.5 Validation of *TERC*/*TERT* allele specific multiplex PCR assay

3.2.5.A: Assay sensitivity

To determine the ability of the assay to detect low levels of mutant alleles, serial dilutions of each plasmid construct (section 2.2.5.D, Chapter 2) into genomic DNA were performed. Dilutions were made using the heterozygous mixes made in section 3.2.1.B, which consisted of ±20% mutant alleles. Two fold dilutions were prepared in genomic DNA (40ng/µl) to produce, ±10%, ±5% and ±2% mutant/WT allele mixtures.

These samples, including genomic DNA only were then used as templates in the multiplex allele-specific PCR assay described in sections 3.2.4 A and B, using either the *TERC* (TCMM) or relevant *TERT* master mix (TTMM1 or TTMM2).

3.2.6.B: Assay reproducibility

To determine assay reproducibility, the following known samples of heterozygous mixes of *TERC53C* and *TERT704C*, prepared from section 3.2.1.B, and WT human genomic DNA (section 2.2.2, Chapter 2**)** were used as templates with all three PCR master mixes (TCMM, TTMM1, and TTMM2). The PCR reactions were performed as described in sections 3.2.4.A and B and the assessment was repeated on three separate occasions.

3.2.6.C: Assay accuracy assessment

To determine the accuracy of the assay in identifying mutations, a series of five blinded samples (unknowns) was tested using the three master mix reactions (TCMM, TTMM1 and TTMM2). As controls, the combined mutant controls TCC, TTC1 and TTC2 (section 3.2.1.C) were used as positive controls for the assay being amplified by TCMM, TTMM1 and TTMM2 PCR master mixes respectively. The PCR reactions were performed as described in sections 3.2.4.A and B. This assessment was repeated in triplicate to ensure the reproducibility of the assay.

3.3 RESULTS

3.3.1 Experimental design

Allele-specific primers were designed, such that they were 100% homologous to the mutated sequence. This was accomplished by designing primers with a mutated sequence being present at the 3'end of each forward primer, making it possible to select and amplify only the mutant in a multiplex PCR. The binding positions of the primers for this multiplex assay development were similar to primers used to generate mutants as shown in Figures 2.1, 2.2, and 2.3 of Chapter 2. For the *TERC* multiplex PCR, as the mutations were all within an 800bp fragment, it was possible to again use a common reverse primer to allow amplification with any of the *TERC* allele-specific primers. Therefore the aim was to combine all 4 *TERC* allele-specific mutant forward primers and the common reverse primer (*M-TCR*). These primers are listed in Table 3.1 (section 3.2.2).

For the multiplex analysis of the *TERT* mutations, seven allele-specific forward primers were designed (see Table 3.1). Due to the large area between mutations, it was necessary to create primer pairs for most of the mutations. These primers were *M-TT202* and *M-TT202R* for *TERT*202, *M-TT412* and *M-TT412R* for *TERT*412, *M-TT846* and *M-TT846R* for *TERT*846, *M-TT1015* and *M-TT1015R* for *TERT*1015, and *M-TT1090* and *TT-exon14-15* for *TERT*1090. The only exception was the amplification using the allele-specific forward primers for *TERT*694 and *TERT*704 in exon 5, which both used the common reverse primer *TT-exon5R*. All primer sequences are listed in Table 3.1.

3.3.2 Single-plex PCR assessment for *TERC* mutations

3.3.2.A: Testing of allele-specific primers on the plasmid positive controls

The first step in the development of the multiplex assay was to confirm the ability of the allele-specific primers to amplify the mutant target and to find a common PCR program that would be suitable to amplify products using each of the primer sets. The PCR program initially used included 40 seconds for annealing, 90 seconds for extension, and a range of 40- 64^{0}C for the annealing temperature for 30 seconds and 35 cycles. The experiments were performed as described in section 3.2.3, using 25pmol of the primers and plasmid constructs: *TERC*178C, *TERC*180C, *TERC*110C and *TERC*53C as templates.

Using primers *M-TC178* and *M-TCR* to amplify from the plasmid *TERC*178C yielded a single, correct-sized product (445bp) over the 40- 64^{0}C range of annealing temperatures. Figure 3.1.A shows the amplification of the *TERC*178 mutant amplicon over this range. Primers *M-TC180* and *M-TCR* also produced a single, correctly sized amplicon (450bp) and very good amplicon yield across the temperature gradient tested, when using *TERC*180C as a template. Figure 3.1.B shows the amplification of the *TERC*180 mutant amplicon over the 40-64^{0}C annealing temperature range. Although not always shown in the figures, a no-template control was always included as a negative control to ensure that reagents were not contaminated.

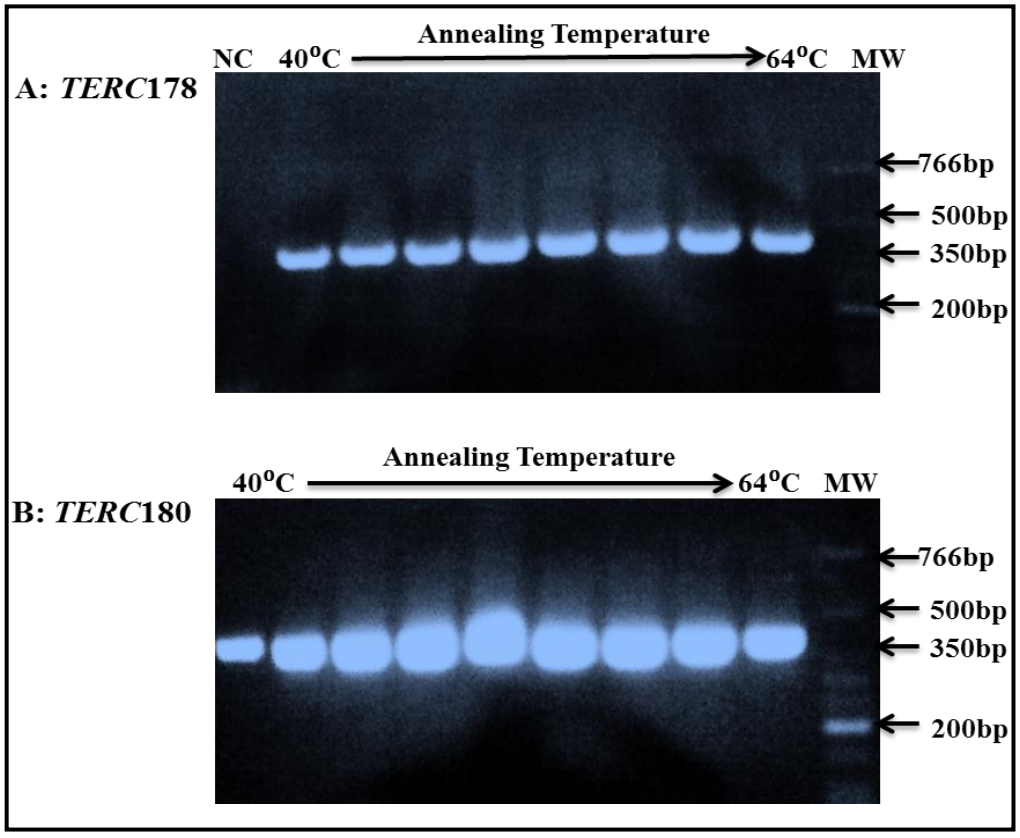

Figure 3.1: Electrophoretic analyses of gradient PCR of allele-specific PCR primers for *TERC*178 and *TERC*180 mutants.

Allele-specific primers for mutations of *TERC* were tested using positive control plasmid constructs in single-plex reactions. The picture above shows the amplicons that were produced when *M-TC178* and *M-TCR* were combined (A) and *M-TC180* and *M-TCR* (B) using an annealing temperature gradient from 40°C- 64°C, using the plasmid constructs *TERC*178C and *TERC*180C respectively, as templates. PCR products were resolved on 2% agarose TAE gel stained with ethidium bromide and visualized with UV light. Lane MW: Low molecular weight ladder (NEB), NC: no-template control.

The *M-TC53* and *M-TCR* primer combination (for the amplification of the *TERC*53 mutation) was tested using the *TERC*53C plasmid construct. Amplification using annealing temperatures above 55^0C produced a specific product of the correct size (554bp) and appropriate yield (shown in figure 3.2.A). However, at 55^0C a fairly negligible non-specific product of a slightly smaller size than the correct product was produced, which increased in yield as the annealing temperature was reduced below 55^0C, while the yield of the expected product was significantly reduced (see Figure 3.2.B). Amplification with primers *M-TC110* and *M-TCR*, using *TERC*110C plasmid construct as a template produced the most specific amplification (470bp) at annealing temperatures below 50^0C, with a smaller non-specific amplification band appearing at higher temperatures (Figure 3.2.C).

Due to the problems experienced with these two reactions, an annealing temperature of 55^0C was found to be a suitable compromise to allow specific amplification with all the primer sets. The yield of the non-specific product using the *TERC*110 primers was fairly low compared to that of the specific product at 55^0C and it is likely that using temperatures below 55^0C would be problematic when the primers are combined in a multiplex reaction. In addition, it is possible that the amplification of those non-specific bands not occur the plasmid template is diluted in WT genomic DNA.

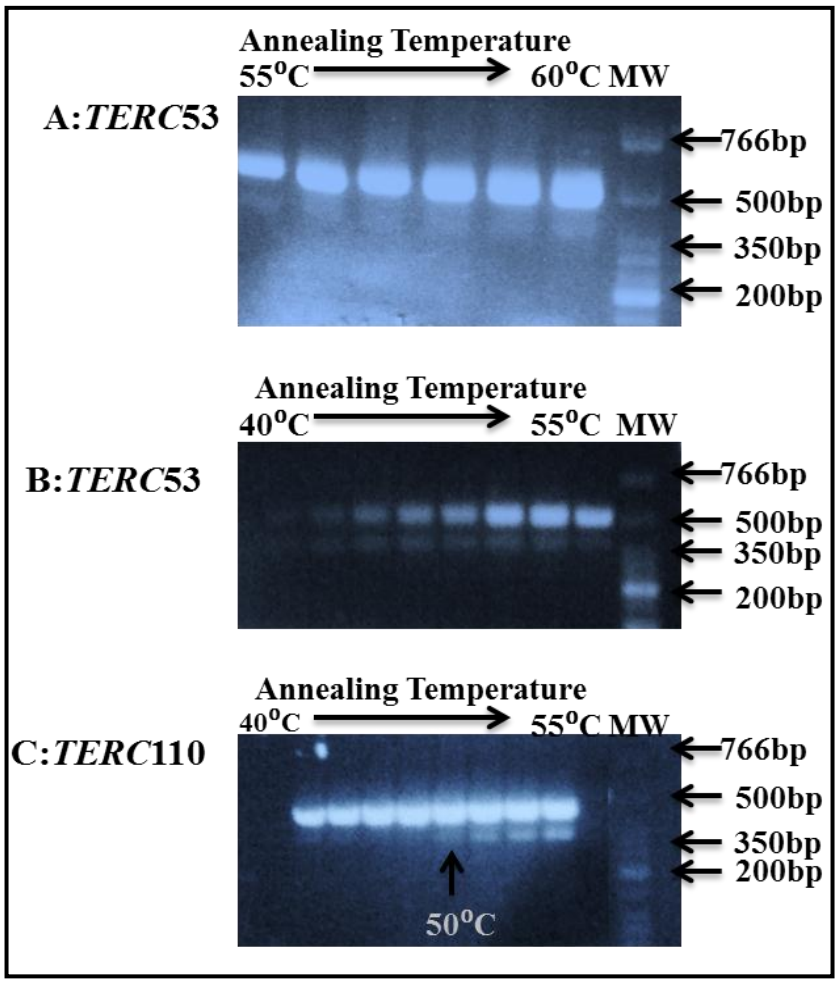

Figure 3.2: Electrophoretic analyses of gradient PCR of allele-specific PCR primers (*M-TC53* and *M-TC110*).

Allele-specific primers for mutations of *TERC* were tested using positive control plasmid constructs in single-plex reactions. The picture above shows the amplicons that were produced when *M-TC53* and *M-TCR* were combined and tested with annealing temperatures of 40°C- 60°C, using *TERC*53C as a template (A) & (B). Panel C shows the amplification using *M-TC110* and *M-TCR*, over the annealing temperature range 40°C- 55°C, using *TERC*110C as a template. PCR products were resolved on a 2% agarose TAE gel stained with ethidium bromide and visualized with UV light. Lane MW: Low molecular weight ladder (NEB).

3.3.2.B: Testing of allele-specificity using WT human genomic DNA as a template

In order to ensure that the allele-specific primers were specific for the mutants and not the WT human genomic DNA under these PCR cycling conditions, we tested each the primer pairs in single-plex reactions using WT genomic DNA as template and as a positive control, the plasmid constructs were used as templates. As an amplification control, we also tested a control primer set (*M-TCTRL* and *M-TCR*) which would amplify a 632bp region of the *TERC* gene regardless of the mutation status of the gene.

This control region was named *TERC*-control. The PCR conditions used were an annealing temperature of 55^0C (as decided above) and 35 cycles, using 25pmol of each primer (Table 3.1) as described in section 3.2.3. Figure 3.3 shows the results of this experiment. The PCR conditions used were an annealing temperature of 55^0C (as decided above) and 35 cycles, using 25pmol of each primer (Table 3.1) as described in section 3.2.3. The correct sized amplicons amplified successfully using the plasmid-based mutants as templates, while no amplification was observed using these primer sets with the genomic DNA. The amplification control fragment (*TERC*-control) (632bp) amplified successfully from the genomic DNA, showing that this could be used as a control for our experiments under these conditions (Figure 3.3). It also showed that in this current experiment the lack of amplification from genomic DNA in the allele-specific reactions was not due to poor quality DNA. Overall, this experiment showed that the *M-TERC* primers were specifically amplifying their target genes from plasmid constructs only in the presence of a mutation, under these specific PCR conditions.

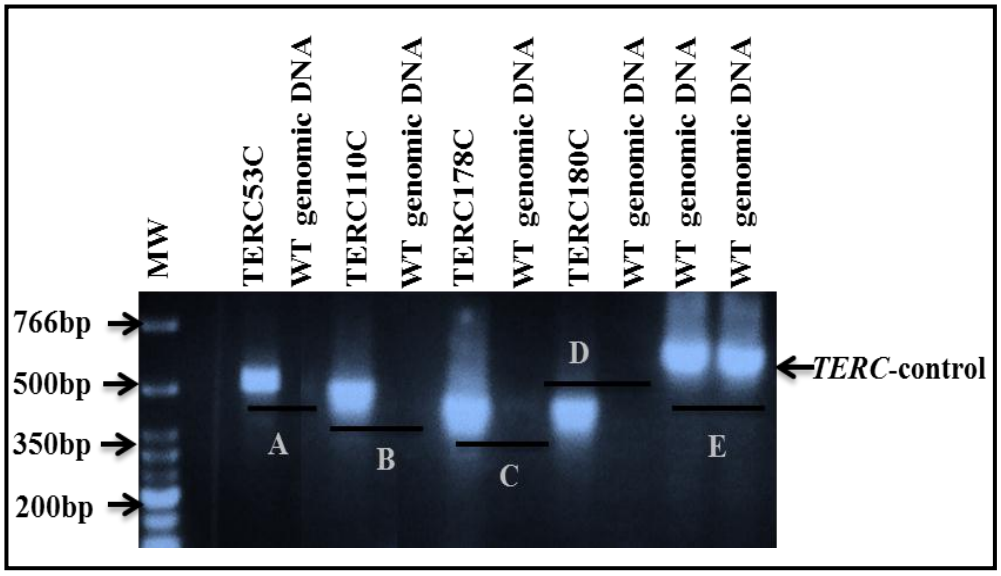

Figure 3.3: Electrophoretic analyses of allele-specificity for *TERC* mutant-specific PCR primers.

Allele specific primers for the 4 *TERC* mutations were tested on both WT human genomic DNA and plasmid constructs in single-plex reactions to check the allele-specific amplification. The photograph above shows the amplification using each primer set, using either a plasmid construct or genomic DNA as a template (as indicated). A: *M-TC53* and *MTCR*, B: *M-TC110* and *M-TCR*, C: *M-TC178* and *M-TCR, D: M-TC180* and *M-TCR*, and *E: M-TCTRL* and *M-TCR.* PCR products were resolved on 2% agarose TAE gel stained with ethidium bromide and visualized with UV light. Lane MW: low molecular weight ladder (NEB).

The correct sized amplicons were amplified very well using the plasmid-based mutants as templates, while no amplification was observed using these primer sets with the genomic DNA. The amplification control fragment (*TERC*-control) (632bp) was amplified very well from the genomic DNA, showing that this could be used as a control for our experiments under these conditions. It also showed that in this current experiment the lack of amplification from genomic DNA in the allele-specific reactions was not due to poor quality DNA. Overall, this experiment showed that the *M-TERC* primers were specifically amplifying their target genes from plasmid constructs only in the presence of a mutation, under these specific PCR conditions.

3.3.3 Multiplex PCR assessment of *TERC* mutations.

3.3.3.A: Simultaneous detection of TERC-control and individual mutants.

Heterozygous mixes prepared in section 3.2.1.B were used as templates to test the simultaneous amplification of each mutant from the plasmid constructs together with the control amplicon (*TERC*-control) from genomic DNA. Each allele-specific forward primer was initially combined separately with the control forward primer (*M-TCTRL*) and the common reverse primer (*M-TCR*) and tested with the relevant heterozygous mix, using this PCR reaction setup: 1X Green GoTaq® Flexi Buffer (Promega, USA), 0.2U GoTaq® Hot Start Polymerase (Promega, USA), 0.8mM dNTPs, 1.5mM magnesium chloride (Promega, USA), 25pmol forward primer for each *TERC* allele-specific forward primer (Table 3.1) and 25pmol common reverse primer (*M-TCR*). The PCR cycling conditions were performed as described in section 3.2.4.A with 35 cycles. While a faint band (Figure 3.4) which corresponds to amplification from the *TERC*180C plasmid was observed, there were no visible amplicons from the other positive plasmid controls when using the heterozygous mixes and this combined primer approach.

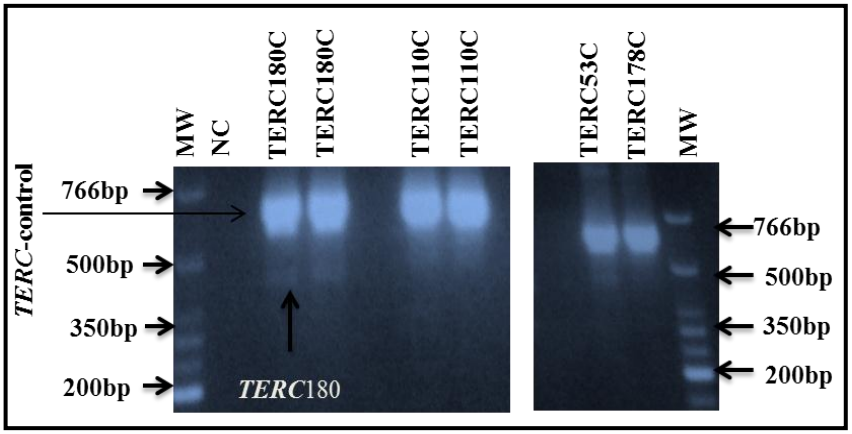

Figure 3.4: Electrophoretic analyses of multiplex PCR for *TERC*-control and each *TERC* mutant amplification.

Multiplexes containing a specific Allele-specific primer (25pmol), *M-TCTRL* (25pmol) and *M-TCR* (25pmol) were used to amplify their specific targets using the heterozygous plasmid/genomic DNA as templates. Each lane name refers to the specific plasmid present in the heterozygous template mix (*TERC*180C and *TERC* 110C were done in duplicate) that was used. PCR amplicons were resolved on a 2% agarose TAE gel stained with ethidium bromide and visualized with UV light. Lane MW: molecular weight ladder (NEB), and NC: no-template DNA control.

In order to improve the simultaneous amplification of each mutant from their specific plasmid in the presence of the control amplicon (*TERC*-control), the concentration of *M-TCTRL* (control forward primer) was reduced in an attempt to reduce the amplification of this product. A single-plex PCR reaction series using only the control primer set (*M-TCTRL* and *M-TCR*), with 40ng WT genomic DNA as a template was performed, where the concentration of forward primer *M-TCTRL* ranged from 25pmol- 2.5pmol and the PCR conditions were those described in section 3.2.3, using 55°C annealing temperature and 35 cycles of PCR. Amplification of *TERC*-control decreased gradually as *M-TCTRL* concentration was reduced as expected (results not shown). The use of 3.1pmol of *M-TCTRL* for all further experiments was decided upon as it showed to allow a reasonable amplification of *TERC*-control. The use of the reduced *M-TCTRL* (3.1pmol) concentration was then tested with each set of allele-specific primers, using this PCR reaction conditions setup: 1X Green GoTaq® Flexi Buffer (Promega, USA), 0.2U GoTaq® Hot Start Polymerase (Promega, USA), 0.8mM dNTPs, 1.5mM magnesium chloride (Promega, USA), 25pmol forward primer for each *TERC* allele-specific forward primer (Table 3.1) and 25pmol common reverse primer (*M-TCR*), using either each specific heterozygous mix prepared from section 3.2.1.B or a WT genomic DNA as a control.

The cycling PCR conditions were performed as described in section 3.2.4.A with 35 cycles. *TERC*53 (Figure 3.5.A), and *TERC*110 (results not shown) were successfully amplified well using the corresponding heterozygous mixes as PCR templates, showing clear bands for the control amplicon (*TERC*-control) and amplification of the relevant mutant product. Amplification of the *TERC*178 mutant (Figure3.5.B) from the heterozygous mix was slightly less efficient, but of the correct size, while amplification using primer *M-TC180* of the *TERC*180 multiplex gave multiple products when using the heterozygous mix (*TERC*180C/genomic DNA) as a template (Figure 3.5.C).

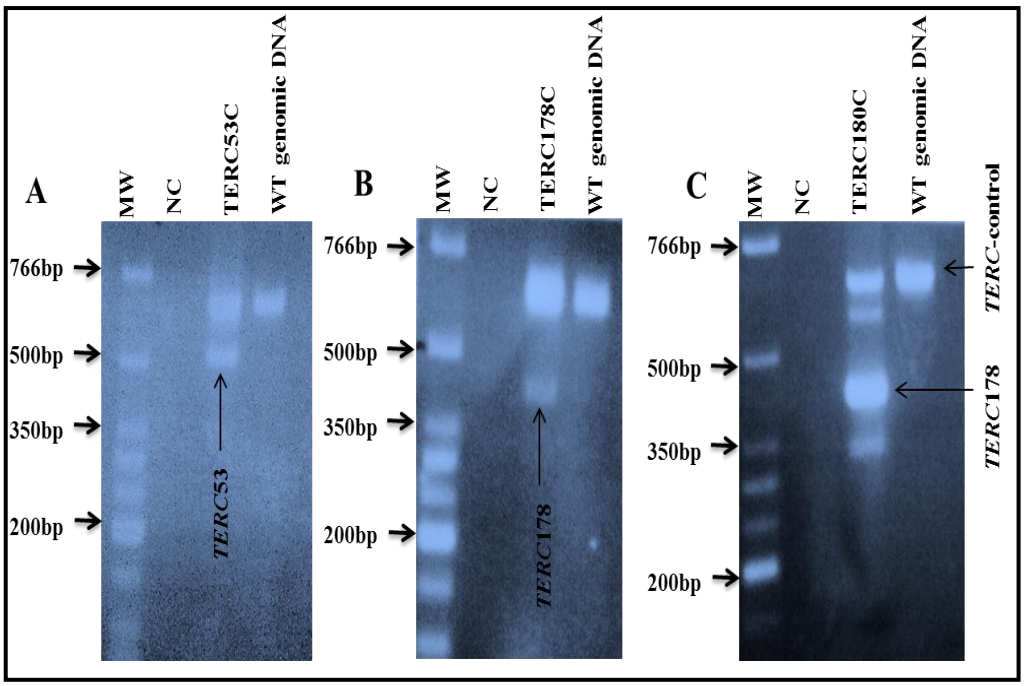

Figure 3.5: Electrophoretic analyses of multiplex for *TERC*-control and each *TERC* mutant amplification using 3.1pmol *M-TCTRL*.

Multiplexes containing each specific allele-specific primer (25pmol), *M-TCTRL* (3.1pmol) and *M-TCR* (25pmol) were used to amplify their specific targets using the heterozygous plasmid/genomic DNA or WT genomic DNA as templates. A: primers *M-TC53*, *M-TCR* and *M-TCTRL* using *TERC*53C/WT genomic DNA mix or WT genomic as a template, B: primers *M-TC178*, *M-TCR*, *M-TCTRL* using *TERC*178C/WT genomic DNA mix or WT genomic DNA, C: primers *M-TC180*, *M-TCR* and *M-TCTRL* using *TERC*180C/WT genomic DNA mix. PCR products were resolved on 2% agarose TAE gel stained with ethidium bromide and visualized under UV light. Lane MW: molecular weight ladder (NEB), lane NC: no-template control.

To further improve the amplification of the *TERC*178 mutant, its allele-specific primer (*M-TC178*) was increased to 37.5pmol while maintaining all other PCR parameters and reagents as described above and using a heterozygous mix (*TERC*178C/WT genomic DNA) prepared from section 3.2.1.B. The cycling PCR conditions were performed as described in section 3.2.4.A with 35 cycles. After increasing this primer concentration, the amplification of mutant *TERC*178 from a heterozygous mix (*TERC*178C/WT genomic DNA) was greatly improved, while still allowing amplification of the control fragment (*TERC*-control) (as seen in Figure 3.6).

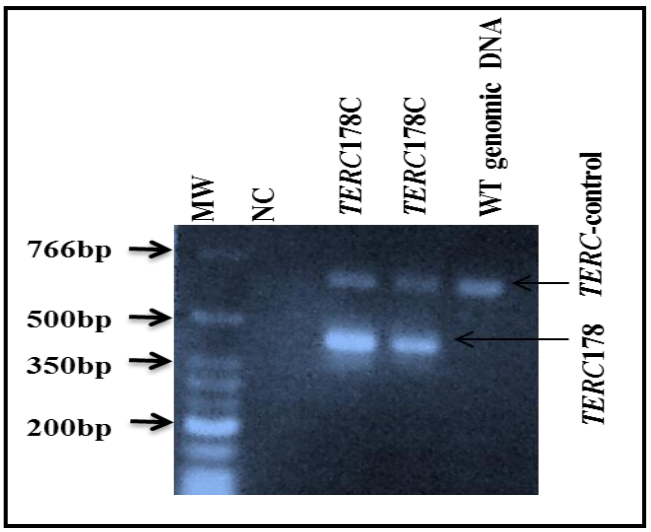

Figure 3.6: Electrophoretic analyses of multiplex PCR for *TERC*-control and *TERC*178 amplification with altered primer concentrations.

The effect of combining the following primers: *M-TC178*, *M-TCTRL* and *M-TCR* in the respective quantities: 37.5pmol, 3.1pmol and 25pmol, was tested using the *TERC*178C/WT genomic DNA heterozygous positive control and WT genomic DNA as templates (done in duplicate). The PCR products were resolved on 2% agarose TAE gel stained with ethidium bromide and visualized under UV light. Lane MW: molecular weight DNA ladder (NEB), lane NC: no-template control.

Thus far, by changing the ratios of the various primers, we could achieve the successful dual amplification of most of the *TERC* mutants along with the *TERC*-control fragment. Several different approaches were tested to try to eliminate the non-specific amplification that was observed with the *M-TC180*, *M-TCR* and *M-TCTRL* primer combination, which include varying the annealing temperature range from 55°C to 60°C to try to improve primer binding specificity, adjusting the extension time from 90 seconds to 120 seconds, and decreasing the concentration of the *M-TC180* allele-specific primer and anticipated that it would minimize the non-specific binding of primers. However this did not improve the result either.

However, further optimization revealed that the non-specific bands were only produced the plasmid construct *TERC*180C was used as a template. If either a purified SDM PCR product (*TERC*180 SDM) or WT genomic DNA was used as a template with these specific primers, the non-specific amplification was not observed (results not shown). This showed that the problem was caused by sequences present on the plasmid. Since these sequences would not be present in the context of the finalised diagnostic assay, we decided to proceed with other experiments to develop the multiplex PCR for *TERC* mutations.

3.3.3.B: Multiplex PCR of all allele-specific primers for TERC mutations

The 4 allele-specific forward primers: *M-TC53* (25pmol), *M-TC110* (25pmol), *M-TC178* (37.5pmol), and *M-TC180* (25pmol) for the *TERC* mutants together with a 3.1pmol for control forward primer (*M-TCTRL*) and 25pmol of the common reverse primer (*M-TCR*) (Table 3.1) were combined into one reaction tube, using this PCR condition: 1X Green GoTaq Flexi Buffer (Promega, USA), 0.2U GoTaq® Hot Start Polymerase (Promega, USA), 0.8mM dNTPs, 1.5mM magnesium chloride (Promega, USA), using each of the 4 heterozygous mix of individual mutant prepared from section 3.2.1.B as a template. The PCR cycling was performed as described in section 3.2.4.A with 35 cycles at 55°C.

Unexpectedly, this primer combination produced at least one non-specific amplicon when any of the heterozygous templates were used (data not shown). We postulated that the multiple products could potentially be caused by non-specific binding of unrelated primers to newly synthesized product that occur later on in the cycling process. To test this theory and improve the specificity, the number of PCR cycles was reduced to a range of 25 and 30 cycles to determine a suitable cycling condition using PCR conditions as described above.

Figure 3.7 shows the effect of reducing the number of cycles on the amplification of *TERC*53 and *TERC*110. As can be seen, reducing the number of cycles to 30 cycles improved the specificity of the amplification, producing only the expected *TERC*53 and *TERC*-control amplicons (Lane 1) when using the *TERC*53C/WT genomic DNA heterozygous mix. However, the alteration of this parameter did not improve the non-specific amplification observed when using the *TERC*110C/WT genomic DNA heterozygous mix (Lane 2). In the case of *TERC*178 and *TERC*180, the desired products were amplified, with no non-specific bands, when *TERC*178C and *TERC*180C heterogeneous mixes were used, using 30 cycles of PCR (results not shown).

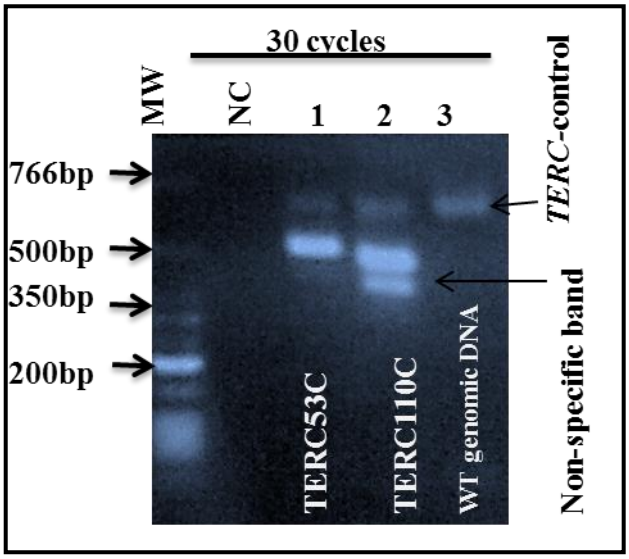

Figure 3.7: Electrophoretic analyses for the effect of reduced PCR cycle number on non-specific amplification using multi-primer master mix.

The amplification of *TERC* mutants and the control (*TERC*-control) was performed using a range of PCR cycles to try and eliminate the non-specific product. The picture above shows the amplification of *TERC* mutants and *TERC*-control using *TERC*53C or *TERC*110C heterozygous mixes and WT genomic DNA as templates, as indicated on the figure. Multiplex PCR analysis of *TERC*53 and *TERC*110 and *TERC*-control amplified using the multiple primers set: *M-TC53* (25pmol), *M-TC110* (25pmol), *M-TC178* (37.5pmol), *M-TC180* (25pmol), *M-TCTRL* (3.1pmol) and *M-TCR* (25pmol), using 30 cycles.

Since the amplification of *TERC*110 displayed a non-specific amplicon despite reducing the cycles, further optimization was necessary. To this end, the use of various additives was tested in the PCR reaction. These additives were tested individually on the multiplex PCR and included 0.8µg/µl BSA (0.3µl of 66.6µg/µl BSA in 25µl reaction), 5% DMSO (0.8µl of 100% DMSO in 25µl reaction) and 3% glycerol (0.75µl of 100% glycerol in 25µl reaction). Markoulatos *et al.* (2002) suggested that using these additives may improve the PCR efficiency and reduce non-specific product amplification in a multiplex PCR setting.

These additives were added separately to the multiplex master mix containing all the primers: *M-TC53* (25pmol), *M-TC110* (25pmol), *M-TC178* (37.5pmol), and *M-TC180* (25pmol) for the *TERC* mutants together with a 3.1pmol for control forward primer (*M-TCTRL*) and 25pmol of the common reverse primer (*M-TCR*) and were tested using each of the heterozygous mixes as templates (prepared from section 3.2.1.B). The PCR reaction conditions used were: 1X Green GoTaq® Flexi Buffer (Promega, USA), 0.2U GoTaq® Hot Start Polymerase (Promega, USA), 0.8mM dNTPs, 1.5mM magnesium chloride (Promega, USA). The PCR cycling was performed as described in section 3.2.4.A with 30 cycles at 55°C annealing temperature.

The addition of DMSO or BSA did not improve the specificity of amplification of the *TERC*110 mutant (data not shown). On the other hand, the addition of 3% glycerol improved the specificity, eliminating the non-specific band. This is illustrated in Figure 3.8 below, which shows the amplification of the control *TERC* fragment (*TERC*-control) in all of the reactions, as well as the amplification of each relevant mutant corresponding to the multiplex reaction mix.

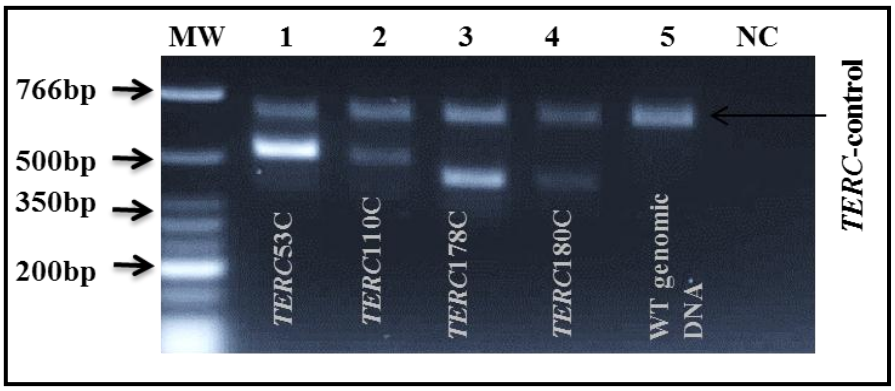

Figure 3.8: Electrophoretic analyses of *TERC* multiplex using 3% glycerol in PCR master mix.

All *TERC* allele-specific forward primers were combined with *M-TCTRL* and *M-TCR*, in a master mix containing 3% glycerol. This master mix was then tested with the specific heterozygous mutant positive controls or genomic DNA as templates (as indicated on the figure). PCR conditions included 55°C annealing temperature, 30 cycles, 25pmol of all primers (*M-TC53, M-TC110, M-TC180* and *M-TCR* (25pmol) of *TERC* mutants except 3.1mol of *M-TCTRL* and 37.5pmol of *M-TC178*. The PCR amplicons were resolved on a 2% agarose TAE gel stained with ethidium bromide and visualized under UV light. Lane MW: molecular weight ladder (NEB), NC: no-template control.

3.3.3.C: TERC Assay positive control (TCC)

Heterogeneous mixes of *TERC*, containing all the plasmid constructs and genomic DNA, were combined together to be used as a multiple mutant positive control for the assay (TCC). While the amplification of the control fragment (*TERC*-control) serves as an internal control for the quality of the genomic DNA used in the assay, a second positive control is needed to ensure that each prepared master mix, containing the multitude of primers is capable of detecting each mutant in every experiment. Failure of either of these controls would render the patient result invalid.

This positive mutant control mix was tested as a template using the master mix described in section 3.2.4.A, with the 3% glycerol added using 25pmol of all primers except 37.5pmol for *M-TERC178* of *TERC*178 mutant and 3.1pmol for *M-TCTRL* of the control fragment (*TERC*-control), using 30 cycles of PCR. As can be seen in Figure 3.9, the positive control produced *TERC*53 mutant, *TERC*110, *TERC*178, *TERC*180 and *TERC*-control bands, as expected (Figure 3.9, Lanes 1 and 2), showing that even combinations of mutations could be detected simultaneously using this methodology (not expected to occur in a patient though).

However, because *TERC*53 and *TERC*110 mutants are very similar in size, they were resolved as one band. The *TERC*178 and *TERC*180 mutant bands were also resolved as one band because of the same reason, but the intensity of both mutants is lower than what is desirable and further optimization would be required, but due to time constraints, we anticipated that further experiments would improve it. Lanes 4 – 7 represent amplification using the individual heterozygous mixes as templates and all four produced the expected size bands.

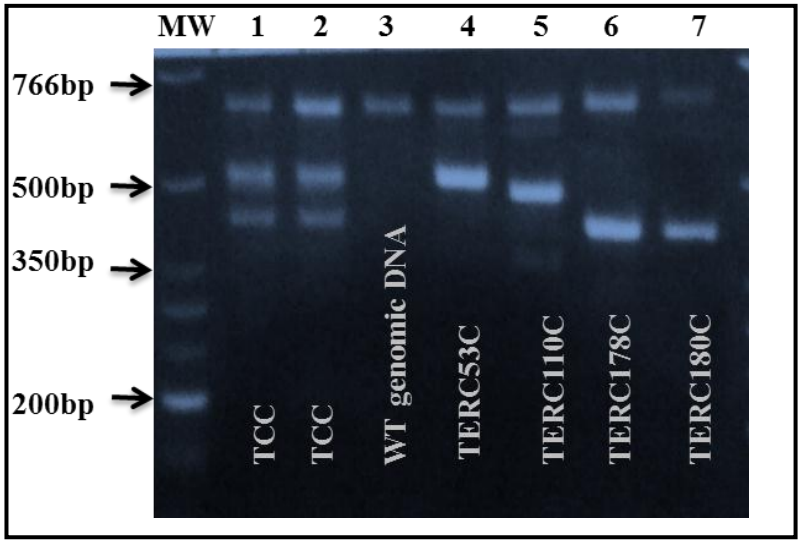

Figure 3.9: Electrophoretic analyse of *TERC* multiplex PCR with controls.

All *TERC* allele-specific forward primers: *M-TC53, M-TC110 M-TC180 and M-TC178* were combined with *M-TCR* and *M-TCTRL*, in a master mix containing 3% glycerol. This master mix was then tested with the specific heterozygous mutant positive controls, genomic DNA or combined mutant positive control TCC as templates (as indicated on the figure, done in duplicate). PCR conditions included 55°C annealing temperature, 30 cycles, 25pmol of all primers except 3.1mol of *M-TCTRL* and 37.5 pmol of *M-TC178*. The PCR amplicons were resolved on a 2% agarose TAE gel stained with ethidium bromide and visualized under UV light. Lane MW: molecular weight ladder (NEB).

Therefore, the development of the multiplex PCR to detect 4 *TERC* mutations *TERC*53, *TERC*110, *TERC*178 and *TERC*180 using a single master mix was achieved. A total of 5 forward primers and a common reverse primer was used to detect either a control fragment (*TERC*-control) alone or a mutant amplicon and control fragment combination. The final PCR cycling conditions and reaction set-up for the multiplex were a common annealing temperature of 55°C for primers, 30 cycles for PCR cycling, 25pmol of all primers except 37.5pmol of *M-TERC178* and 3.1pmol of *M-TCTRL* and the addition of 3% glycerol (forming the *TERC* master mix - TCMM). The controls that were used for the assay included a mutant negative WT genomic DNA, a no template control (NC) and a combined mutant positive control (TCC).

3.3.4 Single-plex assessment for *TERT* mutations

3.3.4.A: Testing of allele-specific primers on the plasmid positive controls and WT genomic DNA

In order to develop a single platform assay to test for mutations in both *TERC* and *TERT* genes, it was important that the PCR cycling conditions that were used for the *TERC* multiplex assay should also be used for the development of the *TERT* multiplex. As with the development of the *TERC* multiplex, it was necessary to first test the ability of the multiplex allele-specific primers of *TERT* to bind only to their target mutants and not the WT genomic DNA sequence. The amplification was performed as described in section 3.2.3 using the *TERC* PCR cycling conditions described above. The multiplex primers were tested in single-plex reactions, using either the corresponding plasmid positive controls or WT genomic DNA as templates. All primers were tested at 25pmol and the relevant primer sequences are listed in Table 3.1.

The primer set *M-TT412* and *M-TT412R*, successfully amplified the 180bp product from the *TERT*412C plasmid construct, the *M-TT846* and *M-TT846R* primer set amplified the expected 240bp amplicon from the *TERT*846C plasmid construct, and the primer pair *M-TT1015* and *M-TT1015R* amplified a 393bp amplicon from the plasmid construct *TERT*1015C as expected. The correct amplicon was also achieved for the other primer pairs with their corresponding plasmid positive controls: *M-TT1090* and M*TT-exon14-15R* yielding a product size of 211bp, *M-TT694* and *TT-exon5R* producing a product sized 353bp.

The amplification from *TERT*704C using *M-TT704* and *TT-exon5R* resulted in the correct-sized amplicon (323bp), as well as some additional amplicons. Unfortunately, when these primer sets were tested using WT genomic DNA as a template, under the *TERC* cycling conditions, amplification of similar sized products was observed for some of the primer sets, while others gave multiple amplicons (Figure 3.10.B). This result indicated that under these PCR conditions the primers were not binding in an allele-specific manner.

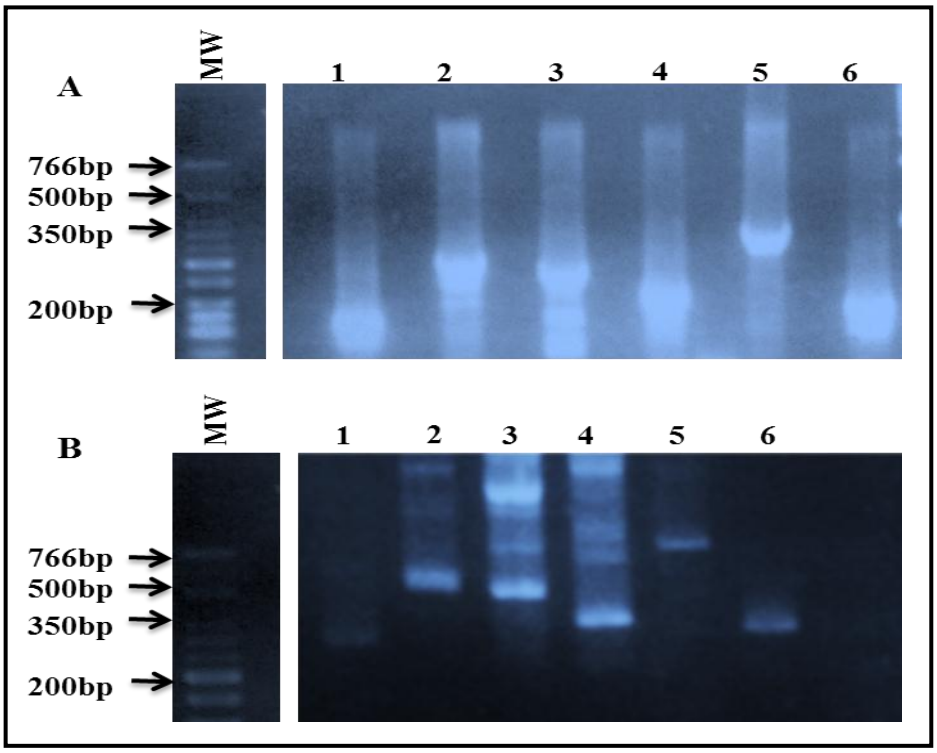

Figure 3.10: Electrophoretic analysis of PCR to test specificity of *TERT* allele-specific forward primers.
Allele-specific primers for mutations of *TERT* were tested using positive control plasmid constructs or WT genomic DNA in single-plex PCR reactions. The picture above shows the use of each primer set to test the ability and specificity to amplify their targets from plasmid constructs in Panel A or WT genomic DNA in panel B. Lanes 1: primer set *M-TT412* and *M-TT412R*, 2*: M-TT694* and *TT-exon5R*, 3: *M-TT704* and *TT-exon5R*, 4: *M-TT846* and *M-TT846R*, 5: M-TT1015 and *M-TT1015R*, 6: *M-TT1090* and *TT-exon14-15R*. PCR products were resolved on 2% agarose TAE gel stained with ethidium bromide and visualized with UV light. Lane MW: Low molecular weight ladder (NEB).

Figure 3.10.A shows that the primer set *M-TT412* and *M-TT412R* (Lane 1), successfully amplified the 180bp product from the *TERT*412C plasmid construct, the *M-TT694* and *TT-exon5R* (Lane 2) produced a product sized 353bp from *TERT*694C, the primer set *M-TT704* and *TT-exon5R* (Lane 3) produced a correct-sized amplicon (323bp) from *TERT*704C, as well as some additional non-specific amplicons, the *M-TT846* and *M-TT846R* primer set (Lane 4) amplified the expected 240bp amplicon from the *TERT*846C plasmid construct, the primer pair *M-TT1015* and *M-TT1015R* (Lane 5) amplified a 393bp amplicon from the plasmid construct *TERT*1015C as expected, and a correct amplicon was also achieved for the other primer pair with its corresponding plasmid positive controls: *M-TT1090* and *TT-exon14-15R* (Lane 6) yielding a product size of 211bp from *TERT*1090C plasmid construct.

Unfortunately, when these primer sets were tested using WT genomic DNA as a template, under the *TERC* cycling conditions, amplification of similar sized products was observed for some of the primer sets, while others gave multiple amplicons (Figure 3.10.B). This result indicated that under these PCR conditions the primers were not binding in an allele-specific manner.

3.3.4.B: Allele-specific primer specificity optimization

A study by Markoulatos *et al* (2002) showed that reducing the magnesium chloride prevents non-specific binding of primers thus enhancing the specific amplification. Therefore, the first step of optimization was to improve the specificity of the primers by varying the magnesium chloride concentrations at 1.5mM, 1mM, 0.8mM, and 0.5mM. The PCR reactions were performed as described in section 3.2.3, with various magnesium chloride concentrations, using the PCR program described in section 3.2.3 with 55°C annealing temperature and 30 cycles. Individual primer sets of *TERT* mutants were tested separately using either a WT genomic DNA or a plasmid mutant construct. Reducing the magnesium chloride to 0.5mM improved the specific amplification, allowing the allele-specific forward primers to only bind to their mutated target sequence and amplify from the plasmid controls only, with no amplification from the WT human genomic DNA (Figure 3.11).

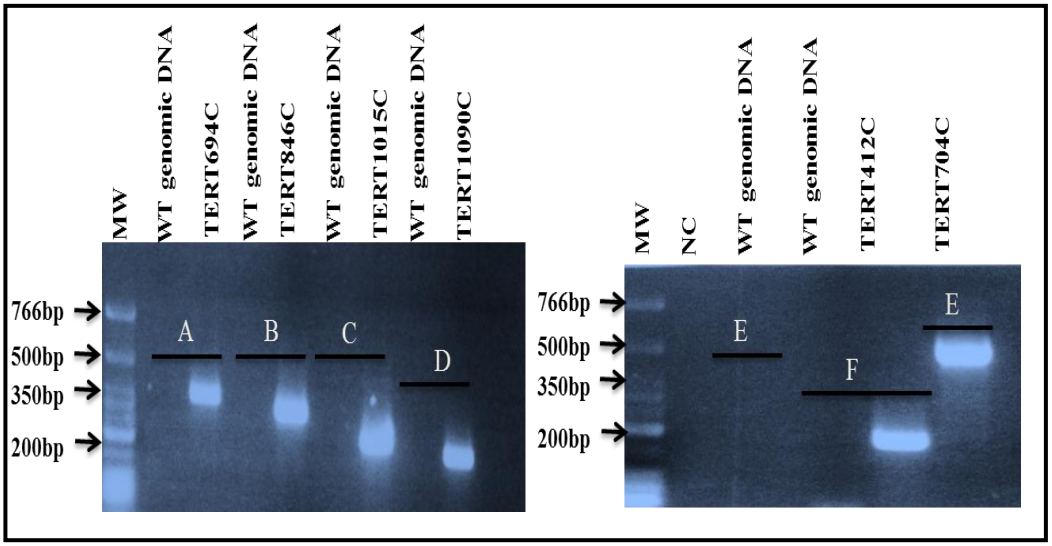

Figure 3.11: Electrophoretic analysis of *TERT* allele-specific primers using 0.5mM magnesium chloride. Allele-specific primers for mutations of *TERT* were tested using positive control plasmid constructs or WT genomic DNA in single-plex PCR reactions, using 0.5mM magnesium chloride in the PCR reaction mix. The picture above shows the amplification results using the following primers set, A: primer set *M-TT694* and *TT-exon5R,* B: *M-TT846* and *M-TT846R, C: M-TT1015* and *M-TT1015R,* D: *M-TT1090* and *TT-exon14-15R,* E: *M-TT704* and *TT-exon5R,* F: *M-TT412* and *M-TT412R*. PCR products were resolved on 2% agarose TAE gel stained with ethidium bromide and visualized with UV light. Lane MW: Low molecular weight ladder (NEB), lane NC: non-template control.

3.3.5 Multiplex PCR assessment of *TERC* mutations

3.3.5.A: Combination of primer pairs for TERT gene : phase 1

Due to the large number of primers that were needed to amplify all of the *TERT* mutants, the primers were combined into two sets. The primers *M-TT412, M-TT412R, M-TT846, M-TT846R, M-TT1015, M-TT1015R, M-TT1090 and MTT-exon14-15R* were used in the first primer set to amplify mutants: *TERT*412, *TERT*846, *TERT*1015, and *TERT*1090, and the primers *M-TT694, M-TT704, TT-exon5R, M-TT846* and *M-TT846R* were used in the second set to amplify mutants *TERT*694, *TERT*704, and *TERT*846. The templates that were used in this analysis were the heterogeneous plasmid/genomic mixes and WT genomic DNA. The PCR cycling was performed as described in section 3.2.4.2, using 25 pmol of each primer and 0.5mM magnesium chloride in each reaction mix without the addition of DMSO.

The primer set 1 reaction mix successfully resulted in the amplification of the correct amplicons for mutant *TERT*412 (180bp), *TERT*846 (240bp), *TERT*1015 (393bp) and *TERT*1090 (211bp), when the corresponding heterozygous mixes were used as templates, while no amplification was observed using WT genomic DNA as a template (Figure 3.12.A). However, in the second primer set only *TERT*846 was successfully amplified. The use of *TERT*694C and *TERT*704C as templates resulted only in the production of significant potential primer-dimers (Figure 3.12.B)

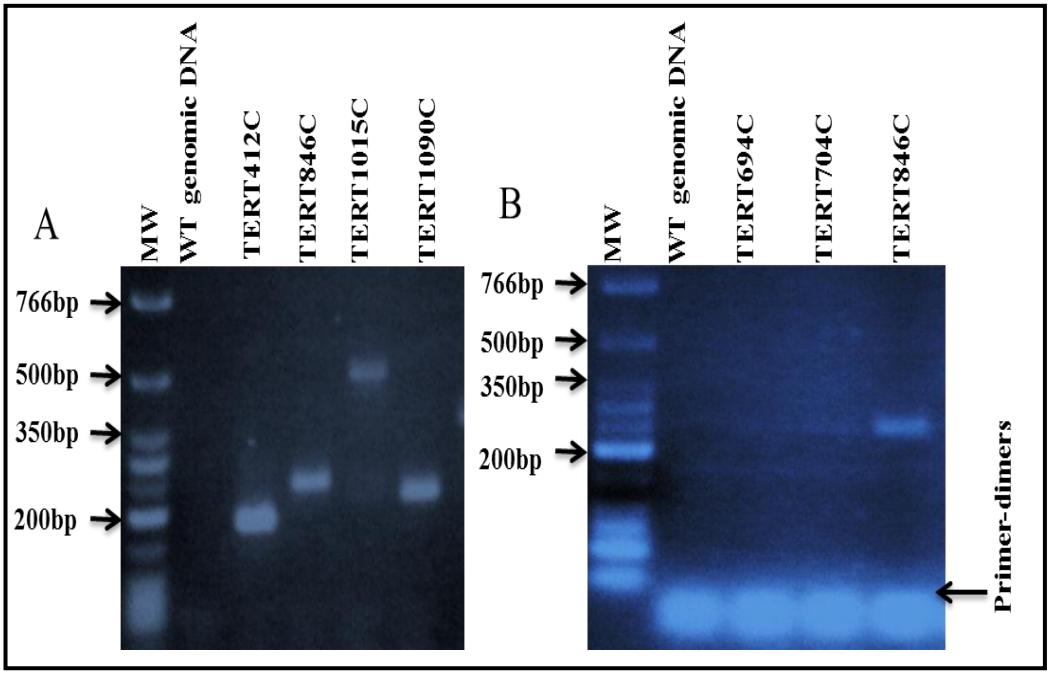

Figure 3.12: Electrophoretic analysis of multi-primer for *TERT* PCR: primer sets 1 and 2.

Primers of *TERT* were combined into primer sets 1 and 2 and used to amplify their sequence targets using either the relevant heterozygous plasmid mixes or WT genomic DNA as templates (as indicated on the figure) Panel A: Amplification using primer set 1: *M-TT412* and *M-TT412R, M-TT846 and M-TT846R, M-TT1015 and M-TT1015R, M-TT1090 and MTT-exon14-15R*. Panel B: Amplification using primer set 2: *M-TT694* and *TT-exon5R, M-TT704 and TT-exon5R, M-TT846* and *M-TT846R.* The picture above shows the amplicons resolved on 2% agarose TAE gel stained with ethidium bromide and visualized under UV light. Lane MW: molecular ladder (NEB), lane WT genomic DNA: negative control for allele-specific primers.

Due to the lack of PCR product in *TERT*694 and *TERT*704, we attempted various approaches to improve the amplification of these mutants. Firstly, we increased the concentration of each allele-specific forward primer (*M-TT694* and *M-TT704*) from a range 25 pmol to 75pmol in order to increase primer binding. The effect of increasing the amount of Taq DNA polymerase from 2U to 4Uwas also investigated in order to maximize the DNA synthesis during cycling. In addition, 5% DMSO was also added into the reaction containing 25pmol primers/ 2U Taq polymerase to try to decrease the melting temperature of primers and improve their binding affinity, a method that was previously described by Henegariu *et al* (1997).

The increase of the forward primer concentration or DNA polymerase or the addition of DMSO, all yielded large amounts of amplicon with no obvious increase in yield compared to one another (Figure 3.13). For the amplification of *TERT*694, an increase in the concentration of primer *M-TT694* increased the yield, as did the addition of DMSO to the master mix. It was therefore decided that both of these changes should be made to aid in the amplification of the *TERC*694 mutant.

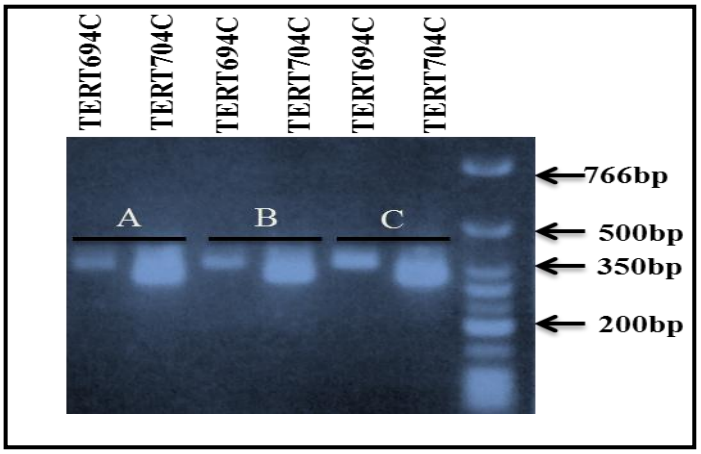

Figure 3.13: Electrophoretic analysis for the effect of increased primer, enzyme and DMSO on the amplification of *TERT*694 and *TERT*704 using primer set 2.

Primer set 2 containing: *M-TT694, TT-exon5R, M-TT704, TT-exon5R, M-TT846* and *M-TT846R* was used to amplify *TERT*694 and *TERT*704 mutants using heterogeneous mixes as templates, using either an increased amount of Taq DNA polymerase (4U) (A), increased forward primer (M-TT694/M-TT704) (75pmol) (B) or the addition of 5% DMSO to the reaction mix (C). The photograph above shows the amplicons of *TERT*694 and *TERT*704 resolved on 2% agarose TAE gel stained with ethidium bromide and visualized with UV light. Lane MW: molecular weight ladder (NEB).

Compared to the result observed in Figure 3.12, simply keeping all reagents on ice improved the amplification of the *TERT*704 mutant using the corresponding heterogeneous mix as template, with no primer-dimer formation observed. Increasing the forward primer concentration or DNA polymerase or the addition of DMSO, all yielded large amounts of amplicon with no obvious increase in yield compared to one another (Figure 3.13). For the amplification of *TERT*694, an increase in the concentration of primer *M-TT694* increased the yield, as did the addition of DMSO to the master mix. It was therefore decided that both of these changes should be combined in the amplification reaction to aid in the amplification of the *TERC*694 mutant.

3.3.5.B: Combination of primer pairs for TERT gene: phase 2

Once the problem associated with the inefficient amplification of *TERT*694 and *TERT*704 was solved, the next phase of the assay development was started. This involved the combination of the following primers: *M-TT412, M-TT694, M-TT704, M-TT846, M-TT1015, M-TT1090* forward primers, and *M-TT412R, TT-exon5R, M-TT846R, M-TT1015R,* and *M-TT-exon14-15R* reverse primers into one PCR master mix to amplify the following mutants: *TERT*412, *TERT*694, *TERT*704, *TERT*846, *TERT*1015 and *TERT*1090. The *TERC* PCR conditions were used, with a reaction master mix containing 0.5mM magnesium chloride, 25pmol for all primers except 75pmol for primer *M-TT694* and the addition of 5% DMSO, as well as setting up of the reaction on ice as described in section 3.2.4.B, using cycling conditions of *TERC* multiplex as described in section 3.2.4.A.

These reaction conditions were tested using each heterogeneous mix and WT genomic DNA as templates. The results of this experiment are shown in Figure 3.14, which shows an overwhelming amplification of a primer-dimer product (+/-70bp) when any of the templates were used with this multiple primer reaction mix. Despite this, mutants *TERT*412, *TERT*846 and *TERT*1090 were amplified to some extent using the corresponding heterogeneous mixes as templates.

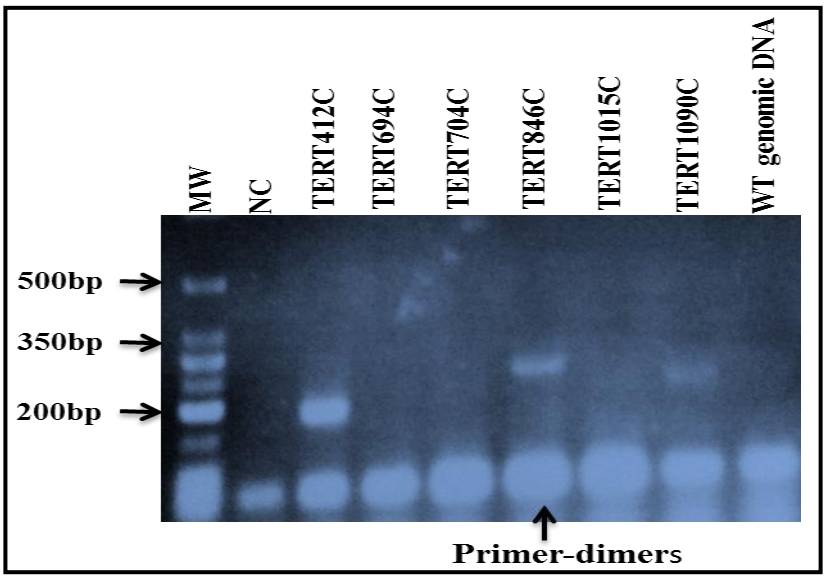

Figure 3.14: Electrophoretic analysis of multi-primer *TERT* analysis: amplification of *TERT* mutants in the presence of 6 allele-specific forward primers.

Primers: *M-TT412, M-TT694(75pmol), M-TT704, M-TT846, M-TT1015, M-TT1090, M-TT412R, TT-exon5R, M-TT846R, M-TT1015R,* and *M-TT-exon14-15R* were combined and used to amplify their respective targets from the heterogeneous plasmid mixes as indicated on the figure (in the presence of 5% DMSO). All primers were 25pmol except *M-TT694* as indicated above. The picture above shows the amplicons resolved on 2% agarose TAE gel stained with ethidium bromide and visualized with UV light. Lane MW: low molecular weight ladder (NEB), lane NC: no-template control.

In order identify the primer set that caused the primer-dimer formation, each set of primers was introduced separately into the reaction mix and tested against the heterogeneous mixes. The *M-TT694* was found to be the main cause of the primer dimer product formation when added to the other primers (results not shown). When *M-TT694* was excluded from the reaction mix, the remaining primers successfully amplified the *TERT*412, *TERT*704, *TERT*846, *TERT*1015, and *TERT*1090 mutants using the corresponding positive heterozygous mixes and showed the lack of amplification of any product when WT genomic DNA was used (Figure 3.15). No primer-dimer amplification was observed.

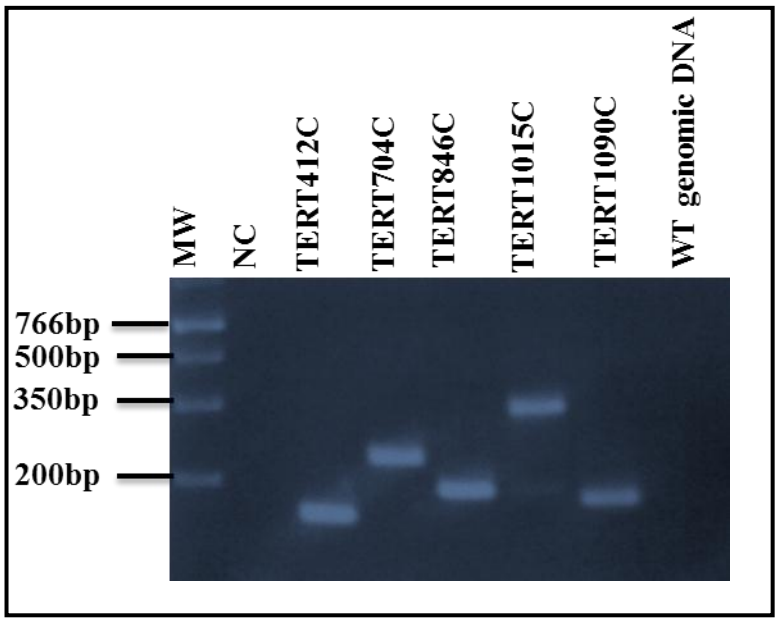

Figure 3.15: Electrophoretic analysis of multi-primer *TERT* analysis: amplification of *TERT* mutants in the presence of 5 allele-specific forward primers (excluding *M-TT694*).

Primers *M-TT412, M-TT704, M-TT846, M-TT1015, M-TT1090, M-TT412R, TT-exon5R, M-TT846R, M-TT1015R,* and *M-TT-exon14-15R* were combined and used to amplify their respective targets from the heterozygous plasmid mixes or WT genomic DNA as indicated on the figure (in the presence of 5% DMSO). The picture above shows the amplicons resolved on 2% agarose TAE gel stained with ethidium bromide and visualized with UV light. Lane MW: low molecular weight ladder (NEB), lane NC: no-template control.

3.3.5.C: Optimization of TERT202 and TERT694 detection

Mutant *TERT*202 was excluded from the multiplex PCR experiments performed above because its original forward primer (5' CTGGGATGCGAACGGTA 3') was designed to amplify an 812bp fragment using a common reverse primer (*M-TT412R)* from exon 2. The common reverse primer for both *TERT*202 and *TERT*412 was used to reduce the number of primers to be used in one master mix when the primers are combined in the multiplex. The amplification of the *TERT*202 fragment was achieved using this primer on the plasmid mutant *TERT*202C, using 0.5mM magnesium chloride, 25pmol of primers, 55^0C annealing temperature and 30 PCR cycles as described in section 3.2.3**.** The PCR cycling conditions were found not to be effective for the amplification of such a large fragment. Thus to rectify this, we designed another reverse primer (*M-TT202R*) (Table 3.1) so that a smaller fragment could be amplified, which would amplify a 233bp fragment if the mutation was present.

This primer was integrated into a *TERT* master mix containing primers to amplify these mutants: *TERT*412, *TERT*704, *TERT*846, *TERT*1015, and *TERT*1090. The *TERT*202 from the *TERT*202C/genomic mix and *TERT*846 or *TERT*1090 from their respective positive control plasmid/genomic mixes were successfully amplified. However, there was no amplification for either *TERT*412 from *TERT*412C or *TERT*1015 from plasmid construct *TERT*1015C (Figure 3.16).

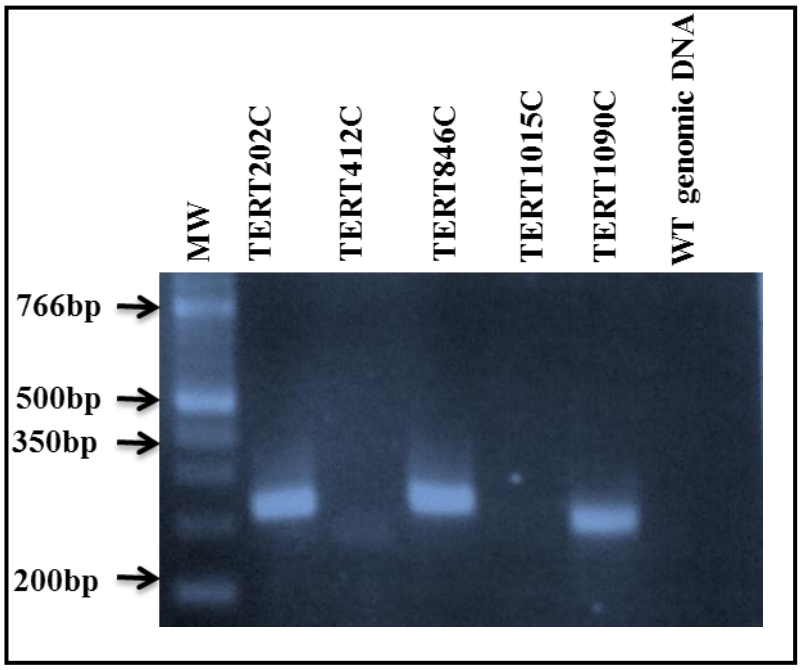

Figure 3.16: Electrophoretic analysis for the incorporation of *TERT*202 specific primer pair into multi-primer assay.

Primers *M-TT202, M-TT202R M-TT412, M-TT412R, M-TT704, TT-exon5R, M-TT846, M-TT846R, M-TT1015* and *M-TT1015R* were combined (in the presence of 5% DMSO) and tested against their respective heterogeneous plasmid templates or WT genomic DNA (as indicated on the figure). The picture above shows the resulting amplicons resolved on 2% agarose TAE gel stained with ethidium bromide and visualized with UV light. Lane MW: low molecular weight ladder (NEB).

Due to the failure of the forward primer (*M-TT694*) used to amplify *TERT*694 from plasmid construct *TERT*694C when incorporated with other allele-specific primers in a multiplex PCR, two separate reaction mixes were prepared to amplify the *TERT* mutations. The first PCR reaction mix consisted of 10 primers: *M-TT412, M-TT704, M-TT846, M-TT1015, M-TT1090, M-TT412R, TT-exon5R, M-TT846R, M-TT1015R,* and *M-TT-exon14-15R* to amplify mutants: *TERT*412, *TERT*704, *TERT*846, *TERT*1015 and *TERT*1090 in one PCR tube and this was referred to as *TERT* master mix one or TTMM1. The second PCR reaction mix contained 4 primers: *M-TT202, M-TT202R, M-TT694* and *TT-exon5R* to amplify mutants *TERT*202 and *TERT*694 was named *TERT* master mix two or TTMM2.

The PCR reactions and cycling conditions described in section 3.2.4.B were used: using 25pmol of all primers, except *M-TT694* (75pmol), 5% DMSO in each master mix, 0.5mM magnesium chloride in both to maintain the primer specificity and reactions setup on ice. All five mutants were amplified successfully, producing single amplicons of the expected size: *TERT*412 (180bp), *TERT*704 (323bp), *TERT*846 (240bp), *TERT*1015 (393bp) and *TERT*1090 (211bp), with no primer dimerization and no amplification when only WT genomic DNA was used. The mutant products *TERT*694 (353bp) and *TERT*202 (233bp) were also successfully amplified a PCR product of the correct band sizes using TTMM2. Therefore use of two master mixes thus allowed the successful specific amplification of 7 *TERT* mutations (Figure 3.17).

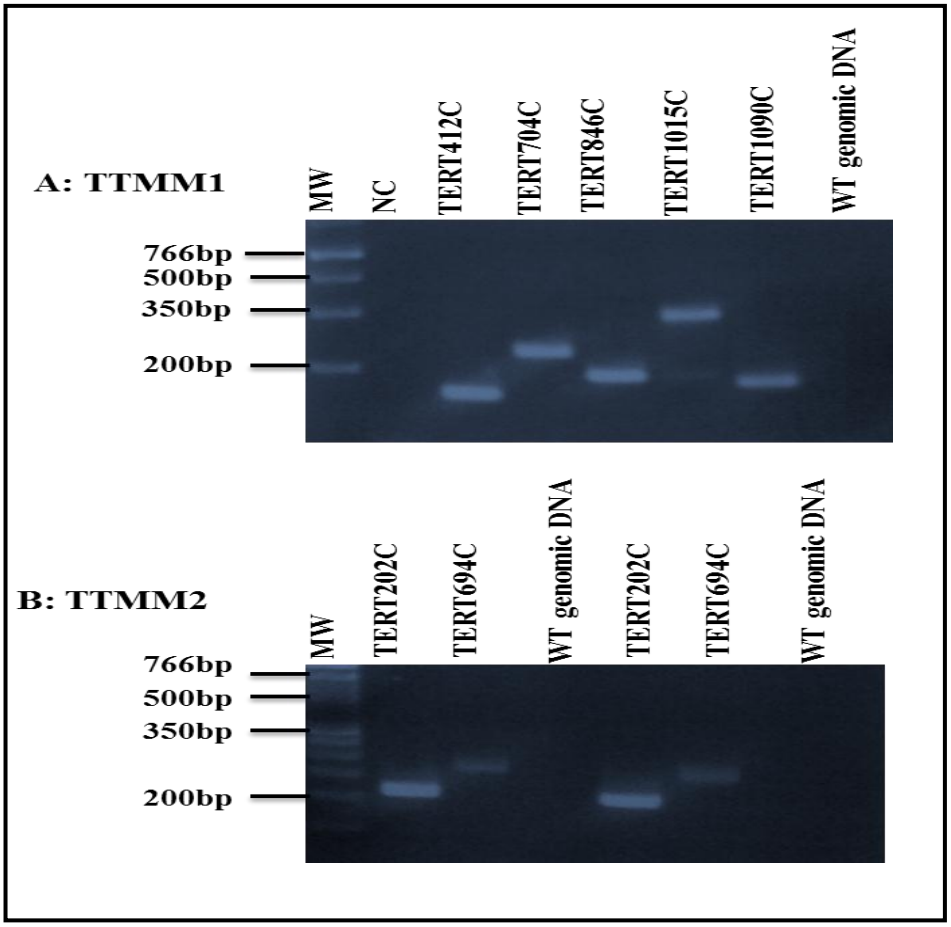

Figure 3.17: Electrophoretic analysis of multi-primer combinations TTMM1 and TTMM2 used to amplify their respective *TERT* mutations.

Two primer master mixes (TTMM1 and TTMM2) were used to amplify each mutant of *TERT* using either heterogeneous mixes or WT genomic DNA as templates (as indicated on the figures). Panel A: Amplification using TTMM1, which contained: *M-TT412, M-TT412R, M-TT704, TT-exon5R, M-TT846, M-TT846R, M-TT1015, M-TT1015R, M-TT1090* and *M-TT-exon14-15R.* Panel B: Amplification using TTMM2, which contained: *M-TT202, M-TT202R, M-TT964* and *TT-exon5R.*perfomed in duplicate. The picture above shows the amplicons resolved on 2% agarose TAE gel stained with ethidium bromide and visualized under UV light. Lane MW: molecular weight ladder (NEB), lane NC: no template control.

3.3.6 Control amplicon for TERT mutation detection assay

The design of our assay was such that the allele-specific primers would only bind if the mutations were present and thus an amplification product would only be produced in the presence of a mutation. In case of genomic DNA being tested that does not contain mutations (WT genomic DNA) and therefore there would be no amplicons produced. Unfortunately, the lack of an amplicon could also be due to PCR failure and thus lead to false negative results. Failure of PCR amplification could be due to different reasons, such as the lack of DNA added as template in the PCR reaction, poor quality DNA, a reagent missing from the master mix and also failure of the PCR machine itself. Therefore, it is very important to be able to avoid these false results. This is achieved by allowing the amplification of a control product simultaneously with the amplicon of interest. This control amplification has to be amplified in the presence or absence of the mutation to ensure that the PCR reaction conditions were favorable.

Due to two master mixes being used for the amplification of the *TERT* mutations (TTMM1 and TTMM2), two controls were required. For the TTMM1 mix, the first control forward primer to be tested was designed to exon 2 to yield an amplicon of 900bp. A single-plex PCR was attempted using 25 pmol of this forward primer: 5'CAACACGGTGA CCGACGCA 3' with reverse primer *M-TT412R* (Table 3.1) using the PCR conditions described in section 3.2.4.2**,** which included the 0.5mM magnesium chloride, 40ng WT human genomic DNA as a template. This PCR yielded no amplification (results not shown). In order to improve the amplification of the control fragment, the single-plex PCR was performed using different concentrations of control primer ranging from 50pmol, 75pmol and 100pmol, and WT human genomic DNA as a template.

This control amplicon was successfully amplified at 75 pmol and 100 pmol control primer concentration (results not shown). This control forward primer (100pmol) was then added to the TTMM1 reaction mix and tested using either a WT genomic DNA or heterogeneous mixes of *TERT*412C and *TERT*704C as templates. While the amplification of the mutants was successful in the presence of this additional primer, there was no co-amplification of the control fragment. This was also the case when only genomic DNA was used as a template (Figure 3.18.A). The successful amplification using these primers alone indicated that the DNA quality was not preventing amplification (Figure 3.18.B).

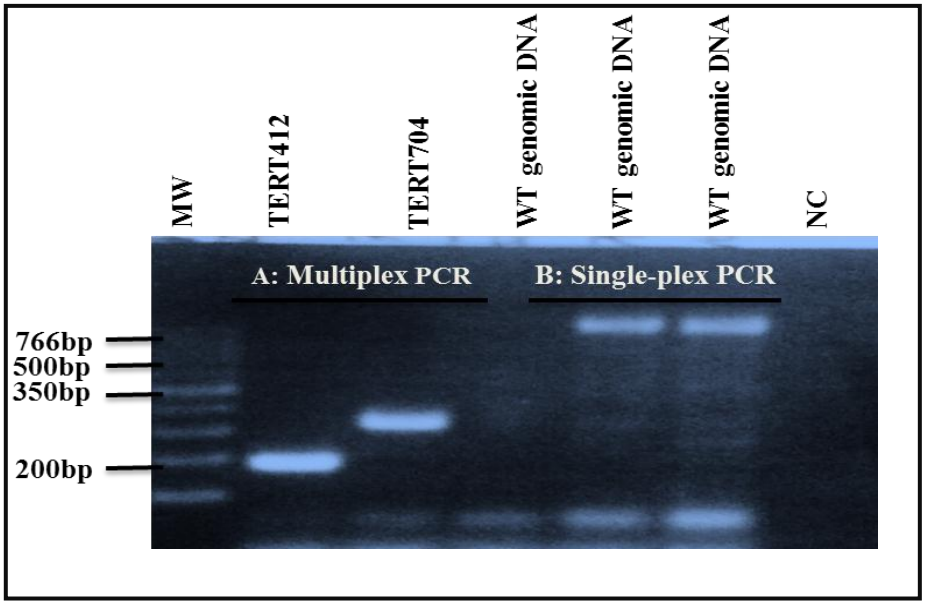

Figure 3.18: Electrophoretic analysis of multi-primer combination (TTMM1) used to amplify *TERT*412 and *TERT*704 separately with control amplification and single-plex PCR for control only.

Multi-primer combination (TTMM1) was used to amplify either *TERT*412 and *TERT*704 with control amplification using heterogeneous mixes (as indicated on the figure) or WT genomic DNA as templates (as indicated on the figures). Single-plex PCR was used to amplify the control amplification using WT genomic DNA as a template. A: Amplification using TTMM1, which contained: *M-TT412, M-TT412R, M-TT704, TT-exon5R, M-TT846, M-TT846R, M-TT1015, M-TT1015R, M-TT1090* and *M-TT-exon14-15R* and control amplification primer. B: Amplification using single-plex PCR which contained: control primer and *M-TT412R.* The picture above shows the amplicons resolved on 2% agarose TAE gel stained with ethidium bromide and visualized under UV light. Lane MW: molecular weight ladder (NEB), lane NC: no template control.

Due to the lack of amplification when the control primer was combined in TTMM1, we designed an additional 3 forward primers from exon 5, 5' CAACACACATGCGGCCAG 3' (for *TERT* exon 5 control 1), 5' TGCGGTGGCTGCGGTGA 3' (for *TERT* exon 5 control 2), and 5' CCCCGTCATC TGAGGAGAG 3' (for *TERT* exon 5 control 3), which would use a reverse primer (*TT-exon5-R*) (for exon 5 primers), from their corresponding exons to produce amplicons. These were designed to amplify control fragments in the region of exon 5 for *TERT* (between 15541bp-16021bp). These primers were tested in single-plex PCRs using either 0.5mM or 1.5mM magnesium chloride in separate master mixes, 25 pmol of each primer, 40 ng WT human genomic DNA as template, 55ºC annealing temperature, and 30 PCR cycles.

There was no amplification in the reaction master mix with 0.5mM $MgCl_2$, but from a master mix with 1.5mM $MgCl_2$, there was a good amplification when these primers were used (Figure 3.19). This indicated that these primers would not be suitable to work either in the TTMM1 or TTMM2 master mixes. As a result, these primers were excluded from the master mixes.

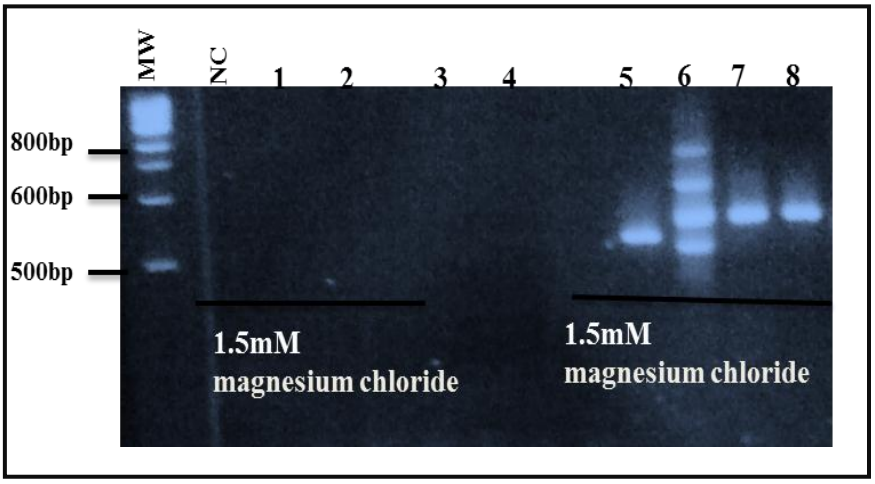

Figure 3.19 Effect of MgCl2 concentrations on the amplification from prospective *TERT* control amplicon primers (exon 5).

Single-plex PCRs were performed using control primers of *TERT* either in a PCR reaction mix with 0.5mM $MgCl_2$ or 1.5mM $MgCl_2$ using WT genomic DNA as a template. Lane MW: 1kb molecular weight ladder (NEB), lane NC: no template control (0.5mM magnesium chloride). Panel of 0.5mM magnesium chloride: lane 1: *TERT* exon 5 control 1primer, lane 2: *TERT* exon 5 control 2 primer, lane 3 and 4: *TERT* exons 5 control 3 primer. Panel of 1.5mM magnesium chloride: lane 5: *TERT* exon 5 control 1 primer, lane 6: *TERT* exon 5 control 2 primer, lane 7 and 8: *TERT* exon 5 control 3 primer. All forward primers were combined with the M-TTexon5R primer. The amplicons were resolved on 2% agarose TAE gel stained with ethidium bromide and visualized under UV light. For the amplification of a control amplicon using the TTMM2 mix, we designed a forward primer (*M-TTCTRL*) (Table 3.1) from exon 2 of *TERT* which would amplify a 450bp amplicon when combined with reverse primer *M-TT202R.* This primer pair was tested in a single-plex PCR using 25pmol of each primer, 0.5mM magnesium chloride, 5% DMSO, 40ng WT human genomic DNA, 55ºC annealing temperature, and 30 PCR cycles, and showed good amplification of the correct sized product (results not shown).

Figure 3.20 shows the result on the amplification of mutants *TERT*202 and *TERT*694 and the control amplicon when *M-TTCTRL* was combined with the other primers in TTMM2, using heterozygous mixes of *TERT*202C and *TERT*694C or WT genomic DNA as templates. The amplification of the mutant products was not inhibited by the addition of this extra primer. The control amplicon was successfully amplified off WT genomic DNA as well as the *TERT*694C heterozygous mix. However, amplification of the control amplicon was not observed when the *TERT*202 mutant amplicon was amplified. This is probably due to the preferential amplification of the smaller product, in light of the fact that both reactions are utilising the same *M-TT202R* reverse primer. The control *TERT* amplicon was then named *TERT*-control.

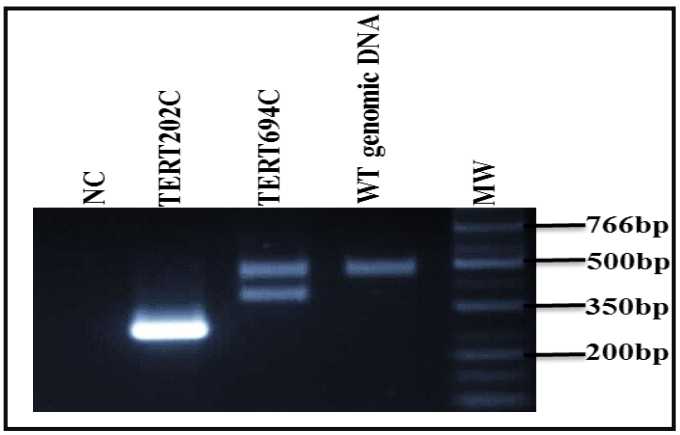

Figure 3.20: Incorporation of M-TTCTRL into TTMM2 to allow for the *TERT* control fragment amplification.

The *TERT*202 and *TERT*694 mutants were individually amplified simultaneously with the control amplicon (*TERT*-control) from heterozygous mixes using a master mix containing 5% DMSO and the following primers: *M-TERT202, M-TERT202R*, M-*TERT694, TT-exon5-R, M-TTCTRL* and *M-TERT202R.* The PCR amplicons were resolved on 2% agarose TAE gel stained with ethidium bromide and visualized under UV light. Lane MW: low molecular weight ladder (NEB) and lane NC: no template control.

As indicated above, each master mix was intended to have a positive control which would be amplified simultaneously with the amplicon of interest. For the *TERC* TCMM mix (section 3.3.3.A) and TTMM2 of *TERT*, the positive control primers were incorporated successfully into the reactions. Unfortunately for TTMM1, none of the designed primers worked in the multi-primer environment (11 primers). As all 3 master mixes would be performed at the same time, we felt that 2 control amplicons were enough to carry on with the assay and would be used to account for issues of poor quality DNA, poor quality reagents or failure of the PCR machine itself.

3.3.7 TERT assay positive controls (TTC1 and TTC2)

Heterozygous mixes of *TERT*, containing 5 plasmid constructs (*TERT*412C, *TERT*704C, *TERT*846C, *TERT*1015C, and *TERT*1090C) and WT genomic DNA in one tube, and another one consisting of 2 mutants (*TERT*202C and *TERT*694C) and WT genomic DNA in another tube were prepared to be used as multiple mutant positive control for the assay (TTC1 and TTC2). As indicated in *TERC* assay, although the amplification of the control fragment (*TERT*-control) works as an internal control for the quality of the genomic DNA used in the assay, a second positive control is necessary to confirm that each master mix used consists of multiple primers that are able to detect each mutant in every experiment. These positive mutant control mixes were tested as templates using the master mixes described in section 3.2.4.A, with the 5% DMSO added using 25 pmol of all primers except 50pmol for *M-TERT694* of *TERT*694 mutant using 30 cycles of PCR.

The amplification of the positive controls with only *TERT*1090 and *TERT*704 mutants was successful, while the amplification of the other 3 mutants (*TERT*412, *TERT*846 and *TERT*1015) from the TTMM1 master mix was not successful (Figure 3. 21). In the second positive control of *TERT*, all mutants were amplified, together with the *TERT*-control fragment, showing that the multiple templates were working accordingly from the master mix TTMM2 (Figure 3.21). As only two mutants were amplified in the first positive control of *TERT*, we felt that there were enough to be positive controls since in this assay we are only interested to detect if the patient would have a mutation or not. Since in the literature, either one of *TERT* mutations has been reported, there is no case where the patient would carry more than one of these mutations.

It was considered that the amplification of only two mutants sufficient to conclude the assay to be used as it has been shown that all mutants are amplified when only one mutant/genomic DNA mix is used. Each of the *TERT* mutations can be detected with the assay.

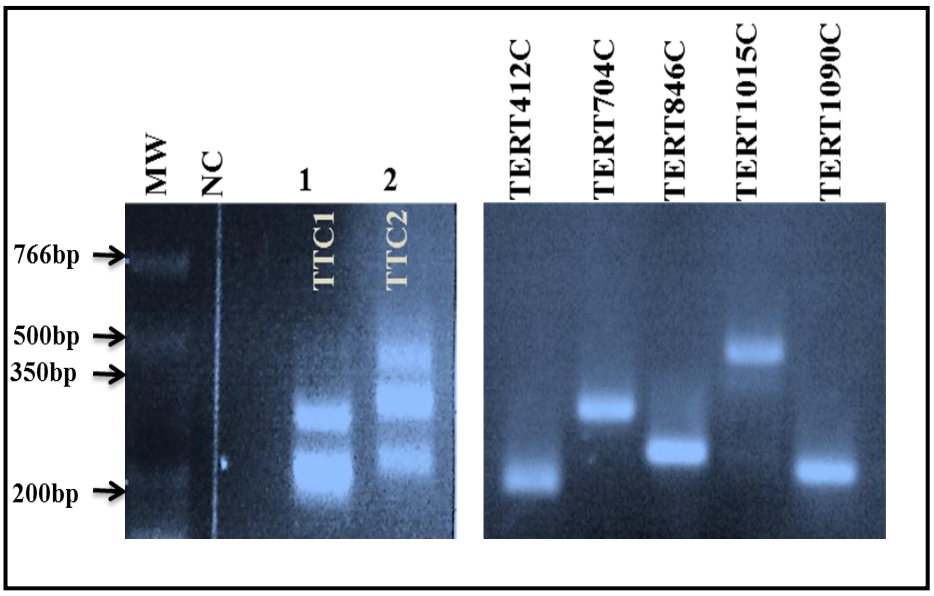

Figure 3.21: Electrophoretic analyse of *TERT* multiplex PCR with controls.

Allele-specific forward primers: *M-TT412, M-TT412R, M-TT704, TT-exon5R, M-TT846, M-TT846R, M-TT1015, M-TT1015R, M-TT1090* and *M-TT-exon14-15R* were combined in the TTMM1 master mix and other allele-specific primers: *M-TT202, M-TT202R, M-TT964* and *TT-exon5R* were combined with *M-TTCTRL* in the TTMM2 master mix, both master mixes contained 5% DMSO. TTMM1 master mix was tested with a combined mutant control TTC1 or specific heterogeneous mutant positive controls, while TTMM2 master mix was tested with a combined mutant positive control TTC2 as templates (as indicated on the figure). PCR conditions were 55°C annealing temperature, 30 cycles, 25pmol of all primers except 50pmol of *M-TERT694*. The PCR amplicons were resolved on 2% agarose TAE gel stained with ethidium bromide and visualized under UV light. Lane MW: Molecular weight DNA ladder (NEB) and lane NC: no template control.

The development of the multiplex PCR to detect 5 *TERT* mutations: *TERT*412, *TERT*704, *TERT*846, *TERT*1015 and *TERT*1090 using a single master mix (TTMM1) and another single master mix (TTMM2) to detect 2 *TERT* mutations: *TERT*202 and *TERT*694 was successfully achieved. Five forward primers and 5 reverse primers in the TTMM1 master mix to detect 5 *TERT* mutants were combined, while also 3 forward primers and 2 reverse primers were also combined successfully to detect either a control amplicon (*TERT*-control) alone or a mutant fragment and control amplicon combination. The final PCR cycling conditions and reaction set-up for the multiplex of *TERT* were a common annealing temperature of 55°C for primers, 30 cycles for PCR cycling, 5% DMSO, two master mixes, TTMM1 (10 primers) and TTMM2 (5 primers) with all containing 25 pmol of all primers with the exception of 50pmol of *M-TERT694.*

The controls for the assay include a mutant negative WT genomic DNA, a negative control (containing no template), and a combined mutant positive controls for the specific master mix, TTC1 for TTMM1 master mix and TTC2 for TTMM2 master mix. The final *TERC*/*TERT* assay will consist of 3 positive controls, TCC, TTC1, and TTC1 which would be used as multiple templates to their specific master mix. TCMM master mix will use positive control TCC, while TTMM1 master mix will use a positive control TTC1 and TTMM2 will use a positive control TTC2 respectively. The no DNA template will also be used as reagents' negative control in one of the master mixes to ensure that reagents are not contaminated. The patient's sample would be analysed in all 3 master mixes.

3.4 Validation of *TERC/TERT* assay

3.4.1 Sensitivity test for the assay assessment

In order for the assay to be used in a clinical diagnostic setting, it is essential to know the limitations of the assay, in relation to the lowest number of mutant alleles that can be detected. As the majority of these mutations in AA are clonal, it is important to report this sensitivity limit in the case of a negative finding. To determine this lower limit of detection a serial dilution of each heterogeneous template (mutant/genomic DNA mix) were performed into WT genomic DNA. Templates containing 10%, 5% and 2% mutant alleles were prepared as described in section 3.2.1.C and then used as templates in the final *TERC* and *TERT* PCR reactions as described in sections 3.2.4.A and 3.2.4.B respectively. Each mutant mix was only tested with its relevant PCR master mix (TCMM, TTMM1 or TTMM2). The results of this dilution series on all of the heterogeneous mixes showed that although the intensity of the amplicon decreased with each dilution (as expected), all of the mutants were still able to be detected when present at a concentration of 2% mutant alleles (98% WT alleles) (Figures 3.22 and 3.23).

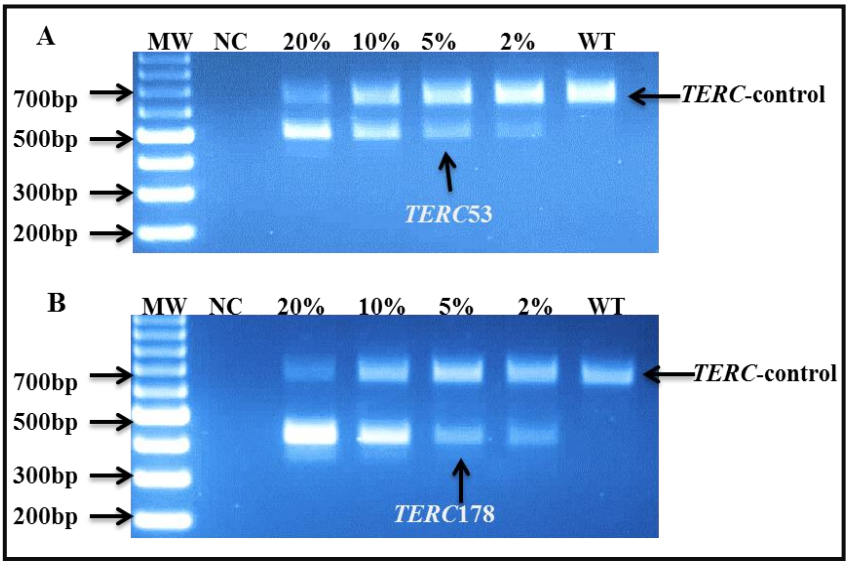

Figure 3.22: Sensitivity assessment for the detection of *TERC* mutations using TCC PCR reaction mix. Plasmids containing the *TERC* SDM PCR products were diluted into WT genomic DNA to produce a dilution series of the mutants (20-2% mutant alleles). These were then used as templates with the TCMM reaction mix. The picture shows the resulting amplicons that were resolved on a 2% agarose TAE gel stained with ethidium bromide and visualized with UV light. Panel A: analysis of the *TERC*53C dilution series, B: analysis of the *TERC*178C dilution series. WT = WT genomic DNA used as a template. NC = no template control, MW: 100bp molecular weight DNA ladder (NEB).

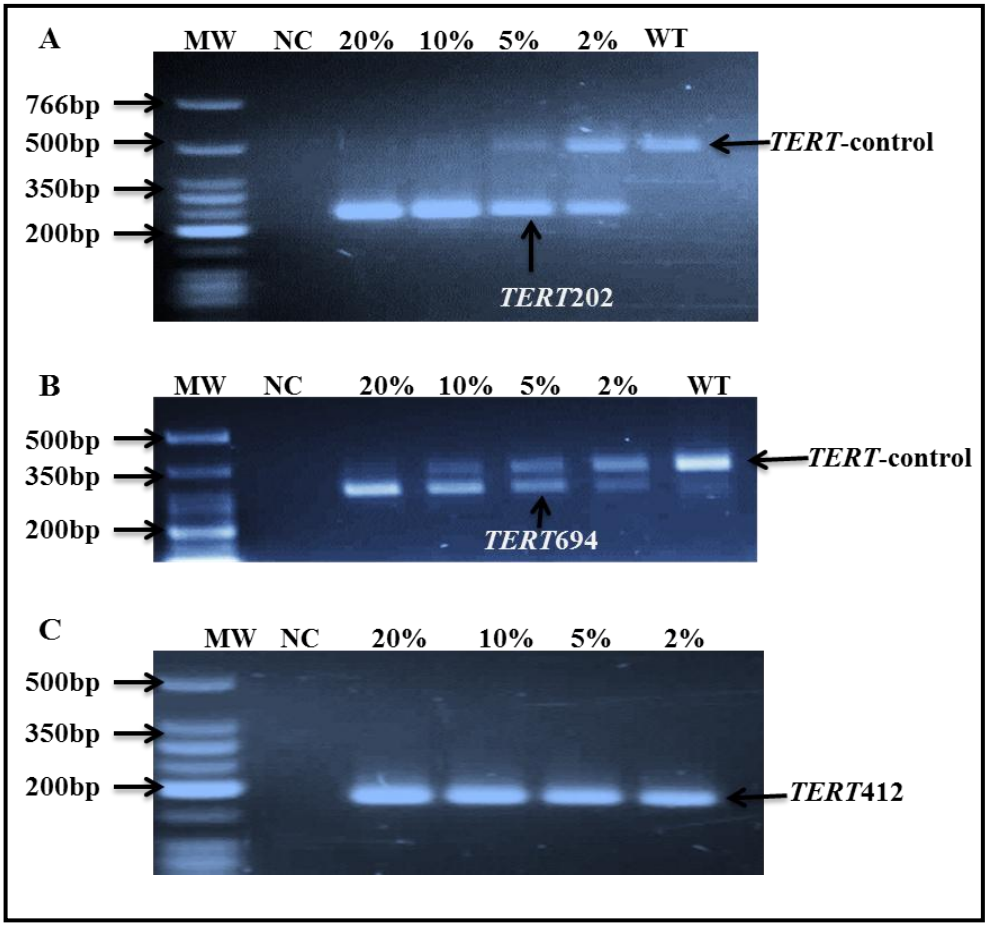

Figure 3.23: Sensitivity assessment for the detection of *TERT* mutations using TTMM1 and TTM2 PCR reaction mixes.

Plasmids containing the *TERT* SDM PCR products were diluted into WT genomic DNA to produce a dilution series of the mutants (20-2% mutant alleles). These were then used as templates with either the TTMM1 or TTMM2 reaction mix. The picture shows the resulting amplicons that were resolved on a 2% agarose TAE gel stained with ethidium bromide and visualized with UV light. Panel A: analysis of the *TERT*202C dilution series, using TTMM2, B: analysis of the *TERT*694C dilution series, using TTMM2. Panel C: analysis of the *TERT*412C dilution series, using TTMM1, WT = WT genomic DNA used as a template. NC = no template control. Lane MW: low molecular weight DNA ladder.

3.4.2 Assay reproducibility assessment

In order to measure the reproducibility of the assay, 3 samples of heterozygous mixes, *TERC*53C and *TERT*704C (prepared in section 3.2.1.B), and WT genomic DNA (prepared in section 3.2.1.A) alone were tested in the assay. Each sample was used as a template in each of the 3 assay reactions, TCMM1, TTMM1 and TTMM2 as described in section 3.2.6.B and the experiment was repeated 3 times to check precision and reproducibility of the results.

The correct results were achieved in triplicate with only *TERC*53 mutant and *TERC*-control amplicons (Figure 3.24.A, Lane 1) produced, using heterogeneous mix *TERC*53C/WT genomic DNA from TCMM1 reaction, while TTMM2 reaction produced only *TERT*-control (Figure 3.24.A, Lane 3) and no amplicon (Figure 3.24.A, Lane 2) produced from TTMM1 reaction as expected. Using heterogeneous mix *TERT*704C/WT genomic mix produced only correct amplicons of *TERC*-control (Figure 3.24.B, Lane 4) from TCMM1 reaction, *TERT*704 mutant (Figure 3.24.B, Lane 5) from TTMM1 reaction, and *TERT*-control (Figure 3.24.B, Lane 6) from TTMM2 reaction as expected.

Using the WT genomic DNA alone as a template into 3 reactions of the assay produced only *TERC*-control amplicon (Figure 3.24.C, Lane 7) from TCMM, no amplicon (Figure 3.24.C, Lane 8) from TTMM1, and only *TERT*-control amplicon (Figure 3.24.C, Lane 9) from TTMM2. After repeating this experiment 3 times, the same results were produced, confirming that the assay is reproducible amplifying only correct amplicons from their respective reactions of the assay. The repeat of these experiments is shown in Table 3.2 with symbols representing amplicons amplified from a correct reaction or no amplification, using either heterogeneous mixes or WT genomic DNA, demonstrating reproducibility.

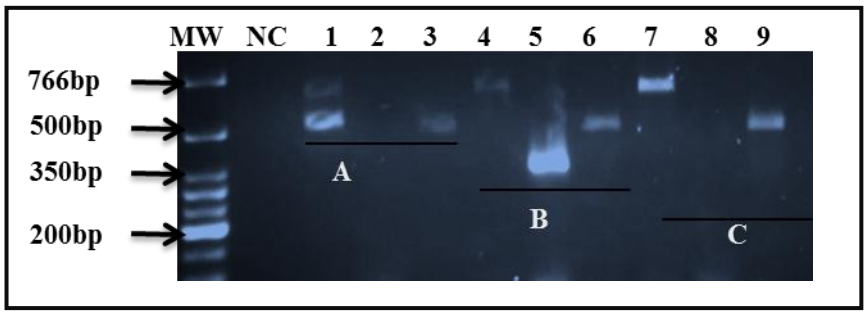

Figure 3.24: Reproducibility assessment for the detection of *TERC*/*TERT* mutations or control amplicons using TCMM, TTMM1 and TTM2 PCR reaction mixes.

Heterogeneous mixes (*TERC*53/WT genomic DNA and *TERT*704/WT genomic DNA) and WT genomic DNA were used as templates to each reaction mix for the detection of only their targets. Panel A: analysis of *TERC*53/WT genomic DNA mix, using TCMM, TTMM1 and TTMM2. Panel B: analysis of *TERT*704/WT genomic DNA mix using TCMM, TTMM1 and TTMM2. Panel C: analysis of WT genomic DNA using TCMM, TTMM1 and TTMM2. The picture shows the resulting amplicons that were resolved on a 2% agarose TAE gel stained with ethidium bromide and visualized with UV light. Lane MW: low molecular weight DNA ladder (NEB), lane NC: no template control.

Table 3.2: Scoring of reproducibility for *TERC*/*TERT* assay

TERC/TERT assay									
	First run$^{\Sigma}$			Second run $^{\Omega}$			Third run$^{\$}$		
amplicons$^{\pi}$	TCMM	TTMM1	TTMM2	TCMM	TTMM1	TTMM2	TCMM	TTMM1	TTMM2
*TERC*53	√	x	x	√	x	x	√	x	x
TERC-control	√	x	x	√	x	x	√	x	x
*TERT*704	x	√	x	x	√	x	x	√	x
TERT-control	√	x	√	√	x	√	√	x	√

$^{\pi}$: PCR products produced from each reaction mix of the assay

$^{\Sigma}$: First experiment of the assay performed using selected heterogeneous mixes (*TERC*53C/WT genomic DNA and *TERT*704C/WT genomic DNA) and WT genomic DNA alone as templates.

$^{\Omega}$: First repeat of first experiment

$^{\$}$: Second repeat of first experiment

✓: Shows the amplification of amplicon from a reaction

x: Shows that there was no amplification of amplicon from a reaction

3.4.3 Assay accuracy assessment

To ensure that the *TERC*/*TERT* assay could be done accurately and consistently in different settings, as would be the case for a diagnostic assay, two different set up of experiments were performed. In the first experiment, 4 unknown samples (blind samples) were used, while in the other, 5 known negative WT samples (prepared in section 3.2.1.D) as templates were used. Both known and unknown samples were tested separately, using the whole *TERC*/*TERT* assay consisting of 3 PCR reactions (TCMM, TTMM1, and TTMM2). As controls, each experiment had 3 positive controls mixes TCC, TTC1, and TTC2 (prepared in section 3.3.7) to ensure that the assay was working correctly and samples (known and unknown) can be compared with known positive control on every experimental run. The experiments were conducted as described in section 3.2.6.C (methods).

Figure 3.25 below shows the *TERT*/*TERC* multiplex analysis of the 4 blind samples: the 3 positive controls and no template controls. The 4 blind samples also showed the correct amplification of both control amplicons: *TERC*-control and *TERT*-control (Figure 3.25), indicating that the DNA was of adequate quality to yield a result. Table 3.3 shows how the results of this analysis were scored compared to the subsequently revealed identity of the samples. Two unknown samples were found to be *TERT* positive while other 2 were WT samples (Figure 3.25). The known negative samples were WT genomic DNA (Figure 3.26) as expected.

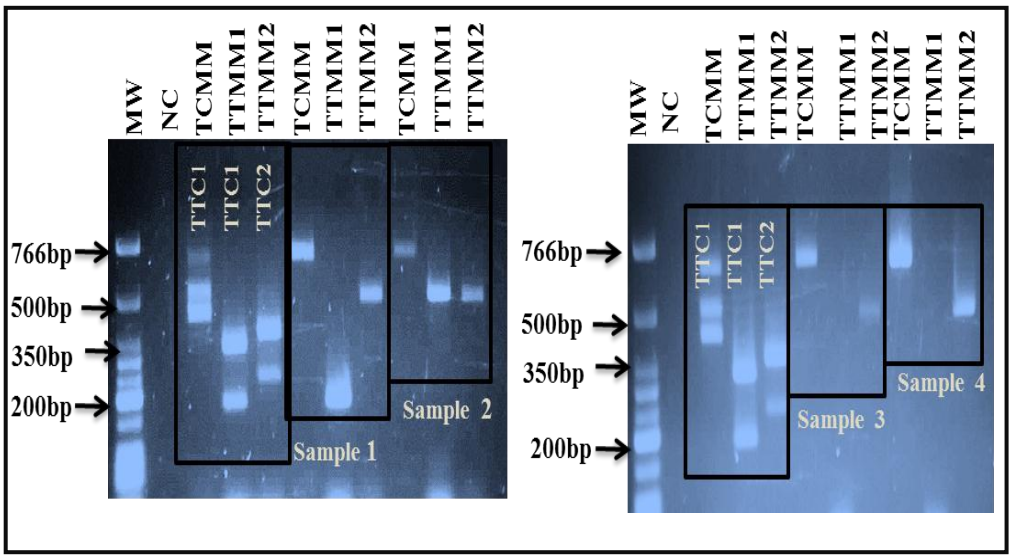

Figure 3.25: Electrophoretic analysis of unknown samples amplicons detected using *TERC/TERT* multiplex PCR assay for the assessment of accuracy.

Four unknown samples were assayed for mutations using the *TERT*/*TERC* multiplex assay. Each sample was tested on 3 reactions of the assay (as indicted on the figure), with 3 positive controls to check the validity of experiment. Lane MW: low molecular ladder (NEB), lane NC: no template control.

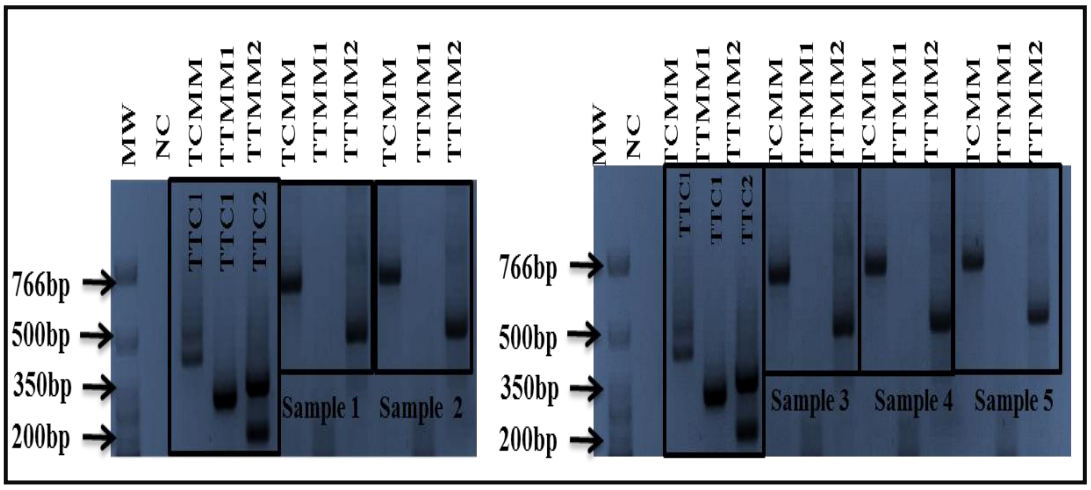

Figure 3.26 Accuracy assessment using healthy individuals DNA as a template in the *TERC/TERT* multiplex PCR assay.

Five samples of healthy individuals were used as templates on the reaction mixes of the *TERC*/*TERT* multiplex PCR (as indicated on the figure) to assess the accuracy amplification of the assay to detect *TERC* and *TERT* mutations. The experiment included positive control (as indicated on the figure) to ensure that the PCR was working correctly. Lane MW: low molecular ladder (NEB), lane NC: no template control.

Table 3.3: Scoring of accuracy assessment for *TERC*/*TERT* assay

Sample[£]	Amplicon[€]
Unknown 1	*TERT* 412
Unknown 2	*TERT* 1015
Unknown 3	WT
Unknown 4	WT
Negative control 1	WT
Negative control 2	WT
Negative control 3	WT
Negative control 4	WT
Negative control 5	WT

£: Unknown samples and known negative WT genomic DNA used as templates for the accuracy assessment in the *TERC*/*TERT* assay.

€: PCR product identified from 3 reactions (TCMM, TTMM1, and TTMM2) of *TERC*/*TERT* assay.

3.5 Cost analysis for the *TERC*/*TERT* assay

The cost and time required to generate a single patient result was calculated using the *TERC*/*TERT* allele-specific multiplex assay and compared to a result that could be generated using DNA sequencing via the standard Sanger sequencing methodology. The costing for the *TERT*/*TERC* assay was based on a total of 7 PCR reactions for a single patient result: 3 positive control reactions, a reagent only control and 3 PCR reactions for the patient (or patient samples in duplicate). The cost of the sequence analysis was based on 1 PCR reaction for the *TERC* gene and 4 PCR reactions for the *TERT* exons that contain the relevant mutations (PCR products 1000bp<). Sequencing would need to be performed with both the forward and reverse primers for each amplicon to confirm the data – thus 10 sequencing reactions in total. The prices of the reagents were based on costs as of August 2013 and the cost of the technologists' time is not included in either scenario. The *TERC*/*TERT* was more cost effective (R409.63) compare to the DNA sequencing (R1148.06) to detect mutations of *TERC* and *TERT*. In addition to the lower cost, performing the experiments and results analysis was also considerable quicker: 4 hours estimated versus 9 hours estimated.

Table 3.4: Cost analysis for the *TERC*/*TERT* assay and time required to produce results

TERC/TERT MULTIPLEX ASSAY COST: 2X PER PATIENT (6 tubes) , CONTROLS (3), AND BLANK (1)			
Reagents	***No. of units***	***cost of each item or ul(R)***	***Sub-total cost (R)*$***
Gloves	**3 Pairs**	**2.44**	**7.32**
Epperndorfs	**10 tubes**	**0.22**	**2.2**
Nuclease free water	**87.2µl**	**0.27**	**23.54**
Primers	**21 items**	**0.42**	**8.82**
Hot Start Taq Polymarase mix	**20U**	**5.01**	**100.2**
DMSO 100%	**25µl**	**0.0064**	**0.16**
Glycerol 50%	**7.5µl**	**2.11**	**15.86**
DNTP's	**60µl**	**3.05**	**183.8**
Filtered tips	**16 items**	**1.18**	**18.88**
Yellow tips	**21 items**	**0.17**	**3.57**
Agarose	**2g**	**22.64**	**45.28**
Total Ω			**409.63**
Time needed			
PCR preparation: 30 minutes			
PCR Cycling: 2.5 hours			
Gel electrophoresis: 1 hour			
Total time∑: 4 hours			

$: Subtotal shows the total of each item used during the assay.

Ω: Total shows the overall total of all reagents and apparatus used to conduct the assay.

∑: Time needed indicates the overall time required to complete the experiment and get results analysis.

Table 3.5: Cost analysis for DNA sequencing to detect *TERC/TERT* mutations

SEQUENCING TERC/TERT MUTATIONS			
PCR Reaction 5 (forward primers), 5 (reverse primers) 1 (Blank)			
Reagents	*No. of units*	*Cost of each item or µl (R)*	*Total cost for each item used (R)*$^{\pi}$
Gloves	2 pairs	2.44	4.88
Epperndorfs	10 tubes	0.22	2.2
Nuclease free water	126.06µl	0.27	34.04
Primers [5(R) & 5(F) 25pmol	10µl	0.42	4.2
Hot Start Taq Polymarase mix	32U	5.01	100.2
DNTP's 10mM	22µl	3.05	67.03
Filtered tips	56 items	1.18	66.08
Yellow tips	16 items	0.17	2.72
Sub-total$^{\beta}$			281.35
PCR product purification (10)			
Qiagen kit	10 columns	43.09	430.09
Nuclease free water	137.5µl	0.27	37.13
Gloves	1 pair	2.44	2.44
Epperndorfs	10 tubes	0.22	2.2
Yellow tips	56 items	0.17	9.52
Sub-total			481.38
Sequencing reactions (10)			
Big Dye reaction mix with dilution buffer	10 reactions	107.79	1077.9
Nuclease free water	115.2µl	0.27	31.1
Primers (forward and Reverse)	16 items	0.42	6.72
Epperndorfs	16 tubes	0.22	3.52
Filtered tips	64 items	0.6	38.4
Gloves	1 pair	2.44	2.44
Sub-total			1160.08
TOTAL$^{\Omega}$	1922.81		
Time needed$^{\Sigma}$			
PCR reactions preparation: 1 hour			
PCR Cycling: 2.5 hours			
PCR product purification : 30 minuntes			
Sequencing cycling : 2 hours			
Sequencing analyisis : 3 hours			
Total time: 9 hours			

π: Total cost for each item shows each item price used during sequencing.

β: Subtotal shows totals for each experiment conducted during DNA sequencing.

Ω: Total indicates the overall cost of the DNA sequencing to get results and analysis.

Σ: Time needed is the time required to perform DNA sequencing and results analysis.

3.6 DISCUSSION AND CONCLUSIONS

This study aimed to develop a simple, rapid and cost-effective diagnostic multiplex PCR assay to detect all *TERT* and *TERC* mutations associated with IST failure in AA. This was to be achieved via the development of a two-tube system – one to detect the relevant 7 *TERT* mutations and the other to detect the 4 relevant *TERC* mutations. These mutations are known to cause telomerase complex dysfunction which leads to the production of shortened telomeres, thus triggering stem cells to apoptosis and leading to the AA phenotype (Verma *et al*, 2002, Young *et al*., 2006). Patients harbouring one of these mutations would indicate that the disease is most likely not caused by the common autoimmune pathogenesis and that such patients are very likely to fail to respond to the standard IST for this form of AA (Yamaguchi *et al*., 2005, Marrone *et al.,* 2007). Thus knowing the mutational status of an AA patient at diagnosis and prior to the initiation of therapy is critical. The IST therapy is very expensive, with significant side effects. It is therefore vital that the likelihood that this type of therapy would be helpful to the patient is determined. Additionally, there is evidence that these mutations can be inherited and that not all persons carrying these mutations develop AA. (Yamaguchi *et al*., 2005). It is therefore important in AA cases where transplantation is being considered that the mutations are not present in the family member donor (Spellman *et al*., 2012).

The current method of choice in most laboratories in western countries to detect these mutations is DNA sequencing. Mutations of *TERC* can be detected easily as this gene consists of a single exon of only 451bp sequence and four of these mutations: *TERC*53, *TERC*110, *TERC*178 and *TERC*180 are found across the gene. However for *TERT* it is not possible to sequence all seven mutations from one PCR reaction, as the mutations span 5 exons (the whole gene is 16 exons). At least 4 separate amplifications (500bp-1kb) are required to detect these mutations via sequencing. Two mutations of exon 2 (*TERT*202, and *TERT*412) can be detected simultaneously, as can *TERT*694 and *TERT*704 in exon 5 and *TERT*1015 and *TERT*1090 in exons 14 and 15 respectively. *TERT*846 in exon 9 requires a 4th reaction. Independent sequencing of these mutations requires more time to analyse with a possibility of detecting clinically irrelevant point mutations (Yamaguchi et al., 2005). In addition, sequencing requires that the amplicons are purified to generate good quality data for better analysis. This requires additional steps in the laboratory, with increased reaction consumables and therefore is rather costly.

Sequencing reactions would also require both forward and reverse primers, used in separate reactions, thus increasing the number of reactions and the time factor, as well as cost. We therefore decided to develop an alternative assay to detect these mutations which will be rapid, simple to interpret and more cost-effective than the current existing DNA sequencing. As allele-specific PCR approach has been successfully used previously in the detection of point mutations and SNPs, and also in a multi-primer/multiplex setting (Walsh *et al.*, 1992). Although an allele-specific PCR approach requires a lot of troubleshooting to find suitable PCR cycling and reaction components (Chamberlain *et al.*, 1988, Schoske *et al.*, 2003), we decided to use this approach with a benefit of primers binding only in the presence of a mutation and detect the gene involved.

Currently there are no commercially available positive controls for these mutations which can be used for assay development. In addition, AA is a rare disease thus few patients are available to be used as a source of DNA with these mutations. Therefore we decided to generate mutants of *TERC* and *TERT* via PCR using SDM primers consisting of a specific mutation or deletions that have been reported to be linked to AA development. These mutants were successfully generated using SDM PCR as reported in Chapter 2. We then designed allele-specific primers to detect the mutants in an allele-specific multiplex PCR, simulating the AA patients sample with one of these mutations by mixing the cloned SDM products with WT genomic DNA. These primers provide a discriminatory factor with the mutations located at the 3'end and binding to its template only in the presence of that specific mutation (Dieffenbach *et al*., 1993, Hirotsu *et al*., 2010). We generated these mutants with the aim to develop and assay which will be used to detect 4 mutations of *TERC* and 7 *TERT* mutations using two PCR reaction mixes.

During the development of the *TERC* multiplex, we found that *TERC*110 amplification had produced non-specific products. But using 3% glycerol we were able to improve the specificity and eliminated non-specific products. Markoulatos *et al.* (2002) suggested that using this additive could improve the specificity of amplification as well as increasing the PCR efficiency, especially in a multiplex PCR setting. Certainly in this study the specificity improved after adding 3% glycerol in a multiplex PCR to amplify *TERC* mutants. Eventually all 4 *TERC* mutants were able to be amplified from one master mix (TCMM) including the experiment positive control amplification amplicon.

However when 55°C and above annealing temperature was used *M-TC53* and *M-TC110* primer for *TERC*53 and *TERC*110 produced non-specific products, but reducing PCR cycling to 30 cycles improved the amplification of *TERC*53. Using 25pmol during the incorporation of a control primer (*M-TCTRL*) into the multi-primer of 4 *TERC* mutants, some mutants were not amplified but reducing the primer concentration to 3.1pmol allowed the amplification of all mutants together with a control amplicon (*TERC*-control). Eventually the detection of all 4 mutants of *TERC* was achieved successfully after all the alterations of PCR cycling and reaction components.

As we wanted to be able to test for the *TERC* and *TERT* mutations at the same time, the cycling conditions generated for the *TERC* amplification had to remain constant for the *TERT* development. Due to this limitation, other parameters had to be changed during the development of the *TERT* multiplex to ensure allele-specificity of the primers and the production of sufficient amplicon. This included the addition of 5% DMSO due to amplification problems with *TERT*694, reaction setup on ice (due to amplification issues of both *TERT*694 and *TERT*704), and different amounts of the various primers. Unfortunately despite several attempts, when primers to amplify all 7 mutants of *TERT* were combined into one reaction mix, we found that the PCR efficiency was not sufficient with some primers causing significant primer dimerization and preventing the amplification of the amplicons of interest. Primer *M-TT694* for *TERT*694 mutant was found to be responsible for the primer dimer formation. The solution was to divide the detection of *TERT* mutations across 2 different PCR reaction mixes: TTMM1 and TTMM2, with *TERT*412, *TERT*704, *TERT*846, *TERT*1015 and *TERT*1090 detected with TTMM1 and *TERT*202 and *TERT*694 with TTMM2.

Hence we finally developed 3 PCR reaction mixes for the entire assay: TCMM for *TERC* mutants, TTMM1 for 5 mutants and TTMM2 for 2 mutants of *TERT*. That could all be used at the same time. In addition, developing assay controls required that mutants which are amplified from one tube of master mix are combined together to be used as multiple templates and these controls were TCC1 for *TERC* and TTC1 and TTC2 for *TERT*. Even when these mutants were combined, the amplicons of interest were still amplified well, showing that they can be used successfully during the assay. Overall the mutants of *TERC* and *TERT* can be detected using this assay.

As indicated in the results section in Chapter 3, when the assay is performed, 2 of 3 reactions mixes, TCMM and TTMM2 consist of the reactions positive controls, *TERC*-control and *TERT*-control respectively. This was done to ensure that there will be an amplicon which will be produced in the presence of mutation or not from the patient's sample that will confirm the quality of DNA template used, and these 2 controls were incorporated successfully to these reactions mixes. Usually, chemical modifications such as locked nucleic acid based primers, zip nucleic based primers or peptide nucleic acid based primers are required to the primers to enhance their viability in a multiplex PCR, however in this assay was developed successfully without them only normal primers were being used in an allele-specific multiplex PCR. Detecting 11 mutations in one simple PCR-gel based assay is noble.

For the assay to be used in the diagnostic environment it requires to be validated. This was partially achieved during this study by performing experiments to investigate accuracy, reproducibility and sensitivity. The accuracy assessment was assessed by analysis 4 "blinded samples" and 5 known WT samples. The results confirmed that the assay was detecting the relevant mutations correctly and did not detect false positives. However, this assessment was flawed in that a very small number of samples were analysed and no "real" AA patient samples were assessed. This was unfortunately due to time constraints and the assay would have to be tested with a much larger sample pool to validate this point before it could be used in the diagnostic arena. This assay is also reproducible, when the same heterogeneous mixes were used in triplicate; the same results were observed − indicating that the assay is consistently reproducing the same amplicons. As for the sensitivity, when plasmid constructs were diluted into genomic DNA making it to 98% WT and 2% mutation, the mutations were still detected, confirming that the assay is sensitive and able to detect even below 20% clinically relevant lower limit of detection when using sequencing. This could help to detect the mutations even if the number of alleles is very low from the DNA sample of a patient, showing that this assay could be more clinically relevant to detect mutations, probably better than DNA sequencing that has 20% sensitivity.

In conclusion, a diagnostic multiplex PCR assay to detect all *TERC* and *TERT* mutations associated with AA was developed successfully. This assay can be applicable to screen these clinically relevant mutations with the advantage of being simple, easy to prepare and able to produce a result within the same day as sample receipt if necessary.

Its cost is significantly cheaper compared to the currently existing methods to detect mutations, considering that in this assay 11 mutations are detected at the same time, with less time required to conduct the experiment. This assay can be applicable not only in AA patient's diagnoses but also can be used in other diseases related to telomerase complex dysfunction, such DKC, IPF and others.

Future work would be to certify *TERC*/*TERT* assay by validating the assay to check accuracy using positive control AA patients or DKC patients, especially those known to have certain *TERC*/*TERT* mutations to ensure that the assay does detect those mutations. In addition, since only 5 healthy individuals were used during the assay development and accuracy test, it would be recommended to test a larger number of healthy individuals from various populations groups in South Africa. This will ensure that mutations investigated during the assay development are not associated with a certain race in the South African population.

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