TELOMERE DYNAMICS IN CHRONIC MYELOID LEUKAEMIA

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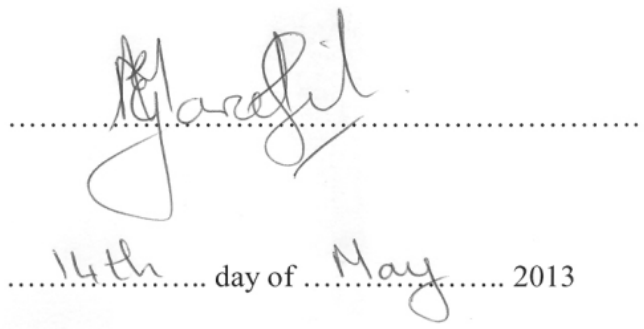
A dissertation submitted to the Faculty of Science, University of the Witwatersrand

in fulfilment of the requirements for the degree of Master of Science

Johannesburg, 2013

Declaration

I, Marcel Eduardo Gil, declare that this dissertation is my own work. It is being submitted for the Degree of Master of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in this or any other University. Ethics approval for research on human tissues used in this study was granted by the University of the Witwatersrand Committee for Research on Human Subjects (Protocol Number M01-11-03 and M07-07-19).



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Publications and Presentations

Material presented in this dissertation has been published in the following journal articles and conference proceedings:

Research Articles

1. **Gil ME and Coetzer TL** (2004). Real-time quantitative RT-PCR for human telomere elongation reverse transcriptase in chronic myeloid leukemia. Leukemia Research, **28**:969-972.

2. **Gil ME and Coetzer TL** (2004). Real-Time quantitative PCR of telomere length. Molecular Biotechnology. Jun; **27**(2):169-172.

Conference Proceedings

1. **Gil ME, Lastrucci RMD, Gunther K and Coetzer T** (2001). Techniques for the measurement of telomere dynamics in chronic myeloid leukaemia. Poster presentation at the IUBMB/SASBMB Special Meeting on the Biochemical and Molecular Basis of Disease, November 2001, Cape Town, South Africa.

2. **Gil ME and Coetzer TL** (2003). Real-time quantitative RT-PCR for human telomere elongation reverse transcriptase in chronic myeloid leukaemia. 3rd Place poster presentation at the 18th Congress of the South African Society of Biochemistry and Molecular Biology, July 2003, University of Pretoria, Pretoria, South Africa.

3. **Gil ME and Coetzer TL** (2004). Diagnostic and prognostic implications of telomere dynamics in Chronic Myeloid Leukaemia. One of five posters short listed for Roche poster presentation award at the Symposium of the South African Molecular and Cell Biology Group, October 2004, University of the Witwatersrand, Johannesburg, South Africa.

4. **Gil ME and Coetzer TL** (2005). Diagnostic and prognostic implications of telomere dynamics in Chronic Myeloid Leukaemia. Oral Presentation at the 19th Conference of the South African Society of Biochemistry and Molecular Biology, January 2005, Stellenbosch University, Stellenbosch, South Africa.

5. **Gil ME and Coetzer TL** (2005). Telomere dynamics in chronic myeloid leukaemia. Poster presentation at the Telomere and Telomerase Meeting, May 2005, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

6. **Gil ME and Coetzer TL** (2005). Telomere dynamics in chronic myeloid leukaemia. Oral presentation at the 45th Conference of the Federation of the South African Societies of Pathology, July 2005, Rietvleidam, Gauteng.

Acknowledgements

This work was initiated in the Research and Development laboratory and then completed in the Red Cell Membrane Unit laboratory, both located in the Department of Molecular Medicine and Haematology at the University of the Witwatersrand Medical School.

My thanks to:

- Prof Thérèsa L Coetzer for supervision.
- Dr Rusla duBreuil for initiating my research.
- Dr Karen Gunther for arranging CML samples and clinical data.
- Dr Pierre Durand for motivational support.
- Mrs Samantha Thomson, Miss Chantel Drummond and Mrs Carol Crowther for providing cell lines.
- Cancer Association of South Africa for research funds and a travelling grant.
- WITS Medical Faculty Research Endowment Fund.
- University of the Witwatersrand, National Health Laboratory Service and the Stella and Paul Loewenstein Charitable and Educational Trust for subsistence allowances.
- Cold Spring Harbor Laboratory for a travel stipend

This dissertation is dedicated to my family and friends who persevered …

Abstract

Telomeres are regions of tandem repeats at the ends of chromosomes ensuring chromosome stability or inducing replicative senescence when critically short. Telomerase extends telomeres and its catalytic subunit, telomerase reverse transcriptase is tightly regulated at multiple levels. Cancerous cells prevent telomere-mediated senescence to attain unlimited proliferation, in most cases by enhancing telomerase activity. Chronic myeloid leukaemia is characterised by the translocation, t(9;22), in haematopoietic stem cells. The resulting fusion protein exhibits constitutive tyrosine kinase activity in the cytoplasm, promoting cellular proliferation, inhibiting apoptosis and impeding cell adhesion. Changes in telomere biology have been observed in chronic myeloid leukaemic cells. The current study aimed to investigate telomere biology in 18 chronic myeloid leukaemia patients at various time intervals from date of diagnosis. Although telomeres were significantly shorter in patients compared to controls, results point to complex telomere dynamics in the malignancy. Increased telomerase activity did not necessarily accompany telomere lengthening and increased transcription of the telomerase catalytic subunit was not necessarily indicative of telomerase activity. Ultimately the current study could not detect any trends between telomere length, telomerase activity and telomerase catalytic subunit expression in chronic myeloid leukaemia patients. Together with inherent patient-to-patient variation and the high cost per assay, measurement of telomere biology does not appear to hold prognostic value in chronic myeloid leukaemia and does not warrant inclusion into a routine test repertoire.

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List of Abbreviations

As in scientific literature, the distinction between a gene, its mRNA transcript and its protein product is made by styling; for example: the Abelson gene is written in lower case italics (*abl*), its mRNA transcript in non-italicised lower case (abl) and its protein product is non-italicised and begins with a capital letter (ABL).

36B4	Acidic ribosomal phosphoprotein
ABL	Abelson protein
AML	Acute myeloid leukaemia
BCR	Breakage cluster domain protein
CML	Chronic myeloid leukaemia
C_T	Cycles to threshold
CV	Coefficient of variation
DIG	Dioxygenin
FCS	Foetal calf serum
*Hinf*I	Restriction enzyme from *Haemophilus influenzae*
hTERT	Human telomerase reverse transcriptase
hTR	Human telomerase RNA component (Also hTERC)
mRNA	Messenger ribonucleic acid
mTERT	Mouse telomerase reverse transcriptase
PBMC	Peripheral blood mononuclear cells
PBL	Peripheral blood leukocytes
PCR	Polymerase chain reaction
Ph	Philadelphia chromosome
PINX1	Pin2-interacting protein 1
PIP1	POT1-interacting protein
PKB	Protein kinase B
PKC	Protein kinase C
POT1	Protection of telomere 1
QRT-PCR	Quantitative real time polymerase chain reaction
RAP1	Transcriptional repressor/activator protein
RO	Reverse osmosis

*Rsa*I	Restriction enzyme from *Rhodopseudomonas sphaeroides*
RT-PCR	Reverse transcription polymerase chain reaction
SD	Standard deviation
ssBP1	Single-stranded DNA binding protein 1
TANK	Tankyrase
TIN2	TRF1-interacting nuclear protein 2
TRAP	Telomere repeat amplification protocol
TRF1/2	Telomeric-repeat binding factor 1/2
TPP1	TIN2-organising protein
T/S ratio	Telomerase:Single gene copy ratio
UBC	Ubiquitin C
WBCs	White blood cells

Chapter 1 Introduction

In vertebrates, telomeres are regions of $(TTAGGG)_n$ repeats forming the ends of chromosomes and conferring protection against degradation, fusion and incomplete replication of the chromosome (Szostak and Blackburn, 1982; Meyne *et al.*, 1998). Telomeres shorten with each cell division and once a critical length is reached, the cell enters replicative senescence. In undifferentiated cells, telomere attrition is reduced by the enzyme telomerase, consisting of a telomere repeat RNA template (hTR) and a reverse transcriptase (hTERT) (Greider and Blackburn, 1985; Greider and Blackburn, 1989).

Constitutive activation of hTERT allows for unlimited cell division and is therefore only transiently activated in a small subset of cells. Regulation occurs at the level of transcription, translation and post-translation. Protein kinase B (PKB or Akt) and protein kinase C (PKC) are serine/threonine kinases that phosphorylate hTERT and enhance its activity. In contrast, phosphorylation of telomerase by c-ABL tyrosine kinase inhibits the enzyme (Cong *et al.*, 2002).

Chronic myeloid leukaemia (CML) is characterised by the Philadelphia (Ph) chromosome - a translocation between chromosomes 9 and 22 resulting in the expression of a *bcr-abl* chimaeric gene. The 210 kDa BCR-ABL fusion protein does not contain the regulatory N terminal cap of ABL, which results in the constitutive activation of ABL kinase and predominant localisation to the cytoplasm. Physiological properties of the fusion protein include: the induction of neoplastic transformation and cell proliferation, growth factor independence, inhibition of apoptosis and the inhibition of cell adhesion (Melo and Barnes, 2007).

A hallmark of cancerous cells is their capacity for unlimited proliferation, so prevention of telomere-mediated senescence is a necessary step for transformation and is achieved in 85% of cancer types by the up-regulation of telomerase (Mergny *et al.*, 2002).

This chapter will provide a brief overview of telomere biology and molecular aspects of CML.

1.1 Telomeres

Human telomeres are nucleoprotein complexes at the ends of chromosomes composed of specialised proteins associated with 9-15kb of (TTAGGG) repeats. Each telomere terminates in a 50-300bp 3' single-stranded G-rich overhang known as a G-tail (Figure 1.1) (Blackburn, 1991; O'Sullivan and Karlseder, 2010). Between telomeres and chromosome coding sequences is a region known as the subtelomere, which acts as a docking site for DNA associated proteins (Mak *et al.*, 2009). Interstitial telomere repeats are blocks of TTAGGG repeats between the centromere and telomere, thought to have originated by telomere-telomere fusions during evolution (Bolzán and Bianchi, 2006; Villasante *et al.*, 2007).

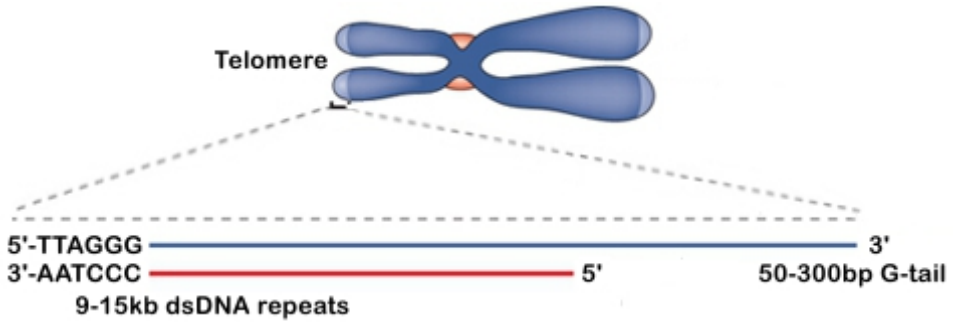

Figure 1.1 Telomeres in humans. Telomeres are composed of double-stranded TTAGGG repeats which terminate in a 3' G-tail of single stranded telomere repeats. Specialized proteins bind along the entire length of telomeres. (Modified from O'Sullivan and Karsleder, 2010)

Heterogeneity of telomere length occurs between individuals and even between chromosomes within an individual (Bolzán and Bianchi, 2006). A mean length however, is maintained with the longest telomere on the long arm of chromosome 4 and the shortest telomere on the short arm of chromosome 17 (Martens *et al.*, 1998; Bolzán and Bianchi, 2006). A gradient of telomere length within tissues also exists, with the longest telomeres belonging to stem cell compartments (Flores *et al.*, 2008).

1.1.1 Telomere proteins

Telomere associated proteins are separated into those with functions limited to the telomere (shelterin and telomerase) and those with functions throughout the chromosome (DNA repair proteins) (Verdun and Karlseder, 2007).

Shelterin accumulates at telomeres throughout the cell cycle and is made up of six subunits: Telomeric-repeat-binding factor 1 and 2 (TRF1 and TRF 2); Transcriptional repressor/activator protein (RAP1); TRF1-interacting protein 2 (TIN2); TIN2-organising protein (TPP1) and Protection of telomeres 1 (POT1) (Figure 1.2) (de Lange, 2005).

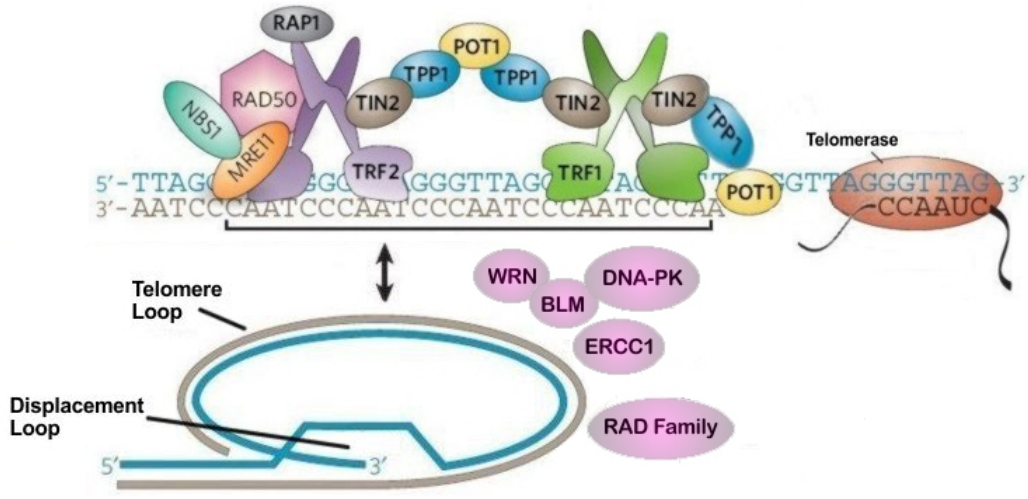

Figure 1.2 Telomere-associated proteins. The architecture of telomeres is facilitated by several proteins with telomere length extended by telomerase. Shelterin (TRF1, TRF2, RAP1, TIN2, TPP1 and POT1) binds double stranded telomere repeats via TRF1 and TRF2 and single-stranded TTAGGG repeats via POT1. Telomere folding into telomere- and displacement-loops is mediated by shelterin, with formation of displacement-loops requiring a recombination event mediated by several DNA repair factors. **TRF1/2** (Telomeric-repeat-binding factor 1 and 2); **RAP1** (Transcriptional repressor/activator protein); **TIN2** (TRF1-interacting protein 2); **TPP1** (POT1/TIN2-organising protein); **POT1** (Protection of telomeres 1); **MRE11** (Meiotic recombination 11 homologue); **NBS1** (Nijmegen breakage syndrome protein); **WRN** (Werner's syndrome protein); **BLM** (Bloom's syndrome protein); **DNA-PK** (DNA-dependent protein kinase); **ERCC1** (Excision repair cross-complementing 1); **RAD family** (RAD DNA-repair protein family). (Modified from Verdun and Karlseder, 2007)

Double-stranded TTAGGG repeats are bound by TRF1 and TRF2, POT1 binds single-stranded telomere repeats with interactions between shelterin subunits mediated by TIN2 and TPP1 (Figure 1.2). Shelterin facilitates the shaping of telomeres and together with DNA repair proteins, protects telomeres from inducing a DNA damage signal, non-homologous end-joining of chromosomes (NHEJ) and homologous recombination (HR) within or between telomeres (de Lange, 2005).

1.1.2 Factors affecting telomere length

Telomere length varies between individuals of the same age (Iwama *et al.*, 1998) Five loci which code for genes involved in DNA repair and DNA synthesis play a role in genetic determination of telomere length (Vasa-Nicotera *et al.*, 2005; Andrew *et al.*, 2006; Mangino *et al.*, 2009.

Histone modification and subtelomeric DNA methylation regulate telomere length by maintaining telomere loops (t-loops) (Blasco, 2007). Telomeres can dictate the assembly of heterochromatic domains in subtelomeric regions by the 'telomere position effect', a mechanism by which telomere silencing of nearby genes is proportional to the length of that respective telomere. This mechanism may be responsible for the preferential elongation of short telomeres by telomerase (Blasco, 2007).

Chronic oxidative stress increases telomere erosion, decreases telomere integrity and induces premature cellular senescence (Kurz *et al.*, 2004). Single-strand breaks occur at a higher frequency in telomeres than elsewhere in the genome since guanine triplets are prone to oxidation and repair of single-strand breaks in telomeres is inefficient (von Zglinicki, 2002; Richter *et al.*, 2007).

Daughter cells can vary in their doubling capacities, with one continuing to divide and the other abruptly entering senescence termed sudden senescence syndrome (Rubelj and Vondraček, 1999). Telomeres of these cells show large-scale deletion events as a result of homologous recombination, failure to repair telomere damage, stalled replication forks and nuclease digestion (Rubelj and Vondraček, 1999).

The C-rich telomere strand is transcribed by DNA-dependent RNA polymerase II into non-coding telomeric-repeat containing RNA (TERRA) (Azzalin *et al.*, 2007). Transcription of human telomeres is dependent on developmental status, telomere length, cellular stress and chromatin structure. The ability of TERRA to inhibit telomerase, coupled with their altered transcription in tumours, points to fundamental roles for the transcripts in cancer and aging (Schoeftner and Blasco, 2007; Redon *et al.*, 2010).

1.1.3 Functions of telomeres

Linear chromosomes require a mechanism to protect chromosome ends from enzymatic attack and DNA repair proteins. Telomeres fulfil this role, preventing a variety of conditions from dementia (Grodstein *et al.*, 2008) to cancer (Murname, 2006).

1.1.3.1 *Absorbing chromosome erosion and protecting chromosome ends*

As a consequence of the end-replication problem, the lagging strand shortens by 50-200bp and leading strand by 15-70bp per cell division (Baird, 2008). Leading strand DNA synthesis generates a blunt end which cannot be processed into t-loops, so G-tails are generated by shelterin-mediated resection of the C-rich telomere strand (Figure 1.3) (de Lange, 2005; Dai *et al.*, 2010). Since telomeres have a finite length, their erosion limits the number of cell cycles a cell undergoes (Hayflick limit).

Eukaryotic cells “cap” chromosome ends to circumvent detection by DNA damage proteins. This process involves the folding of telomeres by shelterin and DNA processing proteins into telomere-loops (t-loops), with G-tails folded into double-stranded telomere repeats termed displacement-loops (D-loops) (Figure 1.3) (Dai *et al.*, 2010).

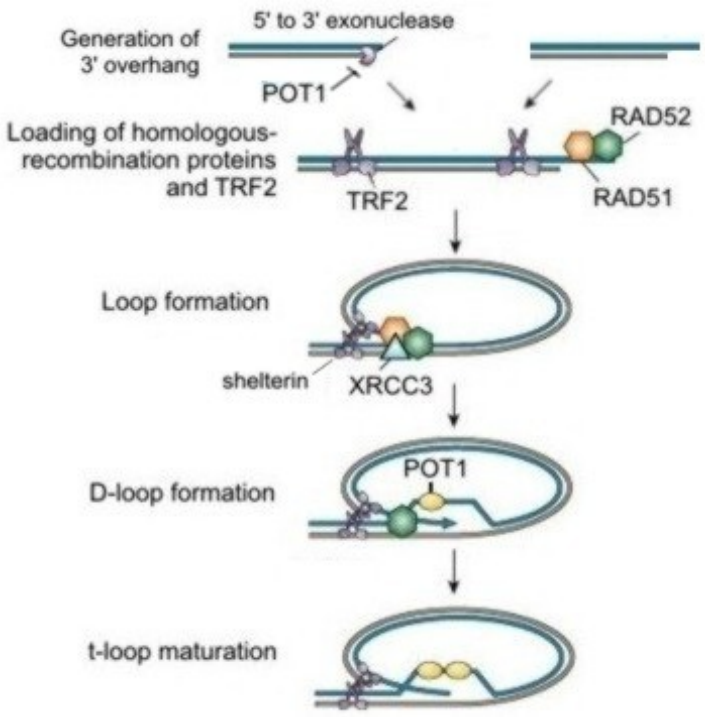

Figure 1.3 Formation of a t-loop. G-tails are required for t-loop formation, so the leading strand of DNA synthesis requires a 5' to 3' exonuclease to process the blunt end. RAD51 and RAD52 are recruited to telomere 3' overhangs, with TRF2 preventing illegitimate invasions into interstitial repeats or telomeres on other chromosomes. Together with shelterin proteins, XRCC3 mediates the invasion of the 3'overhang into double-stranded telomeric DNA to form a D-loop. Removal of recombination proteins and loading of shelterin units along the telomere marks t-loop maturation (modified from de Lange, 2005; Dai *et al.*, 2010).

1.1.3.2 *Protection from recombination and induction of p53*

Telomeres unable to fold into t-loops exert a tumour suppressor effect by initiating replicative senescence or apoptosis via the p53, Retinoblastoma (RB) or both pathways (Deng and Chang, 2007). This has provided insight into the dynamics of cancer promotion and maintenance in the setting of genomic instability, since the two most common abnormalities in cancer are telomere dysfunction and loss of p53 (Deng and Chang, 2007).

Non-homologous end joining (NHEJ) of dysfunctional telomeres usually involves two different chromosomes. The NHEJ proteins DNA-PK_{cs}, XRCC4 and Ku80 are presumed to be involved in recombining blunt ends following G-tail excision by ERCC1/XPF (de Lange, 2005).

Homologous recombination at telomeres that occurs in *cis* results from inadequate stabilisation of D-loops. Branch migration towards the centromere forms two Holiday junctions which, when resolved by the Mre11 recombination repair complex and XRCC3, results in t-loop excision. The ends of the deleted t-loop anneal, generating circular double-stranded telomere repeats, known as telomeric circles (t-circles) and a substantially shortened telomere (de Lange, 2005).

1.1.3.3 *Sister-chromatid identification and resolution*

Sister chromatid cohesion is important for non-sister chromatid recombination and chromosome segregation at meiosis I and II. Mutational studies indicate that telomere repeats together with cytoskeleton proteins anchor chromosomes to the nuclear membrane, facilitating the clustering of telomeres during prophase I and reducing the complexity of homologue searching (Scherthan, 2007).

1.2 Telomerase

Telomerase is an RNA-dependent DNA polymerase which catalyses the addition of telomere repeats to G-tails following DNA replication in S-phase (Greider and Blackburn, 1985; Greider and Blackburn, 1989). Enzyme activity is restricted to cells that undergo rapid expansion such as lymphocytes, keratinocytes, germ cells, stem cell compartments and certain tissues with low cell turnover such as neural tissue (Mergny *et al.*, 2002).

1.2.1 Components of telomerase

Catalytically active telomerase is minimally composed of the reverse transcriptase subunit (TERT), the telomere repeat RNA template (TR) and an accessory protein, either Dyskerin or hsp90 (Greider and Blackburn, 1985; Greider and Blackburn, 1989; Cohen *et al.*, 2007; Mizuno *et al.*, 2007).

1.2.1.1 *Human telomerase reverse transcriptase*

Located on chromosome 5p, *htert* is ~37kb (Genbank Accession number: NG_009265.1), with an mRNA transcript 4015bp long (Genbank Accession number: NM_003219). Composed of 16 exons, *htert* undergoes alternative splicing generating

eight transcripts, one of which is translated into a functional 127kDa reverse transcriptase (Figure 1.4) (Nakamura *et al.*, 1997; Killian *et al.*, 1997; Wick *et al.*, 1999).

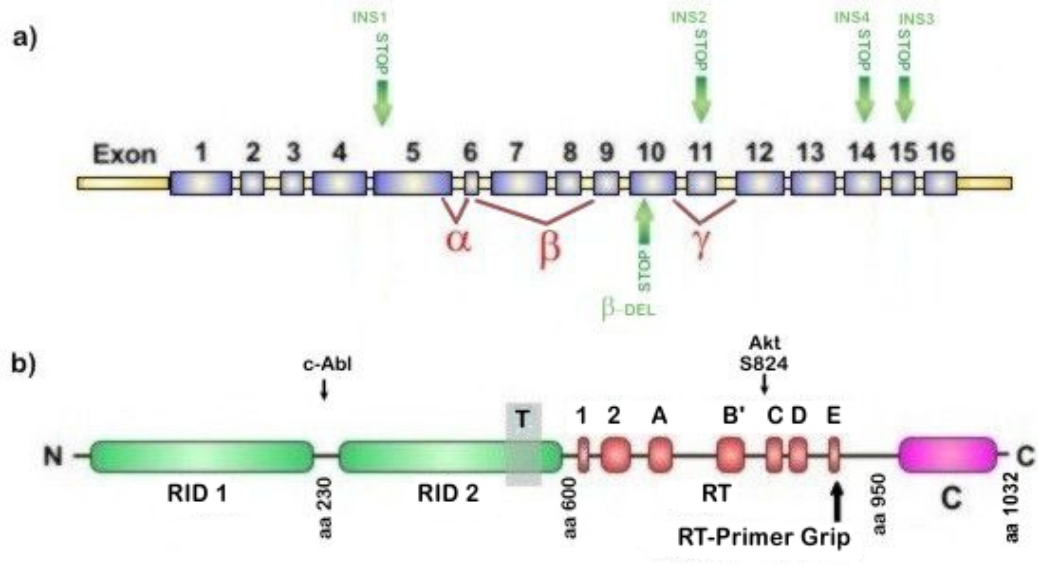

Figure 1.4 Schematic of telomerase reverse transcriptase mRNA and protein. (a) Active hTERT is translated from full length hTERT mRNA (16 exons). Inactive variants with a partial exon 5-partial exon 6 deletion (α-deletion), partial exon 6-exon 7, 8 and 9 deletion (β-deletion), exon 11 deletion (γ-deletion) or combinations thereof are indicated by red lines. The stop codon generated by the β-deletion is indicated. Insertion variants produce mRNA transcripts with premature stop codons as indicated by green arrows. **(b)** Translated hTERT consists of the N-terminal domain (green), reverse transcriptase (RT) domain (red) and the C-terminal domain (purple). The N-terminal domain contains two RNA Interaction Domains (RID1 and RID2), with a telomerase-specific domain known as the T-motif (shaded grey). The catalytic domain of hTERT is made up of seven motifs: 1, 2, A, B', C, D and E. The c-Abl interaction site and Akt phosphorylation site are labelled (Compiled from Nakamura *et al.*, 1997; Killian *et al.*, 1997; Wick *et al.*, 1999; Kelleher *et al.*, 2002; Hisatomi *et al.*, 2003; Huard *et al.*, 2003; Moriarty *et al.*, 2004).

The N-terminal domain of hTERT contains two RNA Interaction Domains (RID1 and RID2) which anchor the telomere (by the T-motif) and hTR (Figure 1.4b) (Moriarty *et al.*, 2004; Wyatt *et al.*, 2007). Additionally, RID2 facilitates the nucleolar localisation of hTERT (Etheridge *et al.*, 2002).

The reverse transcriptase domain is comprised of seven motifs with the reiterative ability of telomerase repeat addition mediated by conformational changes between the A and B' motifs (Lue *et al.*, 2003). Alignment of the telomere within the catalytic domain is facilitated by the RT-Primer Grip domain of the E motif (Wyatt *et al.*, 2007).

The C-terminal domain is implicated in telomerase activity, processivity, dimerisation and telomere length maintenance (Banik *et al.*, 2002; Kelleher *et al.*, 2002; Huard *et al.*, 2003).

1.2.1.2 *Human telomerase RNA component*

The gene coding for the 451 nucleotide human telomerase RNA component (hTR) is located on chromosome 3q26 (Cong *et al.*, 2002). hTR carries the telomere repeat template and has eight conserved regions which facilitate (a) template positioning and translocation during reverse transcription (b) telomerase assembly and enzymatic activity (c) RNA stability (d) nuclear localization and (e) retention in Cajal bodies (Chen *et al.*, 2000; Lukowiak *et al.*, 2001; Jády *et al.*, 2004; Ueda and Roberts, 2004; Theimer *et al.*, 2005).

1.2.1.3 *Dyskerin and hsp90*

Dyskerin is a pseudouridine synthase which facilitates the nuclear translocation of hTR and confers stability to the telomerase complex (Cohen *et al.*, 2007). Originally, dyskerin was isolated as the cause of X-linked dyskeratosis congenita - a rare, inherited disorder characterised by premature ageing, bone marrow failure and malignancy (Heiss *et al.*, 1998). The autosomal dominant form of dyskeratosis congenita results from either a mutation in hTR or haploinsufficiency of hTERT, both of which result in telomere elongation deficiency (Handley *et al.*, 2006).

Hsp90 is an ATP-dependent molecular chaperone which aids in the arrangement of various proteins (Richter and Buchner, 2001). Keppler *et al.* (2006) found that hsp90 not only folds nascent hTERT but also maintains the holoenzyme in a conformation conducive to DNA loading and telomere-repeat catalysis, remaining associated with telomerase during telomere repeat addition.

1.2.2 Catalytic action of telomerase

The molecular chaperones hsp70, hsp90 and p23 play an essential role in assembly and stability of telomerase (Masutomi *et al.*, 2000; Weilbaecher and Lundblad, 1999; Collins, 2006). Once assembled and loaded onto telomere G-tails, telomerase action is divided into three steps: substrate recognition, elongation (telomere repeat addition) and translocation (re-positioning of template for further repeat addition) (Harrington, 2003).

Telomerase is loaded onto a G-tail by alignment with the template sequence of hTR and stabilised by the RT-Primer grip and anchor site (T-motif) (Figure 1.4) (Wyatt *et al.*, 2007). Once aligned, nucleotides are reverse transcribed onto the DNA strand up to the RNA template 5' boundary. The G-tail is then re-positioned for further telomere repeat addition or released for t-loop formation (Harrington, 2003; Lue, 2004).

Dimerisation of telomerase is not required for catalytic activity but is necessary for processivity – the ability to translocate the G-tail within the enzyme (Moriarty *et al.*, 2004). Each telomerase protomer acts on a single G-tail resulting in the lengthening of two telomeres simultaneously (Rivera and Blackburn, 2004).

1.2.3 Regulation of telomerase activity

The importance of telomerase activity regulation has necessitated the evolution of multiple levels of control (Mergny *et al.*, 2002)

1.2.3.1 *Regulation of htert transcription*

A synergistic relationship exists between genetic and epigenetic factors regulating *htert* transcription (Liu *et al.*, 2004; Flores *et al.*, 2006).

Binding of c-Myc/Max to the *htert* promoter correlates with histone acetylation and increased *htert* expression, whereas deacetylation of histones represses *htert* transcription (Szutorisz *et al.*, 2003; Ge *et al.*, 2006). Silencing of *htert* transcription follows the recruitment of histone deacetylase to the *htert* promoter by Sp1 or by MAPK-triggered H3 phosphorylation (Ge *et al.*, 2006).

A variety of signalling proteins involved in apoptosis, cell proliferation and DNA damage repair interact with the *htert* promoter. By various repression and enhancing effects, these proteins induce cell type and cell context-specific expression patterns of *htert* (Goueli and Janknecht, 2004; Flores *et al.*, 2006; Anderson *et al.*, 2006).

1.2.3.2 *Alternative splicing of hTERT*

Although catalytically inactive, hTERT isoforms may have other functions as evidenced by the cell type-specific production of hTERT splice variants. In hepatocellular carcinoma cell lines, most hTERT transcripts are full length or β-deletion variants with 10% being γ-deletion variants (Hisatomi *et al.*, 2003). In activated lymphocytes, c-MYC inhibition causes a shift in hTERT splice-variant production in favour of α- and β-deletion variants (Jalink *et al.*, 2007) and in gastric tissues, increased transcription of full-length hTERT is correlated with increased c-MYC expression (Li *et al.*, 2008).

1.2.3.3 *Phosphorylation of* hTERT

Protein kinase C (PKC) is a serine/threonine kinase involved in cellular proliferation, differentiation and gene expression. Phosphorylation of hTERT by PKC enhances telomerase activity and is counter-balanced by protein phosphatase 2A (Li *et al.*, 1998; Cong *et al.*, 2002). The serine/threonine kinase Protein kinase B (Akt) is activated by various growth and survival factors and is an effector of the phosphatidylinositol-3-kinase signalling pathway. A potential mechanism for telomerase activation by PKC/Akt is translocation of hTERT from the cytoplasm to the nucleus following phosphorylation (Liu *et al.*, 2001).

The non-receptor tyrosine kinase ABL is localised to several subcellular sites, mediating a range of cellular processes from cell growth to DNA-damage response and cell migration (Hantschel and Superti-Furga, 2004; Li, 2005). Consistent with a role in cellular senescence, nuclear ABL phosphorylates hTERT, translocating telomerase from the nucleus to the cytoplasm (Kharbanda *et al.*, 2000).

1.2.3.4 *Controlling telomerase access to telomeres*

Excess TRF1 and TRF2 act in *cis* to repress telomere elongation by stabilising t- and D-loops thus preventing telomerase accessing telomeres (Smogmorzewska and de Lange, 2004). As telomeres elongate, the amount of POT1 bound increases as does the chance that POT1 will bind the 3'-overhang and effectively block telomerase access to the G-tail (Etheridge *et al.*, 2008).

Telomerase access to telomeres can also be blocked by embedding chromosome ends in the nuclear membrane. An isoform of TIN2, termed TIN2L, retains the ability to bind TRF1, TRF2 and associates strongly with the nuclear matrix (Kaminker *et al.*, 2009).

1.2.3.5 *Localisation of telomerase*

The localisation of hTERT and hTR varies throughout the cell cycle and affects telomerase assembly, disassembly and degradation (Collins, 2006). There are two levels of telomerase localisation in cells, cytoplasm-nucleus shuttling and nucleolus-nucleoplasm shuttling (Seimiya *et al.*, 2000; Etheridge, *et al.*, 2002; Tomlinson *et al.*, 2006).

Cytoplasmic-nuclear shuttling of hTERT is mediated by the balance of the hTERT nuclear localisation signal and nuclear exclusion signal (Seimiya *et al.*, 2000). Additionally the 14-3-3 signal proteins bind the C-terminal of hTERT and enhance nuclear localisation (Seimiya *et al.*, 2000).

During G1 and G2 of the cell cycle, hTR is localised to Cajal bodies and hTERT to distinct nucleoplasmic foci (Khurts *et al.*, 2004; Lin and Blackburn, 2004). PinX1 inhibits telomerase activity by sequestration of hTERT in a catalytically inactive state within the nucleolus (Lin and Blackburn, 2004; Lin *et al.*, 2007). In S-phase, the hTERT nucleoplasmic foci associate with Cajal bodies and telomerase assembly is mediated by the SMN complex - a ribonucleoprotein assembly complex (Gubitz *et al.*, 2004; Fu and Collins, 2006; Tomlinson *et al.*, 2006). Assembled telomerase is shuttled to chromosome ends to extend telomeres (Jády *et al.*, 2006).

1.2.3.6 *Effects of hormones and growth factors on telomerase activity*

The *htert* promoter contains two oestrogen response elements which induce *htert* transcription and telomerase activation in oestrogen receptor positive cells (Kyo *et al.*, 1999; Cong *et al.*, 2002). This could be why telomeres tend to be longer in woman than men of the same age (Gudmundsson *et al.*, 2000; Nawrot *et al.*, 2004).

Epidermal growth factor promotes cell proliferation in several cell types and increases telomerase transcription through two specific motifs in the *htert* promoter. Androgen plays a role in mediating induction or repression of telomerase activity via insulin-like growth factors (Bayne and Liu, 2005).

1.3 Telomere biology in cancer

The accumulation of cellular damage can lead to cellular dysfunction (cancer) or cellular loss (senescence) (Mimeault and Batra, 2009). Senescence protects against cancer by triggering cell cycle checkpoint mechanisms in response to phenotypic and functional changes in cells (Figure 1.5) (Mathon and Llyod, 2001; Collado *et al.*, 2007).

Cellular immortality is blocked by two checkpoints - replicative senescence and crisis. Replicative senescence is triggered when a few critically short telomeres accumulate within a cell, thus preventing further telomere loss and genomic instability. Being p53 and Rb dependent, cells can bypass this checkpoint by inactivation of p53 or Rb, or both (Collado *et al.*, 2007).

Cells which bypass replicative senescence undergo several rounds of replication and reach crisis, characterised by dysfunctional telomeres and unstable chromosomes. Telomeres are stabilised by either activation of telomerase or telomerase independent-telomere recombination (Alternative lengthening of Telomeres, ALT) (Deng and Chang, 2007).

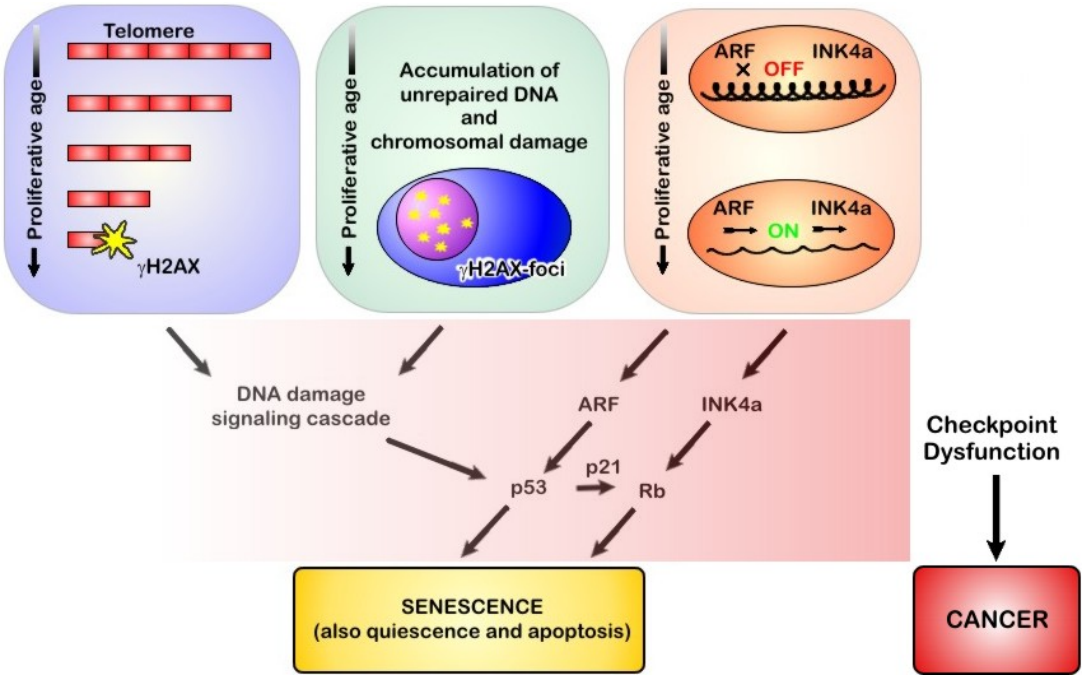

Figure 1.5 Basic mechanisms in senescence induction. The interplay between ageing and cancer is controlled by balancing the repair of cellular damage and the prevention of excessive proliferation. Hayflick Factors are mechanisms by which a cell records the number of cycles undergone - a mitotic clock which, once expired, induces senescence, quiescence or apoptosis. The three best studied Hayflick Factors are telomere shortening, accumulation of DNA damage and activation of the *INK4a/ARF* locus. Their main effectors are p53 and Rb which are essential for tumour suppression (Modified from Collado *et al.*, 2007)

Defects in DNA damage response, DNA replication, chromosome segregation and dysfunctional telomeres cause the accumulation of genetic changes necessary for cancer development (Murname, 2006). There are two classes of dysfunctional telomeres: (1) loss of capping function is characterised by the inability to form t-loops and (2) complete telomere loss results from either a deficiency in telomere maintenance proteins, or following dysfunctional DNA replication (Murname and Sabatier, 2004).

1.3.1 Telomere loss and chromosome rearrangements

Telomere loss promotes chromosome rearrangements through extensive chromosome fusions (Capper *et al.,* 2007). The fused sister chromatids are dicentric, so they form an anaphase bridge during mitosis as a result of the two centromeres being pulled in opposite directions (Murname, 2006). Under mechanical tension, the fused sister chromatids break within fragile sites generating chromosomes which lack telomeres on one end. The process generates terminal deletions, DNA amplification and chromosomal fusions (Desmaze *et al.,* 2003, Murname and Sabatier, 2004; Bailey and Murname, 2006).

Endless fusion cycles lead to the massive cell death observed in crisis, therefore cells that escape crisis exert a level of control over fusion cycles through telomerase activation or recombination events. Overall, the loss of a single telomere in the presence of cell cycle dysregulation can generate sufficient chromosomal instability to drive tumourgenesis (Murname and Sabatier, 2004).

Early stages of cancers of lung, gastric and liver tissues up-regulate TRF1/2 expression resulting in enhanced protection of short telomeres (Cookson and Laughton, 2009). Increased production of TRF1/2 may induce chromosome instability by inhibiting replication fork movement, introducing double-strand breaks in telomeric regions. If not efficiently repaired, chromosome rearrangements occur by fusion cycles (Murname, 2006). Already, evidence is emerging showing that telomere-binding proteins change roles from tumour initiation to progression (Cookson and Laughton, 2009).

1.3.2 Non-canonical functions of hTERT in cancer

Shay and Wright (2005) caution in affording hTERT a primary role in non-telomere lengthening functions in light of the complex interplay between telomere biology and other cellular metabolic pathways.

Over-expression of hTERT rescued cells with short telomeres from senescence and apoptosis even in the presence of anaphase bridges (Röth *et al.,* 2005). Under mild oxidative stress, hTERT prevents mitochondrial induction of apoptosis, by inhibiting

the release of Cytochrome c and apoptosis-inducing factor (Ahmed *et al.*, 2008). However, hTERT has the ability to promote apoptosis by altering mitochondrial membrane potential or metal homeostasis in mitochondria (Cong and Shay, 2008).

Microarray analysis indicate that the ectopic expression of hTERT results in up-regulation of growth promoting genes and genes involved in DNA damage repair with down-regulation of growth inhibitory genes (Cong and Shay, 2008). These include; up-regulation of p21 (Li *et al.*, 2006), cyclin D1 (Jagadeesh and Banerjee, 2006) and CDC6 expression; hyperphosphorylation of pRB (Yang *et al.*, 2008) and modulation of p53 (Lai *et al.*, 2007).

1.3.3 Alternative lengthening of telomeres

Telomere maintenance by recombination events is collectively termed alternative lengthening of telomeres (ALT) (Figure 1.6). ALT is observed in cancers of neuroepithelial or mesenchymal origin (Muntoni and Reddel, 2005; Lafferty-Whyte *et al.*, 2009) and in a small subset of primitive haematopoietic cells in CML (Samassekou *et al.*, 2009).These cells display heterogeneous telomere lengths ranging from 2kb to >50kb and exponential telomere length increase in one replication cycle (Muntoni and Reddel, 2005).

Inter-telomeric homologous recombination (Figure 1.6a) and intra-telomeric recombination (Figure 1.6b) generate cells containing a mix of chromatids with exceptionally short and long telomeres (Scheel and Poremba, 2002; Muntoni *et al.*, 2009). A proportion of these cells contain ALT-associated PML bodies - subnuclear structures comprised of PML protein, linear and circular telomeric DNA and the MRN recombination complex proteins (Mre11-Rad20-Nbs1) (Muntoni and Reddel, 2005; Brouwer *et al.*, 2009). Telomeric circles can be quite large and extend telomeres in a roll-and-spread mechanism (Figure 1.6c). Circular telomeric DNA is used as a template to generate long telomeres in a single step reaction, facilitated by DNA polymerases and recombination proteins (Tomaska *et al.*, 2004; Brouwer *et al.*, 2009).

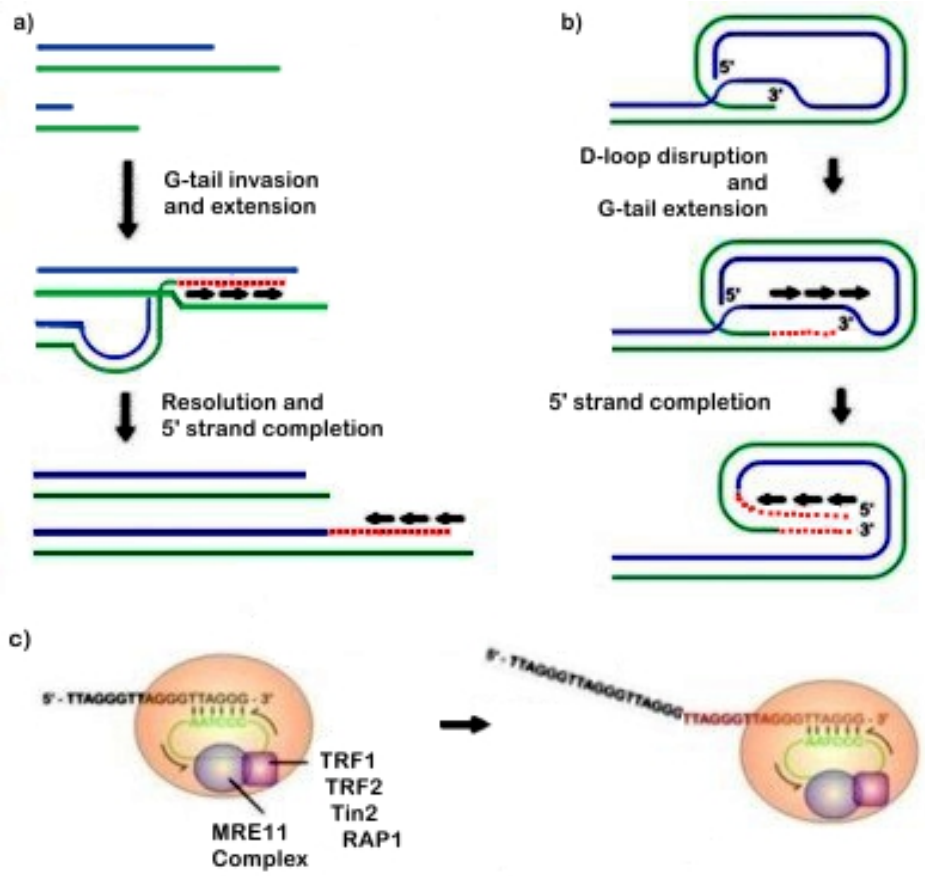

Figure 1.6 Mechanisms of Alternative Lengthening of Telomeres. (**a)** Inter-telomeric homologous recombination involves the invasion of sister or non-sister telomeres. The invading telomere is extended using the invaded telomere as a template followed by dissociation and extension of the 5' strand. **(b)** Intra-telomeric recombination occurs when dysfunctional d-loops allow G-tail extension followed by dissociation and completion of the 5'strand. **(c)** Roll-and-spread mechanism involves circular telomeric DNA acting as a template for telomere addition. Pictured is the ALT-associated PML body mediated telomere extension, but roll-and-spread telomere extension mediated outside these bodies has also been suggested. (Compiled and modified from Hartig and Kool, 2004; Muntoni and Reddel, 2005; Muntoni *et al.*, 2009).

1.3.4 Targeting telomeres and telomerase for anticancer therapy

The complexity of telomere biology and its regulation allows for the targeting of various levels in cancer therapeutics: (1) inhibition of telomerase/ telomere function, (2) telomerase-promoter-directed gene therapy and (3) telomerase immunotherapy (Keith et al, 2002; Kelland, 2007).

Inhibition of telomerase/telomere function includes targeting *htert/htr* transcription, telomerase transcripts, holoenzyme assembly, telomerase-telomere interaction and telomerase activity. Telomerase-promoter-directed gene therapy introduces pro-drug activating systems, apoptotic mediators or toxin genes under the control of a telomerase promoter into tumour cells. Telomerase immunotherapy involves the treatment of antigen-presenting cells with antigenic telomerase peptides to increase cytotoxic T-lymphocyte specificity for cancer cells expressing telomerase (Keith *et al.*, 2002; Kelland, 2007; Cookson and Laughton, 2009).

Imetelstat (GRN163L) is an inhibitor of telomerase currently in Phase II clinical trials for the treatment of solid tumour and hematological malignancies (Geron Corporation, Menlo Park, CA, USA). The agent is a 13-mer thio-phosphoramidate oligonucleotide which acts as a telomerase RNA template antagonist (Kelland, 2007). An appealing property of the inhibitor is its ability to cross the blood-brain barrier and exert an anti-tumour effect on intracerebral tumours (Hashizume *et al.* 2008).

Telomerase inhibitors pose unique problems for use as antitumour agents. Telomerase-dependent tumours could be induced to utilise ALT and agents which cause telomere erosion have a delayed effect, so are unsuitable for use in tumours with long telomeres and toxicity will occur in normal telomerase-positive cells (Mergny *et al.*, 2002; Shay and Keith, 2008).

Safer alternatives to conventional pharmacological agents targeting telomerase are also under investigation. A study by Park *et al.* (2009) investigated the mechanisms responsible for the anti-tumour effect of Korean red ginseng. The extract was shown to progressively down-regulate the expression of hTERT and limit the proliferative potential of U937 human leukaemia cells.

1.4 Chronic Myeloid Leukaemia

Chronic myeloid leukaemia (CML) is associated with the Philadelphia (Ph) chromosome, [t(9;22)(q34;q11)]. The translocation causes the fusion of the 3' sequence from *c-abl* (9q34) with the 5' sequence from *bcr* (22q11). The 210kDa BCR-ABL fusion protein is the causative agent of CML, and promotes cellular proliferation, apoptosis inhibition and impediment of cell adhesion (Melo and Barnes, 2007). The CML chronic phase stem cell exhibits a transcriptional profile of normal myeloid progenitor cells with the exception of the ability to enter quiescence (Bruns *et al.*, 2009).

Most patients are diagnosed in the chronic phase which can last for several years and is characterised by myeloid cell accumulation in bone marrow, peripheral blood and extramedullary sites. Chronic phase is characterised by a high peripheral white blood cell (WBC) count and the presence of peripheral blast cells (Savona and Talpaz, 2008). Occasionally, an intermediary accelerated phase occurs, marked by an increase in total WBCs and peripheral blast cells, with the appearance of new clonal cytogenetic abnormalities (Ren, 2005; Melo and Barnes, 2007). Blast crisis is the terminal phase of the disease lasting a few months and characterised by a rapid expansion of myeloid or lymphoid blast cells in bone marrow and peripheral blood and accumulation of secondary cytogenetic changes (Melo and Barnes, 2007) (Figure 1.7).

The transition to blast crisis is characterised by differentiation arrest, dysfunctional DNA damage repair, and loss of tumour-suppressor functions (Melo and Barnes, 2007; Oehler *et al.*, 2009). BCR-ABL exerts a dose-dependent effect reducing apoptosis susceptibility and enhancing proliferative potential and differentiation arrest in blast crisis (Melo and Barnes, 2007; Bueno *et al.*, 2008)

The most common secondary changes are +Ph, +8 and i(17q), with p53 mutations involved in some cases. Two infrequent blast crisis-associated translocations are t(3;21)(q26;q22) which induces accumulation of differentiation-arrested blast cells and t(7;11)(p15;p15) which promotes expansion of stem progenitor cells insensitive to differentiation-inducing stimuli (Calabretta and Perrotti, 2004).

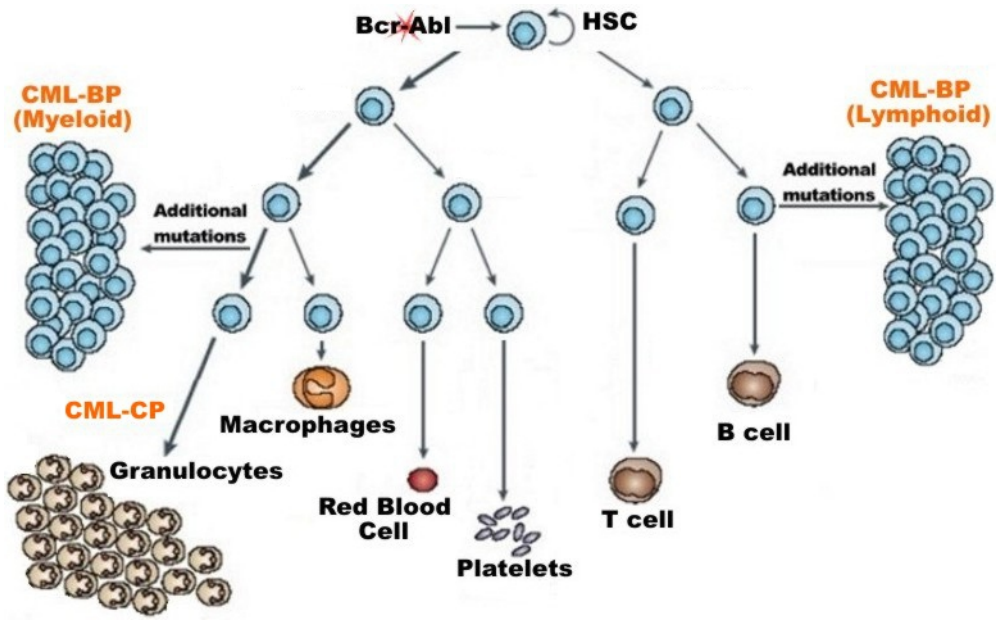

Figure 1.7 Chronic myeloid leukaemia. Chronic myeloid leukaemia (CML) is a biphasic disease initiated by expression of the *bcr-abl* fusion gene in haematopoietic stem cells (HSCs). HSCs can differentiate to produce myeloid cells (Granulocytes, Macrophages, Red blood cells, Platelets) or lymphoid cells (T and B cells). The initial chronic phase of CML (CML-CP) is characterised by expansion of granulocytes. Acquisition of additional mutations causes progression to blast phase (CML-BP), characterised by accumulation of myeloid (66% patients) or lymphoid blast cells (33% patients) (Adapted from Ren, 2005).

Allogenic bone marrow transplant is the only curative treatment of CML beyond 10 years follow up. Traditional treatment involved the chemotherapeutic agents hydroxyurea and α-interferon but the rational design of Imatinib has taken CML treatment success a leap forward (Merx *et al.*, 2002). Imatinib blocks the catalytic domain of the BCR-ABL tyrosine kinase, stabilising ABL in an inactive form (Merx *et al.*, 2002). Patients show a low rate of disease progression (Melo and Barnes, 2007), however, long term usage is related to drug resistance so second generation compounds, such as Nilotinib and Dasatinib, have been developed to treat patients resistant to Imatinib (Melo and Chuah, 2008; Kantarjian *et al.*, 2011).

1.4.1 The Philadelphia chromosome

1.4.1.1 *Structural organisation of ABL, BCR and BCR-ABL*

The breakpoint cluster region protein (BCR) is a multifunctional signal transduction molecule involved in cell cycle regulation (Shchemelinin *et al.,* 2006). The N-terminal contains a coiled-coil motif which facilitates dimerisation of BCR and a serine/threonine kinase domain. The central region enhances RAS activity and the C-terminal contains a GTPase activating protein (Chopra *et al.,* 1999) (Figure 1.8a).

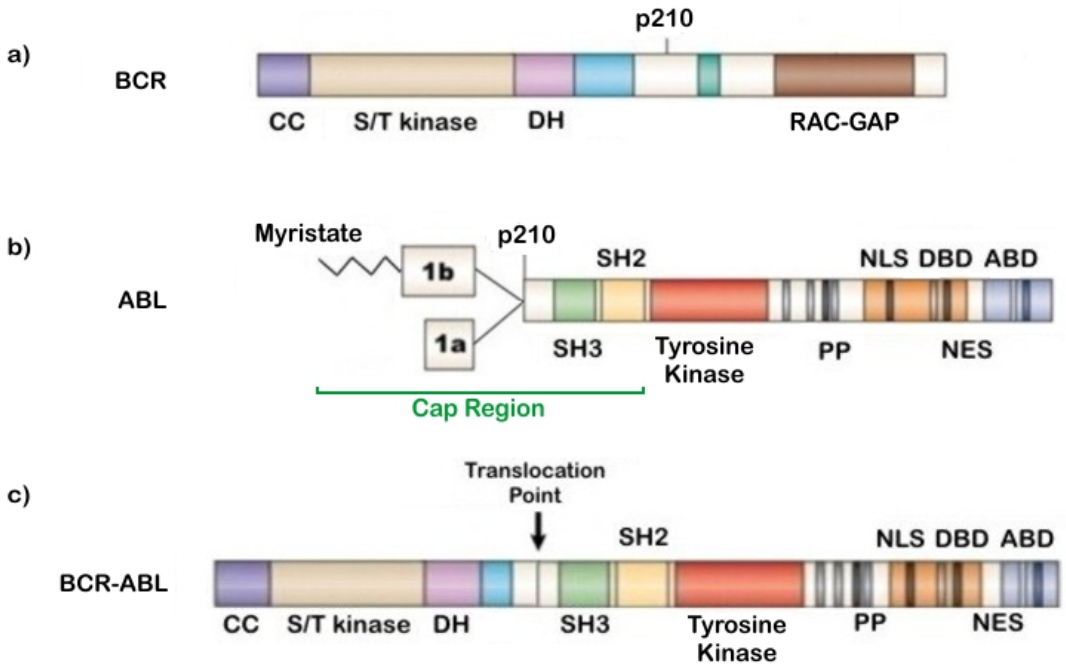

Figure 1.8 Structural domains of BCR, ABL and BCR-ABL. (a) BCR contains a coiled-coil domain (CC), a serine-threonine kinase and a Dbl/CDC24 guanine-nucleotide exchange factor homology domain (DH). The carboxyl terminal contains a RAC guanosine triphosphate-activating protein domain (RAC-GAP). The p210 fusion point is indicated. **(b)** The amino-terminal of ABL includes a myristate moiety (absent in certain splice variants), and contains tandem SRC homology 3 (SH3), SH2 and the tyrosine-kinase domains. The carboxy-terminal region contains proline-rich SH3 binding sites (PP), nuclear localisation signals (NLS), a nuclear exporting signal (NES), a DNA-binding domain (DBD) and an actin-binding domain (ABD). The p210 fusion point is indicated. **(c)** The t(9;22)(q34;q11) reciprocal translocation is translated into the pathogenic p210 BCR-ABL protein. The p210 BCR-ABL protein has constitutive tyrosine kinase activity, serine-threonine kinase activity and a variety of domains which interact with cellular signalling pathways. (Modified from Hantschel and Superti-Furga, 2004; Ren, 2005).

ABL is a non-receptor tyrosine kinase expressed in most tissues in response to DNA damage and oxidative stress (Hantschel and Superti-Furga, 2004). ABL interacts with proteins regulating cell growth, cell survival, DNA-damage responses, actin dynamics and cell migration (Hantschel and Superti-Furga, 2004). The amino terminus contains a 'Cap' region which is essential for autoinhibition of tyrosine kinase activity. The carboxy-terminal region contains several domains involved in subcellular localization of ABL and interactions with cellular molecules, as shown in Figure 1.8b (Hantschel and Superti-Furga 2004).

The *abl* breakpoint occurs 5' of exon 2 resulting in the loss of exon 1 and the *bcr* breakpoint occurs within the Major Breakpoint Cluster Region resulting in the loss of the first 9 or 10 *bcr* exons (Ren, 2005). Fusion of *bcr* to *abl* results in constitutive tyrosine kinase activation by a combination of a gain-of-function mutation mediated by the coiled-coil dimerisation motif in BCR and a loss-of-function by the loss of the inhibitory N-terminus of ABL (Figure 1.8 c). Together with its cytoplasmic location, the constitutively activated tyrosine kinase confers the transforming ability of BCR-ABL (Chopra *et al.*, 1999; Melo and Barnes, 2007; Hantschel and Superti-Furga, 2004).

1.4.1.2 *Role of p210 BCR-ABL in CML*

The tyrosine kinase is central to oncogenesis since most patients enter disease remission when treated with Imatinib (Hantschel and Superti-Furga, 2004; Chu *et al.*, 2004). BCR-ABL exerts its tumourgenic activity by (a) inducing cell proliferation and growth factor independence; (b) inhibiting apoptosis and adhesion to marrow stroma (Chopra *et al.*, 1999) and (c) generating genome instability by down regulation of DNA repair proteins (Calabretta and Perrotti 2004).

Many signalling proteins interact with BCR-ABL or become phosphorylated in BCR-ABL-expressing cells (Chopra *et al.*, 1999; Calabretta and Perrotti, 2004; Ren, 2005) (Figure 1.9). Central to the leukaemogenic properties of BCR-ABL is its ability to autophosphorylate following oligomerisation via the 5' coiled-coil motif. The phosphorylation of residues Y177 and Y1294 results in binding to growth factor receptor-bound protein 2 and activation of ABL-tyrosine kinase,

respectively. The Ras and PI3K-AKT pathways are activated via the interaction of BCR-ABL with various accessory proteins, promoting cell proliferation, inhibiting apoptosis and disrupting cell adhesion and migration.

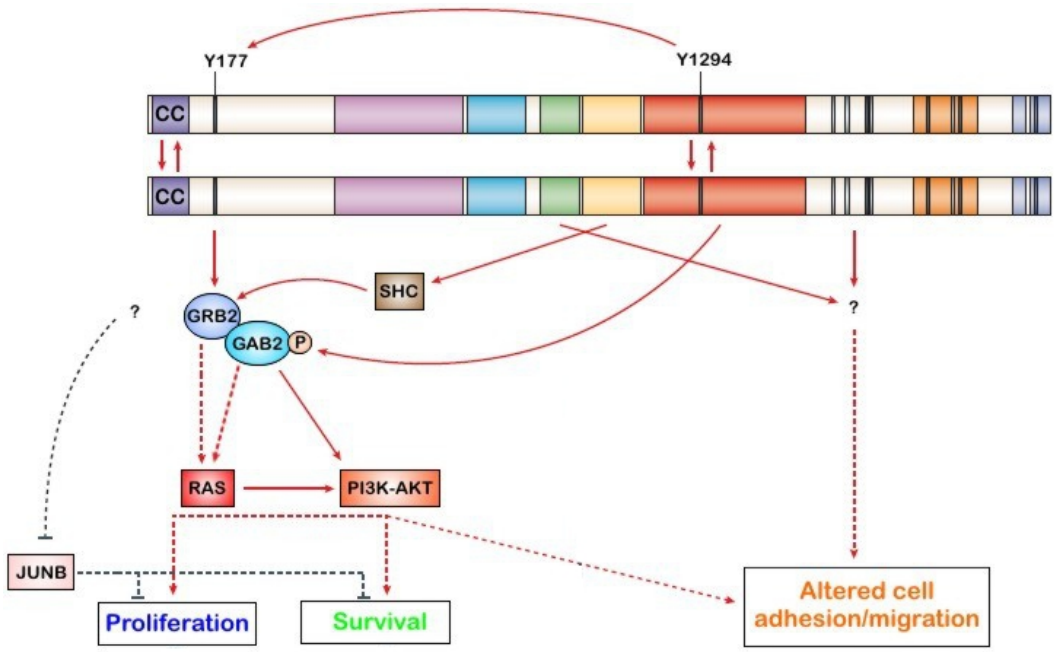

Figure 1.9 Leukaemogenic signaling of BCR-ABL. By forming dimers or tetramers through its coiled-coil (CC) domain, transphosphorylation at position Y177 or Y793 or both occurs in the absence of a growth signal. Phosphorylation at Y177 generates a high-affinity binding site for growth factor receptor-bound protein 2 (GRB2). SH2-SHC-mediated GRB2 recruitment can also occur following phosphorylation at Y1294. Both GRB2 and GAB2 (GRB-associated binder 2) in turn activate RAS or Phosphatidylinositol 3-kinase (PI3K) or both, inducing cell proliferation and inactivating apoptotic pathways. The SH3 domain, proline-rich SH3 domain and the ABL-tyrosine kinase of BCR-ABL disrupt the normal functioning of proteins involved in controlled cell adhesion and migration. Signalling pathways involved in the inhibition of the RAS pathway inhibitor JUNB in BCR-ABL positive cells are currently unknown. The net effect of BCR-ABL mediated cellular signalling is malignant transformation by activation of proliferation, inactivation of apoptosis, disruption of cell adhesion and migration and inhibition of differentiation. (Adapted from Ren, 2005).

1.4.2 Telomere biology in CML

Telomeres are shorter than normal controls and telomerase activity is normal to moderately elevated in most chronic phase CML patients, with a subset of patients having telomere lengths within the normal range. In blast phase further shortening of telomeres is observed and with some patients a shift towards higher levels of telomerase activity occurs (Ohyashiki *et al.*, 1997; Brümmendorf *et al.*, 2000; Drummond *et al.*, 2005).

Telomere biology in CML may contribute to disease pathology and have prognostic potential. Normal telomere length at time of diagnosis correlates to better treatment response and lower incidence of blast phase (Iwama *et al.*, 1997, Brümmendorf *et al.*, 2000 and Boultwood *et al.*, 2000). Continuous telomere shortening is proposed to be a marker for disease progression and cytogenetic changes in CML (Ohyashiki *et al.*, 1997; Drummond *et al.*, 2005). Telomerase activity is detectable in a subset of chronic phase patients and the majority of blast phase patients (Iwama *et al.*, 1997; Ohyashiki *et al.* 1997; Brümmendorf *et al.*, 2000). Interestingly, Campbell *et al.* (2006) measured hTERT down regulation in blast cells with no effect on telomerase activity.

Brümmendorf *et al.* (2000) constructed a generalized model of telomere length changes in CML (Figure 1.10). Outliers of this model have been attributed to: (a) subsets of quiescent CML cells in chronic phase CML resulting in stabilisation of telomere length and decreased telomerase activity, (b) the proportion of cycling versus non-cycling $CD34^+$ naive and differentiated cells, (c) normal and malignant myeloid cells being analysed concurrently, (d) variation in karyotypes between individuals and (e) differing treatment regimens (Ohyashiki *et al.* 1997; Brümmendorf *et al.*, 2000; Drummond *et al.*, 2005).

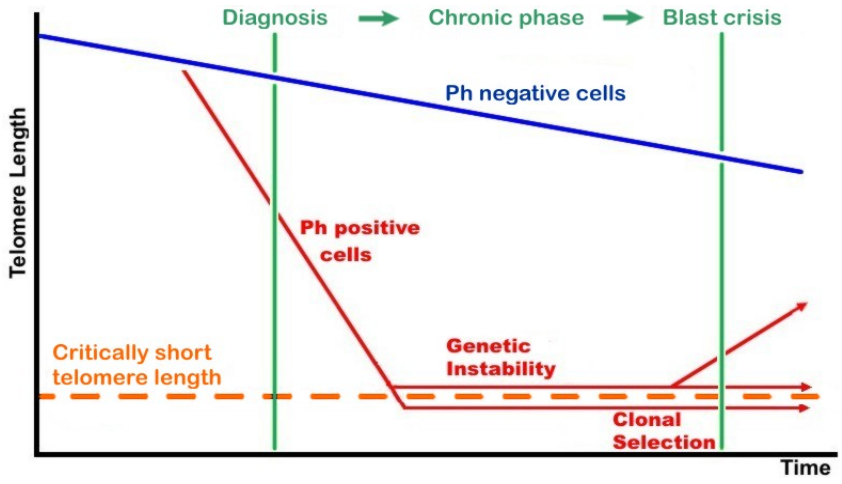

Figure 1.10 Generalised model of telomere length changes in chronic myeloid leukaemia. At time of diagnosis, telomeres are shorter in CML patients when compared to controls with critical telomere length reached just prior to transformation. Transformation to blast phase is characterised by the acquisition of additional cytogenetic changes and is indicative of genetic instability associated with critically short telomeres. Clonal selection of cells with growth and survival advantages occurs whose telomeres either lengthen or stabilise depending on the cellular environment. (Adapted from Brümmendorf *et al.*, 2000).

1.4.3 Comparing studies of telomere biology in CML

Many techniques exist for the measurement of telomere length each with inherent advantages and disadvantages (Appendix E). The technique used can influence the interpretation of telomere biology *in vivo* and yield conflicting results when compared to other studies. Also, the sample analysed must be the same (e.g. $CD34^+$ selected cells or PBMCs).

1.4.3.1 *Influence of cytogenetic changes on telomere biology in CML*

Ohyashiki *et al.* (1997) first suggested that variations in telomere biology between CML patients may result from patient-specific cytogenetic changes.

The loss of a p53 allele as a result of i(17q) is common during progression of CML to blast phase (Johansson *et al.*, 2002). In CML, the combination of p53 loss and

telomere dysfunction may induce progression to blast crisis, acceleration of blast crisis or both (Chin *et al.*, 1999).

In CML, jumping translocations are highly conserved with the pericentric region of chromosome 1 being the donor and telomeres the recipients. The result is a reduction in telomere length far beyond the rate attributed to cell cycling with a dramatic increase in chromosome instability as a direct result of the jumping translocation or altered chromosomal end architecture or both (Berger and Bernard, 2007).

Oehler *et al.* (2009) used available microarray data to identify genes whose expression patterns discriminate between chronic phase and blast crisis. One of the genes identified is that of the cholesterol scavenger receptor which is involved in the binding and internalisation of lipoproteins. The cholesterol specific Receptor C_k is an activator of *htert* transcription and thus a link exists between cholesterol metabolism and *htert* (Sikand *et al.*, 2006).

1.4.3.2 *Influence of oxidative stress on telomere biology in CML*

BCR-ABL increases production and accumulation of reactive oxygen species (ROS) in CML (Sattler *et al.*, 2000; Kim *et al.*, 2005). The effect of ROS on telomere biology in CML has not been directly investigated; however, since: a) ROS is elevated in CML, b) telomeres are sensitive to ROS, c) ROS damage at telomeres is not effectively repaired and d) mechanisms for surveying the genome for DNA damage and repairing these lesions are compromised in CML; it can be inferred that BCR-ABL-mediated ROS production contributes to telomere shortening.

1.4.3.3 *Haematological findings and telomere biology in CML*

Associations between white blood cell, neutrophil and basophil counts, presence of primitive haematopoietic cells in the peripheral blood and telomere dynamics in CML are largely unknown. This lack of knowledge results from studies of telomere dynamics in CML being relatively small and statistical analyses within and between these studies yielding conflicting results (Iwama *et al.*, 1997; Terasaki *et al.*, 2002)

1.4.3.4 *Influence of treatment regimes on telomere biology in CML*

Hydroxyurea is a cytotoxic agent which exerts an anti-proliferative effect on cells by inhibiting ribonucleotide reductase. Apoptosis induced by hydroxyurea was shown to be independent of telomeres and telomerase in K562 cells since over expression of hTERT protected K562 cells from the double-stranded DNA break-inducing agent, etoposide but not hydroxyurea (Akiyama *et al.*, 2002).

In CML, interferon (INF)-α reduces leukaemic cell mass and the occurrence of cells bearing t(9;22) (Carella *et al.*, 1997). Iwama *et al.* (1997) found IFN-α to have a normalising effect on telomere length, not as a result of telomere lengthening but due to a reduction in Ph+ cells with short telomeres. The decrease in Ph+ cells is attributed to repression of the hTERT promoter by INF-α, leading to decreased viability of rapidly dividing Ph+ cells (Xu *et al.*, 2000).

A decrease in telomerase activity, telomere length and *htert* is observed in CML cells treated with Imantinib (Tauchi *et al.*, 2002). The suppressive effect on telomerase activity by Imatinib results from the de-repression of apoptotic pathways (Vigneri and Wang, 2001; Tauchi *et al.*, 2002; Uziel *et al.*, 2005).

Telomere length increases after haematopoietic cell transplant as a result of normal haematopoietic cells being in the majority (Brümmendorf *et al.*, 2000). For the first year following bone marrow transplantation, however, telomere length decreases faster than normal controls and has been attributed to the low number of normal transplanted stem cells trying to regenerate an entire haematopoietic system (Brümmendorf *et al.*, 2000; Rufer *et al.*, 2001).

1.5 Objectives

To better understand telomere dynamics in CML the following objectives were set:

- Comparison of two telomere length assays on the basis of speed and ease of use.

- Development of a real-time fluorescence-based RT-PCR to measure hTERT mRNA expression in CML.

- Determination of telomere length, telomerase activity and hTERT mRNA expression in CML samples taken at several time intervals from date of diagnosis.

- Analysis of telomere biology in CML.

Chapter 2 Materials and Methods

Unless otherwise stated solutions were made to volume using MilliQ water and centrifugation steps carried out at room temperature. Suppliers of laboratory equipment and reagents are given in Appendix B and constituents of buffers and solutions in Appendix C.

2.1 Subjects

Eighteen patients were enrolled into the study. Clinical details and date of each sampling for CML patients are given in Appendix D Table D1. Thirty eight control subjects were enrolled from the Department of Molecular Medicine and Haematology, University of the Witwatersrand Medical School. Age and gender of controls are given in Appendix D Table D7. Ethics clearance was obtained from the University of the Witwatersrand Human Research Ethics Committee (Ref: M070719).

In accordance with ethics requirements, information sheets regarding the study were given to subjects prior to enrolment and informed consent was obtained from all participants (Appendix A). Samples were rendered anonymous by coding CML samples as CML***x_y*** (where ***x*** is a numerical value unique to each patient and ***y*** the number of months elapsed since diagnosis) and controls as C***x*** (where ***x*** is a numerical value unique to each control subject).

Discrimination between chronic, accelerated and blast phase can be made on the percentage of blast cells in the peripheral blood (Vardiman et al., 2002; Melo and Barnes, 2007). Generally, accelerated phase presents with blasts comprising 10% to 19% of peripheral blood white cells and blast phase with blasts comprising 20% or more. These criteria (ratified at WHO annual general meeting in Delhi, 2008; http://www.who.int/topics/classification/en/) were used in the current study to classify samples from patients as being in chronic, accelerated or blast phase (Appendix D Table D1).

The number of patients and number of follow-up samples taken from each patient was limited by the subsequent enrolment of CML patients in an Imatinib clinical trial. Following enrolment in the trial, access to blood samples from patients was not permitted, in accordance with clinical trial regulations.

2.2 Sample collection and preparation

Peripheral blood samples (three EDTA-containing tubes with 5ml capacity) were collected from CML patients in conjunction with monthly routine blood testing, at the Charlotte Maxeke Johannesburg Hospital. In addition, 5ml peripheral blood samples were obtained from healthy volunteers in EDTA-containing tubes, to serve as controls.

Whole blood samples were centrifuged 200 x *g* for 10 minutes using the Jouan BR3.11 centrifuge and buffy coat removed to a 15ml polystyrene tube. Residual red blood cells were lysed by adding 12ml 0.17M NH_4Cl and incubating at room temperature for 15 minutes. White blood cells (WBCs) were pelleted by centrifugation at 3000 x *g* for 10 minutes. The lysis step was repeated and the WBC pellet washed twice with 8ml 0.9% NaCl (Talmud et al., 1991). Washed cells were resuspended in 5ml PBS and quantitated using the Improved Neubauer Haemocytometer. The number of cells counted in 25 fields was substituted into the equation:

$$\text{cells/ml} = \left(\frac{(\text{Number of cells counted})(\text{dilution factor})(10^3)}{(V_c)}\right)$$

[Equation 1]

where 10^3 is the conversion factor converting microlitres to millilitres and V_c is the volume of 25 fields (0.1µl). Therefore, the equation can be simplified to:

$$\text{cells/ml} = (\text{number of cells in 25 fields})(10^4)$$

[Equation 2]

Aliquots of 1 x 10^7 cells were pelleted in 1.5ml Eppendorf tubes and designated for DNA or RNA extraction (Section 2.4.1 and Section 2.4.2 respectively). Pelleted cells

were stored at -70^{o}C, with cells designated for RNA extraction lysed by repetitive pipetting using 1ml TriPure® Isolation Reagent or 600µl RNeasy® Lysis Reagent prior to storage. Aliquots of 2 x 10^5 cells designated for telomerase activity determination were lysed in the kit lysis buffer and stored at -70^{o}C till use (Section 2.6).

2.3 Telomerase positive controls

To validate the assays, two telomerase positive controls were used: HL-60 cell extracts and activated lymphocyte extracts.

Unstimulated peripheral blood T cells and B cells have very low levels of telomerase activity which elevates to detectable levels upon mitogenic stimulation (Hiyama et al., 1995). To generate a telomerase-positive control, peripheral blood lymphocytes isolated from a control subject were activated by mitogenic stimulation using phytohaemagglutinin-P (Igarashi and Sakaguchi, 1996; Yamada et al., 1996; Chebel et al., 2009).

All procedures for cell culturing were performed under sterile conditions in a Labotec BioFlow (Model 660) Laminar flow unit. Blood collected in 10ml heparin-containing tubes was layered onto 10ml Ficoll-Paque™ in a 50ml polypropylene tube and centrifuged at 400 x *g* for 30 minutes (using the Jouan BR3.11 centrifuge). The upper plasma layer was discarded and the middle peripheral blood mononuclear cell (PBMC) layer was washed in PBS at 1000 x *g* for 20 minutes. To remove monocytes and macrophages, isolated PBMCs were resuspended in 20ml peripheral blood lymphocyte isolation medium [RPMI-1640 containing 10% (v/v) foetal calf serum (FCS); 2.0mM L-glutamine and 0.05mM 2-mercaptoethanol] and cultured in 75cm^2 culture flasks for one hour at 37^{o}C in humidified air containing 5% CO_2. Inclusion of 2-mercaptoethanol in the medium induces monocytes/macrophages to adhere to the flask surface. Suspended lymphocytes were poured into 15ml polypropylene tubes and washed twice with RPMI-1640 medium (Igarashi and Sakaguchi, 1996).

Peripheral blood lymphocytes were counted (Section 2.2) and 2 x 10^8 cells were suspended in 20ml culture medium [RPMI-1640 containing 10% (v/v) FCS; 2.0mM

L-glutamine; 2% (v/v) Penicillin-Streptomycin (stock contained: 5,000 units penicillin and 5mg streptomycin per ml)] and incubated in 75cm^2 culture flasks for four hours at 37^{o}C in humidified air containing 5% CO_2. Following stabilisation, lymphocytes were stimulated for 72 hours (without medium change) by incubation at 37^{o}C in humidified air containing 5% CO_2 using 1µg/ml phytohaemagglutinin-M and 10U/ml Interleukin-2.

The HL-60 cell line was selected as an additional telomerase positive control since it has constitutive telomerase activity and raised hTERT mRNA expression (Savre-Train et al., 2000). One millilitre aliquots containing 10^7 HL-60 cells stored in FCS containing 10% DMSO in liquid nitrogen were obtained from Miss C. Drummond and Dr W. Prinz (Department of Molecular Medicine and Haematology, University of the Witwatersrand). Cells were thawed in a 37^{o}C waterbath, suspended in 30ml RPMI-1640 containing 10% (v/v) FCS and pelleted for 10 minutes at 100 x *g*. Pelleted HL-60 cells were washed again followed by resuspension in 5ml culture medium [RPMI-1640 containing 20% (v/v) FCS; 2.0mM L-glutamine; 2.0% (v/v) Penicillin-Streptomycin (stock contained: 5,000 units penicillin and 5mg streptomycin per ml)] and cultured in 25cm^2 culture flasks at 37^{o}C in humidified air containing 5% CO_2. Cultured HL-60 cells were inspected daily for active cell division and viability using the Zeiss ID03 inverted microscope. In the event of increased acidity (culture medium colour changed from red to yellow), cells were pelleted and resuspended in fresh culture medium.

Stimulated lymphocytes and cultured HL-60 cells were aliquotted and stored as detailed in Section 2.2.

2.4 Nucleic acid extraction

2.4.1 *DNA isolation*

Talmud et al. (1991) described a DNA isolation method which dissociates protein-DNA interactions by increasing the alkalinity of the crude extract with sodium hydroxide. To purify crude extracts, protein and RNA were removed as described in Ausubel et al. (2008).

Aliquots of 10^7 cells were lysed by addition of 800µl 0.05M NaOH, boiled for 10 minutes and then neutralised with 100µl 1M Tris-HCl (pH 8.0). Samples were centrifuged at 12000 x *g* for 30 minutes (4°C) in a Sorvall RMC14 centrifuge following the addition of 900µl phenol:chloroform (2:1, pH 8.0). DNA was precipitated from the upper aqueous phase by overnight incubation at -70°C using 1/10 volume 3M sodium acetate (pH 5.2) and 2.5 volumes ice cold ethanol. DNA was pelleted by centrifugation at 12000 x *g* (4°C) for 30 minutes and residual salt was washed away using 70% ethanol. Isolated DNA was air-dried overnight, suspended in 1ml TE buffer and stored at -70°C.

2.4.2 *RNA isolation*

TriPure® Isolation Reagent isolates RNA, DNA and protein from a sample by liquid phase separation and alcohol precipitation (Chomczynski and Sacchi 1987). RNA was isolated from 10^7 PBMCs as per manufacturer's instructions and suspended in a final volume of 50µl RNase-free water. Centrifugation steps were carried out using the Sorvall RMC14 centrifuge and RNA was solubilised by 15 minute incubation at 60°C and then stored at -70°C.

The RNeasy® Mini kit was used for comparative purposes and selectively binds total RNA to a silica-gel-based membrane. Guanidine isothiocyanate in the lysis buffer provides the denaturing environment required to inactivate RNases, and repeated washes with ethanol removes salts and other contaminants. RNA was isolated as per manufacturer's instructions and RNA eluted with a final volume of 50µl RNase-free water and then stored at -70°C.

2.4.3 *Quantitation and electrophoresis of nucleic acids*

DNA and RNA were quantitated using the Beckman DU-65 spectrophotometer. Sample aliquots were diluted in MilliQ water and absorbance measured at wavelengths of 260nm and 280nm following calibration with MilliQ water. Nucleic acid concentration was calculated using the equation:

$$\text{Nucleic Acid Concentration } (\mu g/\mu l) = \frac{(A_{260})(df)(c)}{1000}$$

[Equation 3]

where A_{260} is the sample absorbance at 260nm, df is the sample dilution factor and c is the extinction coefficient of DNA (50 $\mu g.ml^{-1}$) or RNA (40 $\mu g.ml^{-1}$) given an absorbance at 260nm equal to 1.

To assess nucleic acid integrity, 1µg aliquots of isolated nucleic acid were mixed with 1µl DNA/RNA loading dye and loaded on 1% agarose gels in 1x TAE containing 0.5µg/µl ethidium bromide. Nucleic acids were resolved in 1x TAE at 120V for 90 minutes using the EPS301 Power supply. Ethidium bromide, bound to nucleic acids, was visualised using the Chromato-VUE® transilluminator and digitally captured by the Gene Genius Gel-Doc system (GeneSnap v. 2.60.0.14).

2.5 bcr-abl p210 mRNA amplification

A 10µl reverse transcription reaction of 1µg total RNA from CML samples was carried out using the First Strand cDNA Synthesis kit and the supplied random hexamer primer, as per manufacturer's instructions. A PCR containing 4µl cDNA in a 20µl final volume was performed using the PCR Master Mix with primers at a final concentration of 0.4µM each. The PCR primers (van Dongen et al., 1999) flank the translocation point of the bcr-abl p210 mRNA and so discriminate between exon b3 translocation variants. The primers were kindly supplied by Dr P. Willem from the Cytogenetics Unit of the Department of Molecular Medicine and Haematology at the University of the Witwatersrand.

A PCR was performed in the Eppendorf Mastercycler Gradient thermal cycler as described by van Dongen et al. (1999). Products were resolved on 1.5% agarose gels and visualised by ethidium bromide staining as per Section 2.4.3.

2.6 Telomerase activity

The telomere repeat amplification protocol (TRAP) detects telomerase activity in as few as ten cancer cells and is sensitive enough to detect telomerase activity in normal proliferative progenitor cells and activated lymphocytes (Hiyama and Hiyama, 2003).

Cell lysates are incubated with a telomere 3' overhang mimic (TS primer), which is elongated in the presence of telomerase by addition of telomeric repeats. Telomerase-extended primers are amplified using PCR and the extent of telomerase-primer elongation is calculated.

Telomerase activity was measured in the current study using the TeloTAGGG Telomerase PCR ELISA^{PLUS} kit or the TRAPeze ELISA kit according to manufacturers' instructions.

The TeloTAGGG Telomerase PCR ELISA^{PLUS} PCR master mix contained all reagents for *Taq*-polymerase mediated PCR, the TS primer and a 216bp internal control (to control for false-negatives due to PCR inhibition) and respective amplification primers. The TS primer and internal control sense primer each contained a 5' biotin label to facilitate detection of products using an ELISA.

Aliquots of 2 x 10^5 WBCs were lysed with 200µl CHAPS kit lysis buffer containing RNase inhibitor (200U/ml), with volumes equivalent to 3 x 10^3 cells used for each TRAP reaction. A negative control for each sample was prepared by heating a 3 x 10^3 cell aliquot for 10 minutes at 85^{o}C, abolishing any telomerase activity. Following the addition of the PCR master mix, samples and their negative controls were incubated for 30 minutes at 25^{o}C to allow for telomerase-mediated TS primer elongation. The internal control and any elongated TS primers were then amplified by PCR followed by immobilisation of amplification products to streptavidin-coated microtitre plates. Three wells were required per sample: an aliquot of the sample negative control was placed in the first well and aliquots of the test sample were placed in the second and third well. A dioxygenin-labeled probe specific for telomere repeats was added to the negative control and one test sample well, with a dioxygenin-labeled probe specific for the internal control added to the duplicate test sample. Wells were incubated with anti-dioxygenin-horseradish peroxidase antibody and the reaction was stopped after 10 minutes by the addition of HCl. The intensity of yellow colour generated for each sample or control was measured at a wavelength of 450nm (background wavelength 690nm) and absorbance values are reported as (A_{450} - A_{690}).

To control for light absorption by reagents and cellular contents, the absorbance of sample negative controls was subtracted from the absorbance of the sample and from the absorbance of the internal control. For TeloTAGGG Telomerase PCR ELISA^{PLUS} results to be considered reliable the following must apply for each sample:

$$(A_{450} - A_{690})_{negative} < 0.10$$

$$(A_{450} - A_{690})_{internal\ control} - (A_{450} - A_{690})_{negative} > 2.0$$

Samples are considered telomerase-positive if:

$$(A_{450} - A_{690})_{sample} - (A_{450} - A_{690})_{negative} > 2 \times (A_{450} - A_{690})_{negative}$$

[Equation 4a]

In later experiments, telomerase activity was assayed using the TRAPeze ELISA Telomerase Detection kit since a telomerase positive cell pellet is provided and the kit is easier and faster to use than the TeloTAGGG Telomerase PCR ELISA^{PLUS} kit. Cell lysates were prepared using CHAPS lysis buffer, containing RNase inhibitor, as per the TeloTAGGG Telomerase PCR ELISA^{PLUS} kit. The PCR master mix contained a biotinylated TS primer, telomere reverse primer and deoxyribonucleotide mix in a Tris buffer containing $MgCl_2$. The deoxyribonucleotide mix contained dinitrophenyl (DNP)-conjugated dCTP, which allowed for detection of amplification products by ELISA. The absorbance of the sample following ELISA is proportional to the number of DNP-conjugated dCTP nucleotides incorporated into extended TS primers and thus proportional to the level of telomerase activity in the sample.

The PCR master mix was added to samples following the addition of FastStart *Taq* DNA Polymerase to a final concentration of 5 units/µl. Samples and controls were incubated for 30 minutes at 30^{o}C to allow for telomerase-mediated extension of TS primers with products amplified by PCR. Following immobilisation of PCR products to streptavidin-coated plates, wells were incubated with anti-dinitrophenyl horseradish peroxidase, followed by incubation for 10 minutes with peroxidase substrate. The peroxidase-mediated reaction was stopped by addition of HCl. Absorbance of samples

was measured as described above for the TeloTAGGG Telomerase PCR ELISA^{PLUS} kit.

For the TRAPeze ELISA Telomerase Detection kit, a heat-treated 10µl aliquot (85^{o}C for 10 minutes) of each sample functioned as a negative control. Separate tubes containing supplied telomeric-repeat oligonucleotides functioned as a PCR control and a lysate prepared from the supplied telomerase positive cell pellet functioned as a positive control. Results were considered reliable if:

$$(A_{450} - A_{690})_{\text{negative}} < 0.25$$
$$(A_{450} - A_{690})_{\text{lysis buffer}} < 0.2$$
$$(A_{450} - A_{690})_{\text{PCR control}} > 0.8$$
$$(A_{450} - A_{690})_{\text{positive control}} > 0.15$$

Samples are considered telomerase-positive if:

$$(A_{450} - A_{690})_{\text{sample}} - (A_{450} - A_{690})_{\text{negative}} > 0.15$$

[Equation 4b]

2.6.1 *Non-denaturing polyacrylamide gel electrophoresis*

DNA strands containing varying numbers of telomere repeats are generated by TRAP as a function of the level of telomerase activity in a sample. These fragments can be visualised as a ladder of six-base pair increments, termed the telomerase ladder, using non-denaturing polyacrylamide gel electrophoresis. Dalla Torre et al. (2002) described a technique where TRAP products are visualised by silver-staining. A 12% non-denaturing polyacrylamide gel was prepared (Appendix C) and a 25µl aliquot of the 50µl TRAP amplification reaction product was added to 15µl DNA/RNA loading dye and resolved in 1xTBE for 16 hours (constant voltage of 45V).

Following electrophoresis, PCR fragments were fixed by gently shaking the gel in 0.5% (v/v) acetic acid and 10% (v/v) ethanol for 15 minutes. Gels were then stained for 10 minutes by shaking in 0.2% (w/v) $AgNO_3$ and washed three times with large

volumes of reverse osmosis (RO) water. After the final wash, gels were incubated in developing solution [0.1% (v/v) formaldehyde; 3% (w/v) NaOH] until bands were visible. The developing solution was washed off repeatedly with large volumes of RO water to prevent over-staining. Telomere ladders were digitally captured using the Gene Genius Gel-Doc system (GeneSnap v. 2.60.0.14).

In the event of over-staining, chemical reducers were used to remove excess silver as described by Gharahdaghi et al. (1999). Two stock solutions of 30mM potassium ferricyanide and 100mM sodium thiosulphate were made up. Just prior to use, the solutions were added together in a 1:1 ratio, poured over the gel and gently agitated until the desired stain intensity was reached. The reaction was stopped by rinsing in several batches of RO water, followed by shaking for 20 minutes in 200mM ammonium hydrogen carbonate. Gels were washed five times with large volumes of RO water and analysed.

2.7 Telomere length

In the current study, telomere length was measured using real-time quantitative PCR (RTQ-PCR). Originally described by Cawthon (2002) using the ABI Prism system, the method was optimised for use on the Roche Light Cycler. The traditional telomere restriction fragment length (TRF) method, which utilises Southern hybridisation, was performed on 12 samples to compare to telomere length RTQ-PCR.

2.7.1 *Telomere restriction fragment length assay*

Analysis of telomere length using Southern hybridisation was based on the technique described by Harley et al. (1990) and performed using the TeloTAGGG Telomere Length Assay with modifications.

DNA samples (2µg) were digested for 2 hours at 37^{o}C with 2.5U of *Rsa*I and 2.5U *Hinf*I in the reaction buffer supplied in the kit (final volume 50µl). The combination of these restriction endonucleases reduces human genomic DNA to fragments of approximately 125bp but does not recognize telomere repeats. A 2µl aliquot was resolved on a 0.8% agarose gel containing 0.5µg/µl ethidium bromide (120V in 1x

TAE buffer) and visualised using the Chromato-VUE® transilluminator to confirm DNA digestion.

Digested DNA was resolved on a 30cm long 0.8% agarose gel at 125V in 1x TAE until the bromophenol blue dye front had moved 20cm from the wells (approximately 4 hours). A DIG-labeled DNA size ladder supplied in the kit was resolved alongside samples. To facilitate transfer to nylon membrane, resolved telomeres were depurinated by gentle agitation of the agarose gel in 0.2M HCl for 10 minutes. The HCl was poured off and the gel rinsed twice with MilliQ water then washed twice with DNA denaturing buffer (1.5M NaCl, 0.5M NaOH) for 15 minutes. After two washes with MilliQ water, gels were washed twice with neutralization buffer (1.5M NaCl, 1.0M Tris-HCl, pH 7.5) and gentle agitation for 15 minutes. DNA was transferred to positively charged nylon membrane by capillary transfer overnight using 20x SSC and then fixed by baking the membrane at 80^{o}C for 30 minutes.

Membranes were hybridised for 3 hours with a telomere repeat-specific DIG-labeled probe diluted in DIG Easy Hyb at 42^{o}C using the Thermo-Hybaid Shake ‘n Stack set to speed number 3. Unbound antibody was removed by washing twice for 5 minutes with 2x SSC, 0.1% SDS, then twice for 20 minutes at 50^{o}C with 0.2x SSC, 0.1% SDS. Chemiluminescent detection of bound telomere probes was performed using the anti-DIG-alkaline phosphatase antibody and protocol supplied. Membranes were exposed to Lumi-Film Chemiluminescent detection film for 10 and 20 minutes. Developed films were analysed by densitometry using the Discovery Series 1D Analysis Software: Quantity One (Version 4.6.1; BioRad Laboratories, 2008).

Each sample lane of the scanned image is overlaid with a grid as shown in Figure 2.1. For each square, the signal intensity within the box (OD_i) and corresponding length, determined by comparison to the molecular size marker (L_i), are determined.

The mean TRF length for the sample is calculated according to the formula:

$$\overline{\mathrm{TRF}} = \frac{\sum(\mathrm{ODi})}{\sum(\mathrm{ODi/Li})}$$

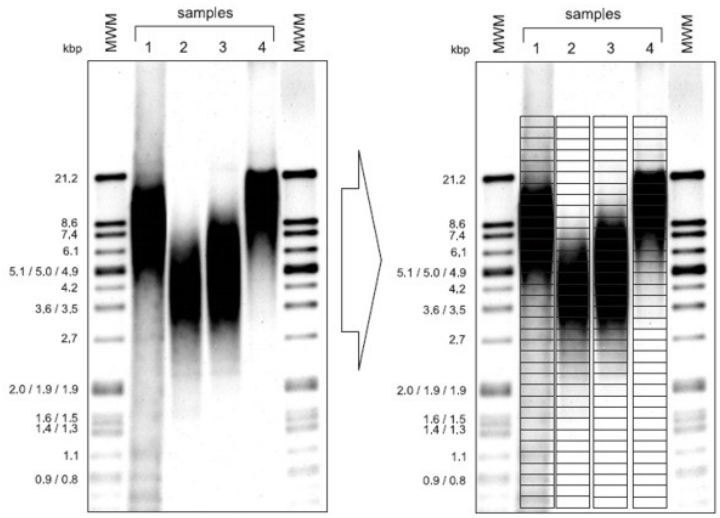

Figure 2.1 Densitometric analysis of telomere restriction fragments. TRF smears are overlayed digitally with a grid and the signal for each square is determined using a densitometer. The signal intensity for each square is related to the size of the marker and the mean value for each sample is reported as the TRF length. (Image taken from TeloTAGGG Telomere Length Assay package insert).

2.7.2 *Telomere length quantitated by real-time PCR*

Real-time PCR combines amplification and detection by collecting data throughout the PCR process. PCR product is detected using DNA-binding fluorophores and correlating fluorescence intensity to product concentration. Real-time PCR is sensitive, rapid and does not involve post-amplification manipulation; however, specialised equipment is required and in-depth understanding of normalization techniques is needed (Wong and Medrano, 2005).

Originally published by Cawthon R. (2002) using the Prism 7700 Sequence Detection System (Applied Biosystems), telomere length RTQ-PCR determines the relative telomere length of a sample by comparing the rate of telomere amplification to that of a single copy gene - acidic ribosomal phosphoprotein PO (36B4). In the current study the method was optimised on the Roche LightCycler 1 using LightCycler Software version 3.5 and Fast-Start DNA Master SYBR® Green (Gil and Coetzer, 2004a) or SYBR® Green Taq ReadyMix™.

Total DNA was isolated from WBCs and quantitated as per section 2.4.3. Primers for telomere (upstream primer tel1b and downstream primer tel2b - RM Cawthon, personal communication) and 36B4 PCR (upstream primer 36B4u and downstream primer 36B4d) were synthesised by IDT Technologies (USA) and stored at -20^{o}C as 100µM stock solutions in MilliQ water (Table 2.1).

Table 2.1 Primer sequences and product sizes for real-time quantitative telomere length PCR

Primer	Sequence (5'-3')	Product Size (bp)
Tel1b	- Cgg TTT g(TTTggg)$_5$ TT -	Variable
Tel2b	- ggC TTg (CCT TAC)$_5$ CCT -	
36B4u	- CAg CAA gTg ggA Agg TgT AAT CC-	74
36B4d	- CCC ATT CTA TCA TCA ACg ggT ACA A -	

Using the SYBR® Green Taq ReadyMix™, each 20µl reaction contained 50ng DNA, $MgCl_2$ at a final concentration of 3.5mM and telomere or 36B4 primers to a final concentration of 0.2µM. Antibody-bound *Taq* polymerase was activated for 30 seconds at 95^{o}C, followed by 25 cycles of 95^{o}C for 5 seconds, 56^{o}C for 20 seconds and 72^{o}C for 60 seconds for the telomere reaction and 30 cycles of 95^{o}C for 5 seconds, 58^{o}C for 20 seconds and 72^{o}C for 30 seconds for the 36B4 reaction.

The transition rates (rate of temperature change between PCR steps) were set to 20^{o}C/second with the exception of the annealing transition rate for the telomere reaction, which was optimised to 4^{o}C/second. Also, standard curves for telomere and 36B4 reactions were generated using a 1.6-fold dilution series of calibrator DNA (genomic DNA extracted from normal lymphocytes) yielding PCR reactions where the input DNA is equal to 100, 59.2, 35.4, 21.1 and 12.6ng per reaction. To compensate for run-to-run variation, triplicate reactions of 50ng calibrator DNA were performed for each run and all readings adjusted automatically by LightCycler Software version 3.5 to the standard curves previously constructed.

Data were generated using LightCycler Software version 3.5 with the following analysis settings (as recommended by the manufacturer):

a) Analysis method Fit-Points method using 3 fit-points.

A straight line is generated on the exponential phase of a PCR reaction using 3 fit-points.

b) Baseline Adjustment Arithmetic

Baseline fluorescence can vary between samples for a number of reasons, such as pipetting errors. Baseline adjustment by the arithmetic method involves the subtraction of the mean of the five lowest fluorescence points from each reaction.

c) Noise band Calculated by LightCycler

The noise band is set to a default of 3 x the standard deviation of the lowest five fluorescence values of the run.

d) Crossing line Set to 0.15 for each experiment

Telomere length quantitation using real-time PCR uses the $2^{-\Delta\Delta C_T}$ method for analysis (Livak and Schmittgen, 2001; Nordfjäll et al., 2005).

The equation that describes exponential amplification of PCR is:

$$X_n = X_o \times (1 + E_X)^n$$

where X_n is the number of target molecules at cycle n of the reaction, X_o is the initial number of target molecules and E_x the efficiency of target amplification.

The threshold cycle ($\mathbf{C_t}$) indicates the fractional cycle number, at which the amount of amplified target reaches a fixed threshold. Thus, for the telomere reaction:

$$X_t = X_o \times (1 + E_X)^{C_{t,X}}$$

where X_t is the threshold number of target molecules, $C_{t,X}$ is the threshold cycle for target amplification. The equation for the reference (36B4u) is similarly written as:

$$S_t = S_o \times (1 + E_S)^{C_{t,S}}$$

where S_t is the threshold number of reference molecules, S_o is the initial number of reference molecules, E_S is the efficiency of reference amplification and $C_{t,S}$ is the threshold cycle for reference amplification.

Normalising the target PCR to a reference gene PCR gives the equation:

$$\frac{X_t}{S_t} = \frac{X_o \times (1 + E_X)^{C_{t,X}}}{S_o \times (1 + E_S)^{C_{t,S}}}$$

In real-time telomere length PCR $X_o = S_o$ and the efficiency of the sample and standard curve reactions are equated by the calibrator of each run (amplification of 50ng DNA used to generate standard curves).

So:

$$\frac{X_t}{S_t} = (1 + E)^{C_{t,X} - C_{t,S}}$$

Thus, normalisation of real-time telomere length PCR to the single copy gene - acidic ribosomal phosphoprotein (36B4) reaction is written as:

$$\frac{(\text{Telomere})_t}{(36B4)_t} = (1+E)^{-\Delta C_t} = T/S\ ratio$$

where $\Delta C_t = C_t^{\text{Telomere}} - C_t^{36B4}$.

The T/S ratio of the test sample is related to the T/S ratio of the standard curve (at 50ng) and is written as:

$$\frac{T/S\ ratio_{(sample)}}{T/S\ ratio_{(standard\ curve)}} = \frac{(1+E)^{-\Delta C_t}{}_{(sample)}}{(1+E)^{-\Delta C_t}{}_{(standard\ curve)}}$$

Since the efficiency of the sample and standard curve reactions are equated by the run calibrator, *E* can be assumed to be equal to 1, so:

$$\frac{T/S\ ratio_{(sample)}}{T/S\ ratio_{(standard\ curve)}} = \frac{2^{-\Delta C_{t\ (sample)}}}{2^{-\Delta C_{t\ (standard\ curve)}}}$$

Since:

$$\frac{2^x}{2^y} = 2^{(x-y)}$$

The T/S ratio of a sample can be simplified to:

$$T/S\ ratio = 2^{-(\Delta Ct_{\text{sample}} - \Delta Ct_{\text{standard curve}})}$$

[Equation 6]

2.8 Telomerase catalytic subunit (hTERT) mRNA quantitation

An RNA transcript can be quantitated using RT-PCR with normalisation to an internal control. This control can be an added transcript which competes for amplification with the cDNA of interest by sharing primers or an endogenous transcript such as a maintenance gene (Wong and Medrano, 2005).

2.8.1 *Conventional RT-PCR*

An endogenous internal control for quantitative RT-PCR must be expressed at constant levels between samples (Warrington et al., 2000). Published housekeeping genes suitable for hTERT mRNA quantitation in CML were selected if they fulfilled the following criteria; (1) Not located in genomic sites known to be fragile in CML and (2) expressed at constant levels in haematopoietic cells. Both mitochondrial single-stranded DNA binding protein 1 (ssBP1) and Ubiquitin C (UBC) fulfill the criteria for hTERT mRNA quantitation and were initially used. Since better results were obtained using ssBP1 as an endogeneous control in multiplex reactions, this gene became the control of choice.

A 20µl reverse transcription reaction of 1µg total RNA with 1.6µg oligo-dT_{15} primer was performed using the First Strand cDNA Synthesis kit. Multiplex PCR of 10µl cDNA was carried out in a 50µl volume using the PCR Master Mix kit with primers for hTERT and ssBP1 (IDT, USA) added to final concentrations of 0.25µM and 0.3µM. These primers flank introns to discriminate between RNA amplification and contaminating DNA amplification and were stored as 100µM stock solutions at -20^{o}C. Primer-pair sequences and the size of amplification products generated are listed in Table 2.2.

Table 2.2 Primer sequences for hTERT conventional and real-time RT-PCR.

Primer[1]	Sequence (5'-3')	Product Size (bp)	
		DNA	mRNA
hTERTs	- Agg AgC TgA CgT ggA AgA Tg -	10906	302
hTERTa	- CTg ACC TCT gAT TCC gAC Ag -		
ssBP1s	- gCg ATC Agg ggA TAg TgA Ag -	2000	194
ssBP1a	- TTg CTT gTC gCC TCA CAT TA -		

[1] (s) sense primer, (a) antisense primer

A "hot-start" at 94^{o}C was followed by 40 cycles of 94^{o}C for 30 seconds, 62^{o}C for 30 seconds and 72^{o}C for 1 minute, using the Eppendorf Gradient Mastercycler. PCR products were resolved using a 1.5% agarose gel in 1x TAE containing 0.5µg/µl ethidium bromide.

Densitometric analysis of PCR products was performed using GeneTools v.2.10.02 on the Gene Genius Gel-Doc system. hTERT mRNA expression was calculated using the equation:

$$\text{hTERT mRNA expression} = \frac{\text{hTERT peak area}}{\text{ssBP1 peak area}}$$

[Equation 7]

Since conventional RT-PCR makes use of end-point detection which has a number of limitations, including poor precision, low sensitivity and a short dynamic range; a real-time RT-PCR was developed.

2.8.2 *Real-time RT-PCR*

cDNA was generated as described in Section 2.8.1. Real-time PCR was carried out on the Roche LightCycler 1 using LightCycler Fast-Start DNA Master SYBR® Green or SYBR® Green Taq ReadyMix™.

Using SYBR® Green Taq ReadyMix™, a 20µl real-time PCR of 4µl cDNA was carried out with $MgCl_2$ added to a final concentration of 8.25mM. Primers for hTERT and ssBP1 (Section 2.8.1) were added to final concentrations of 0.25µM for each primer. *Taq* polymerase was activated for 30 seconds at 95^{o}C, followed by 40 cycles of 95^{o}C for 10 seconds, 58^{o}C for 10 seconds and 72^{o}C for 20 seconds. Transition rates were set to 20^{o}C/second and peak areas were determined by melting curve analysis using the LightCycler Software version 3.5.3.

Melting curves were generated following PCR by heating reaction products at 95^{o}C for 10 seconds followed by incubation at 70^{o}C for 2 minutes to allow all amplification products to anneal and form double-stranded amplicons. Samples were then heated to 95^{o}C at 0.2^{o}C/second with fluorescence measured at each 0.2^{o}C increment. Since the majority of amplicons in solution are of equal length, the largest change in fluorescence will occur at a temperature equivalent to the melting temperature of the amplicon. Melting curves (fluorescence versus temperature) were generated using the

LightCycler Quantitation software version 3.5 and areas under each peak automatically calculated. The hTERT mRNA expression was calculated using Equation 7 (Section 2.8.1).

2.9 Statistical analysis and computational programs

Microsoft Excel 2010 for Windows (Microsoft Corporation, USA) was used for mathematical calculations, plotting of graphs and determination of R^2 values for graphs. In addition the program was used to calculate the mean value, standard deviation (SD) the coefficient of variation (CV) for data sets.

Design of diagrams and labelling of figures were performed using Corel Photo-paint X3 version 13.0.0.667 (Corel Corporation, Ontario Canada).

3.1 Nucleic acid integrity

Following agarose gel electrophoresis, intact genomic DNA was visualised as a single high molecular weight band (Figure 3.1a, Lane 2) and intact RNA as distinct bands of the 28S and 18S ribosome complexes (Figure 3.1a, Lane 3).

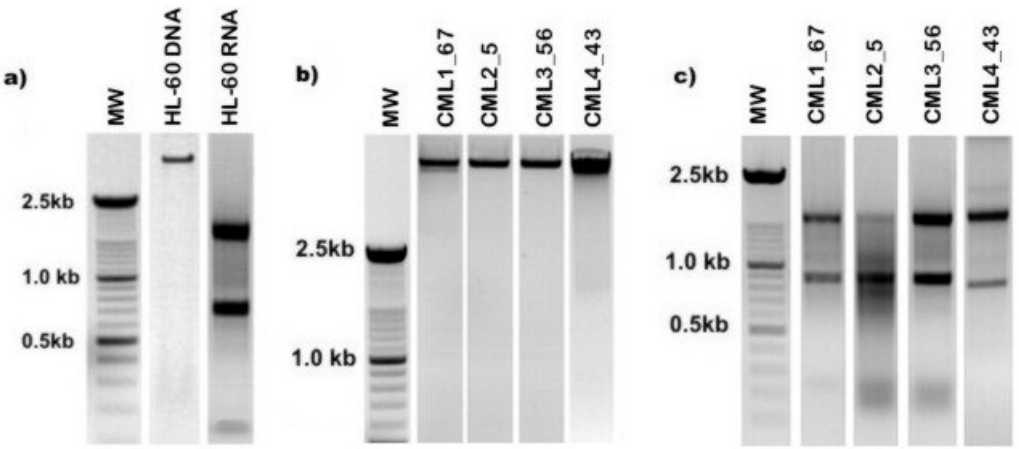

Figure 3.1 Representative DNA and RNA samples. (a) Isolated RNA and DNA were visualised following agarose gel electrophoresis by ethidium bromide staining. Intact DNA resolved as a single high molecular size band (HL-60 DNA). Intact RNA was visualised as two distinct bands - the 28S and 18S ribosome complexes. These complexes resolve as bands equivalent to ~1.7kb and ~0.8kb DNA size fragments respectively, with the 5S band occasionally seen resolving at 0.15kb (HL-60 RNA). Absence of either the 28S or 18S fragments or the presence of low molecular weight RNA indicates degradation. Isolated DNA **(b)** and RNA **(c)** of four CML patient samples are shown. Note the partial degradation in CML2_5, indicated by the pronounced 18S band compared to the 28S band. **MW:** 100bp DNA molecular size marker.

Purity of extracted DNA or RNA was estimated using the absorbance ratio A_{260}/A_{280}, since nucleic acids and proteins absorb at wavelengths of 260nm and 280nm respectively. Pure DNA has a ratio of approximately 1.8 and RNA of 2.0, whereas a sample with contaminating protein will have a ratio less than 1.5. Extracted RNA tends to solubilise less readily than DNA, so low RNA absorption ratios can also result from partial solubilisation. DNA assays were performed only if the A_{260}/A_{280}

ratio of the sample was between 1.6 and 1.9; and in the case of RNA assays, between 1.9 and 2.1. Samples CML5_80 and CML5_83 were not assayed for hTERT mRNA following visual confirmation of RNA degradation.

3.2 bcr-abl p210 RT-PCR

To confirm that patients did in fact have CML, RT-PCR was used to detect the expression of bcr-abl p210. The reaction discriminates between exon b3 variants of p210 by producing a 417bp (exon b3 inclusive variant) or 342bp fragment (excluding exon b3) (Figure 3.2). The exon b3 variants do not exhibit any pathological differences since exon b3 does not contain any functional domains (Inokuchi, 2006). Although several patients were on long-term treatment, all CML patients were positive for the Ph chromosome using RT-PCR, which may be attributed to the ineffectiveness of hydroxyurea and α-interferon to completely eliminate all p210-positive cells (Savona and Talpaz, 2008). Half of the patients harboured the exon b3 inclusive variant and the other half the exon b3 exclusive variant.

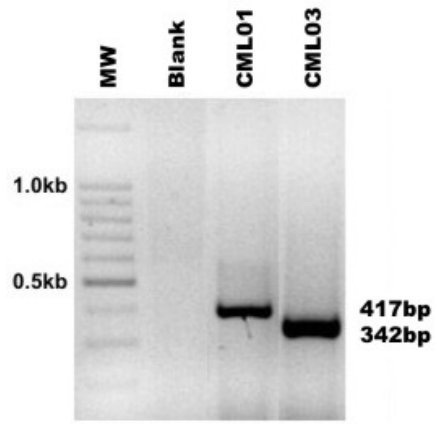

Figure 3.2 Visualisation of bcr-abl p210 variants using RT-PCR. The RT-PCR primers flank the bcr-abl p210 translocation point so exon b3 variants can be discriminated by a product size difference of 75bp. **MW:** 100bp DNA molecular size marker. **Lane 1:** Reaction blank. **Lane 2:** RT-PCR products of a bcr-abl p210 transcript including exon b3 and excluding exon b3 (**Lane 3**).

3.3 Telomerase activity

3.3.1 *Validation of assays using an HL-60 cell line and PHA-stimulated lymphocytes*

In agreement with published data, telomerase activity was detected in PHA-stimulated lymphocytes and an HL-60 cell line, whereas unstimulated lymphocytes had no detectable telomerase activity (Appendix D2) (Hiyama et al., 1995; Savre-Train et al., 2000). The HL-60 cell line exhibited high levels of relative telomerase activity (RTA) in the order of 19x that of PHA-stimulated lymphocytes; pointing to additional levels of control over telomerase activity lost in the HL-60 cell line. The cell isolates where used as positive controls in telomere and telomerase assays.

3.3.2 *Telomerase activity was not detected in all CML patients*

As expected, normal samples had undetectable telomerase activity whereas most CML samples exhibited moderate to high levels of telomerase activity (Appendix D2). All samples taken from the patient in accelerated phase (CML1; Appendix D2) had detectable telomerase activity, unlike samples taken from patients in chronic phase (Appendix D2). Telomerase activity was detected in 10 of 16 samples (63%) with blast cells present in the peripheral blood. Three samples had detectable telomerase activity where blast cells were absent from peripheral blood (CML3_60, CML3_66 and CML15. No result could be obtained for samples CML6_38 and CML6_39 since the internal control failed for the original sample assay and when the assay was repeated (Appendix D2) – probably resulting from PCR inhibitors present in the samples.

Resolving TRAP amplification products by polyacrylamide electrophoresis allows for visualization of the telomerase ladder; with the number of incremental bands being proportional to the telomerase activity of that sample. In Figure 3.3, the telomerase ladder is absent in the control sample, which had undetectable telomerase activity, but visible in sample CML1_67, which was telomerase positive as determined using the TeloTAGGG Telomerase PCR ELISA^{PLUS}.

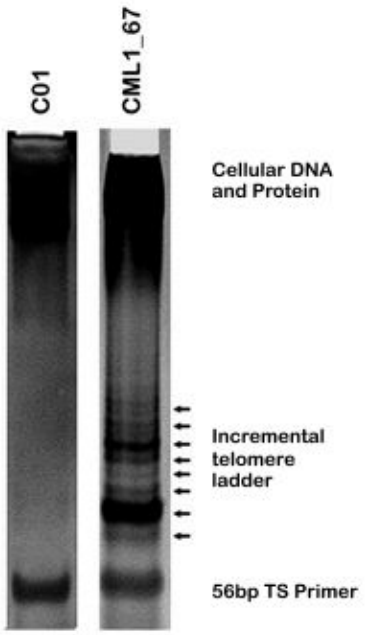

Figure 3.3 Telomerase ladder visualised by silver staining. Using the TRAP assay, telomerase adds hexameric repeats to a primer. Resolution of telomerase positive samples yields an incremental ladder formed by TRAP products, the number of increments being proportional to the telomerase activity of the sample. Telomerase activity was undetectable in sample C01 and present in sample CML1_67. The prominent band observed for CML1_67 is probably a function of shorter TRAP products being preferentially amplified in the PCR reaction. The high molecular size product in both lanes represents genomic DNA and cellular proteins since the TRAP assay is conducted using whole cell extract.

3.4 Telomeres are shorter in CML patients compared to controls

3.4.1 *Telomere restriction fragment length*

Telomere restriction fragments (TRFs) were resolved by electrophoresis (Figure 3.4) and analysed using densitometry with the mean telomere restriction fragment length for each sample calculated (Section 2.7.1; Appendix D3).

The TRF lengths of eight controls were determined and fell within the normal published range of 2-10kb (Lansdorp, 2008). The narrow range reported in this study results from the narrow age range, with the longest TRF measured for a 26 year old (7.69kb, C38) and the shortest in a 40 year old (6.87kb – C35) (Figure 3.4; Appendix

D3). As shown in previous studies, the correlation between TRF and age is poor and is a function of the variation in telomere length between individuals of the same age (Kimura et al., 2008). Discreet bands between 0.8 and 3.5kb were observed in three controls and may represent interstitial telomeric sequences located within centromeres.

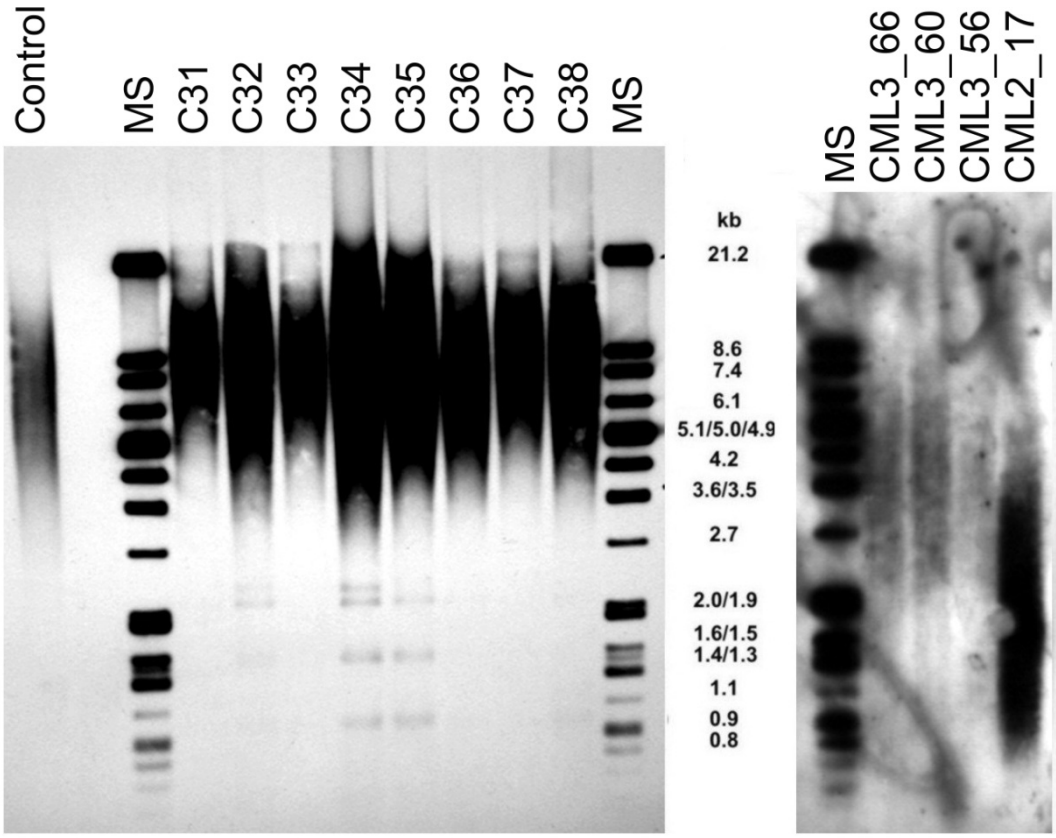

Figure 3.4 Southern hybridisation of telomere restriction fragments. Telomeres within a cell are heterogeneous in length, so hybridisation of telomere restriction fragments with telomere-specific probes are visualised as smears. The TRFs of eight normal individuals (left) were determined and fell within the normal published average of 2-10kb. Conversely, the TRFs of two CML patients fell below the average lengths of the controls and ranged from 2.48kb (CML2_17) to 3.45kb (CML3_60). MS: Molecular Size Marker.

An objective of this study was the optimisation of a telomere length assay for use in a routine laboratory. Southern hybridisation is technically demanding and too time consuming; therefore, a technique utilizing real-time PCR to measure telomere length was explored.

3.4.2 *Telomere length real-time PCR*

Real-time telomere length is calculated by comparison of telomere and a single copy gene (36B4) amplification of a sample to the corresponding standard curves. Since the assay was originally published for the ABI Prism system (Cawthon, 2002), it had to be adapted for use on the Roche LightCycler 1. The optimised assay was published using the LightCycler Fast-Start DNA Master SYBR Green kit (Gil and Coetzer, 2004a) but subsequently all samples were analysed using SYBR Green Taq ReadyMix and are reported here. The assay was optimised using DNA from an HL-60 cell line and PHA-stimulated lymphocytes and unstimulated lymphocytes from a normal control. Results showed that (a) dilution series generated linear curves (Figure 3.5; Appendix D4), (b) replicates did not vary significantly (Figure 3.6a; Appendix D5) and (c) single gene copy amplification products were visualised as a single band and telomere repeat amplification as a smear (approximately 100 – 400bp), following agarose gel electrophoresis (Figure 3.6b).

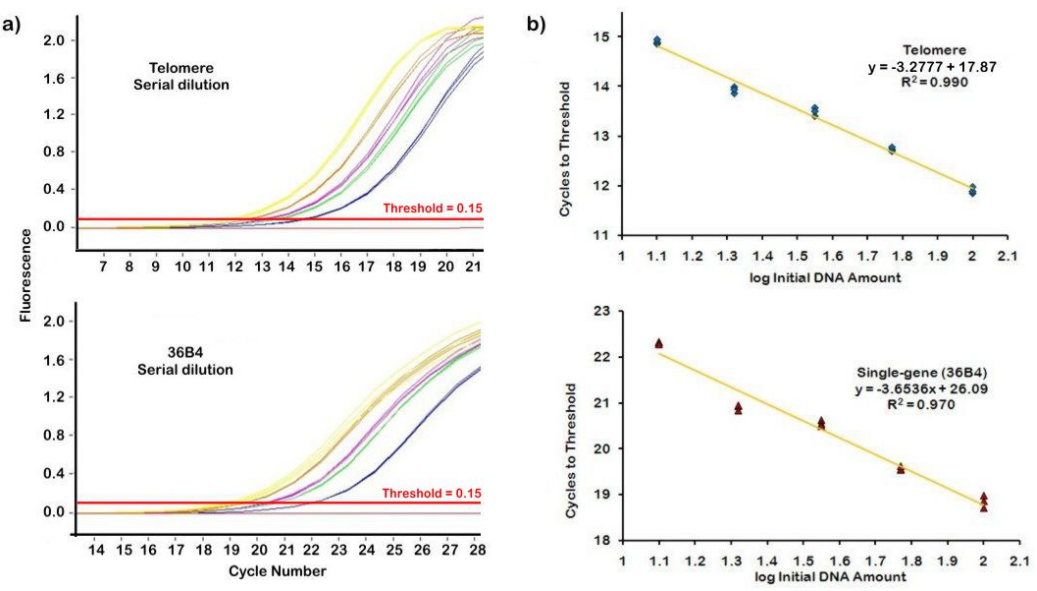

Figure 3.5 Real-time telomere length PCR standard curves. (a) Triplicate telomere (top) and 36B4 (bottom) amplification reactions of 100, 59.2, 35.4, 21.1 and 12.6 ng HL-60 genomic DNA (yellow, orange, purple, green and blue curves respectively) were performed using the Roche Light Cycler 1 and reported as the sample fluorescence following each PCR cycle. **(b)** Standard curves for the serial dilution reactions were generated by plotting the number of cycles required for each DNA amount to reach the set threshold of 0.15. Telomere and 36B4 standard curves had correlation coefficients of 0.99 and 0.97 respectively, with gradients of -3.2 and -3.6 respectively.

Standard curves were constructed by plotting the number of cycles required to reach threshold (Ct) versus the initial amount of DNA used in the reaction. Telomere and 36B4 standard curves had correlation coefficients of 0.99 and 0.97 respectively (Figure 3.5b; Appendix D4); with gradients of -3.2 and -3.6, respectively, which fall within the validity range stipulated by the manufacturer. The similarity of gradients confirmed that the efficiencies of the telomere and 36B4 reactions were similar and therefore comparable.

Assay reproducibility was demonstrated by determining the coefficient of variation (CV) of 17 replicates of 21.1ng HL-60 DNA (Figure 3.6a). Telomere amplification had a CV of 1.12% (mean C_t = 13.37, SD = 0.15) and 36B4 amplification had a CV of 1.53% (mean C_t = 20.80, SD = 0.32) (Appendix D5). The extent of variation between experiments was calculated from the amplification of telomeres and the single-copy gene (36B4) of a 50ng control sample included in six assays. The coefficient of variation of the telomere and 36B4 amplification reactions was 4.03% (mean C_t = 12.41, SD = 0.50) and 3.14% (mean C_t = 22.32, SD = 0.70), respectively (Appendix D6).

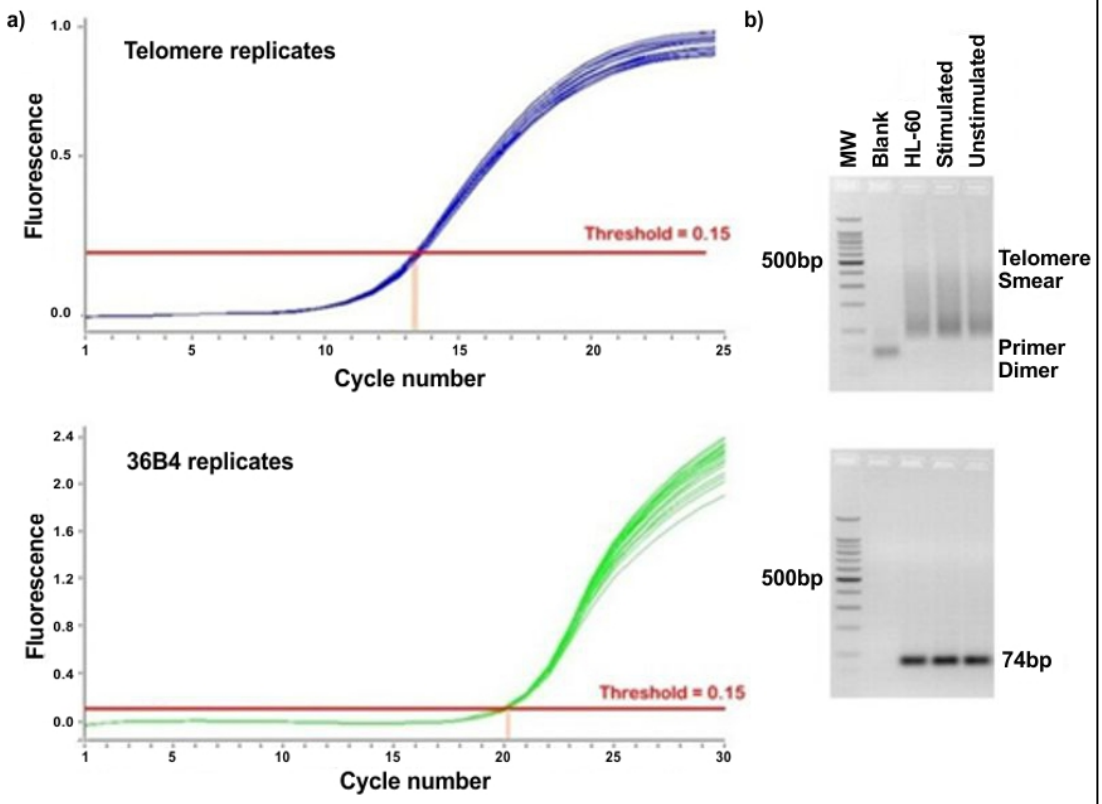

Figure 3.6 Amplification curves of real-time telomere length PCR replicates and visualization of amplification products. (a) Reproducibility of real-time telomere length PCR was demonstrated by showing the coefficient of variation (CV) of 17 replicates subjected to telomere (top) and 36B4 (bottom) amplification to be less than 15%. **(b)** DNA extracted from an HL-60 cell line and normal lymphocytes cultured with PHA (stimulated) or without PHA (unstimulated) were assayed for telomere length using real-time PCR. Agarose gel electrophoresis of products following real-time PCR of the single-copy gene, 36B4, produced a 74bp fragment (bottom). Telomeres, however, are heterogeneous in length and real-time PCR produced a range of fragment sizes, visualised as a smear following agarose gel electrophoresis (top).

To show that telomere lengths calculated using Southern hybridisation and real-time PCR were comparable; Cawthon R (2002) demonstrated a linear relationship between mean TRF length and relative T/S ratios using DNA extracted from 95 healthy individuals. In the current study, since only a small number of samples were assayed a statistical relationship was not determined, but as expected, CML samples had low TRF and T/S ratio values whereas controls had high TRF and T/S ratio values (Figure 3.7).

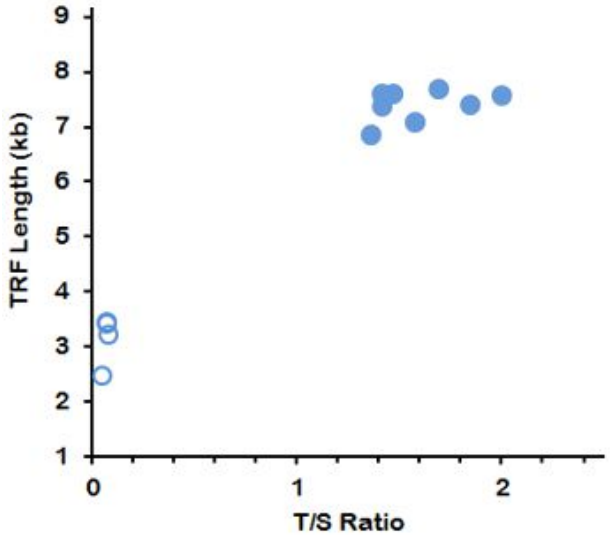

Figure 3.7 TRF lengths versus T/S ratios. As expected, when T/S ratio is plotted against TRF length, control samples (closed circles) cluster towards the top right of the graph (long telomeres, high T/S ratio) and CML samples (open circles) cluster to the bottom left of the graph (short telomeres, low T/S ratio). Note: CML3_60 (3.45kb) and CML3_66 (3.42kb) overlap on the graph.

Using large cohorts and sensitive telomere length assays, the decline of haematopoietic cell telomere length with age in healthy individuals has been shown to be non-linear. Furthermore, the telomere length at any given age is highly variable with the most rapid drop occurring in the early years of life and the attrition rate varying between lymphocytes and granulocytes (Kimura et al., 2008; Lansdorp, 2008). In the current study, the plot of T/S ratios versus age in controls demonstrated a trend of telomere length decreasing with age in a non-linear manner (non-linear R^2 = 0.25 versus linear R^2 = 0.22) (Figure 3.8, Appendix D7). Variation between age-matched controls was largest between samples taken from two 38-year olds (C04: T/S=2.22 and C09: T/S=0.61, variation T/S=0.48) (Appendix D7) and is a result of variation in inherited telomere length and differing rates of telomere shortening between individuals (Chen et al., 2011). A recent study by Eisenberg et al. (2011) found telomere lengths in age-matched controls can vary by as much as 13.54kb. Using the equation relating TRF length to T/S ratios of Cawthon (2002) (y=1910.5x + 4157), this range equates to a T/S ratio of ≈ 4.91.

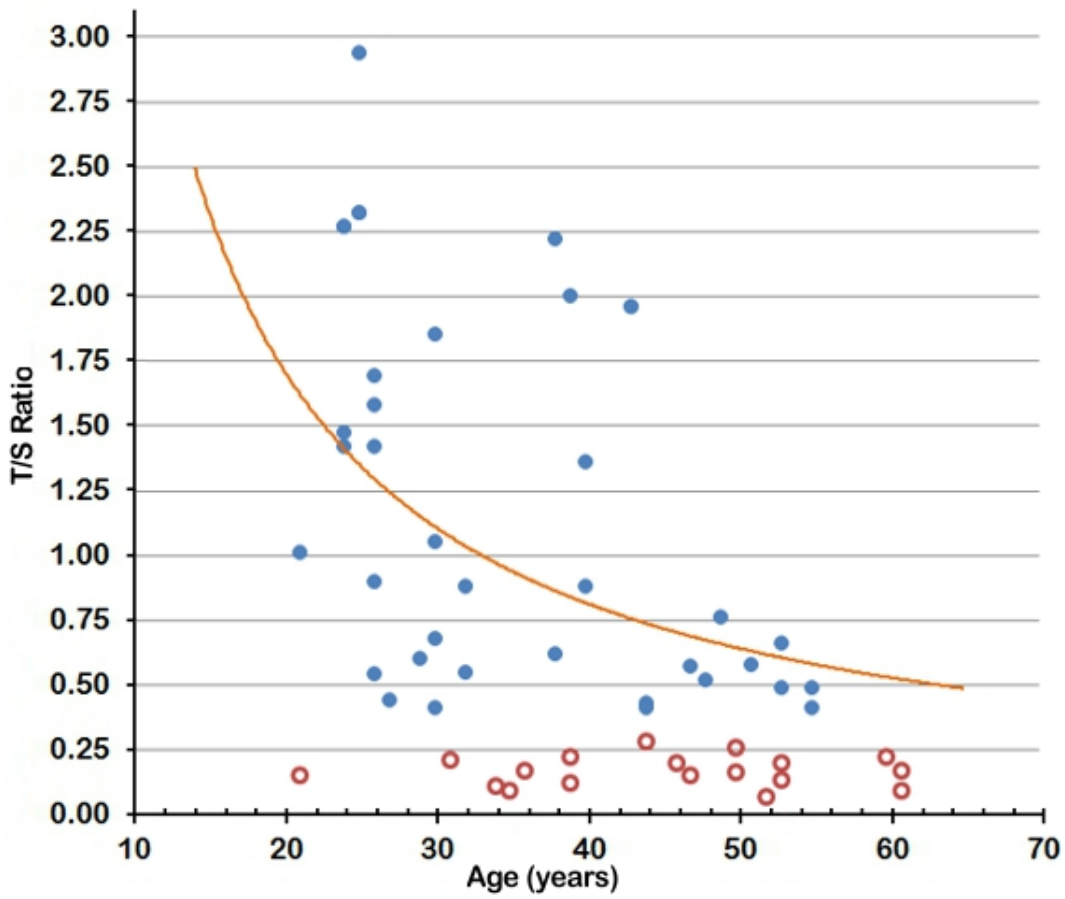

Figure 3.8 T/S ratio versus age of CML patients and controls. Telomere length in healthy individuals (solid blue circles) decreased with age in a non-linear manner (orange trend line).As expected, this exponential relationship showed very poor correlation as a result of telomere length heterogeneity between individuals of the same age. In CML (red open circles), mean telomere length was shorter than healthy controls analysed (T/S ratio 0.2 versus 1.18) with an observable loss of heterogeneity between individuals and age groups. Where multiple samplings were taken from one patient, the mean T/S ratio was plotted.

In agreement with Bodoni et al. (2008) telomeres of the 18 CML patients in the current study were shorter than normal controls (Figure 3.8). The telomere length of two CML samples approached the range measured in normal controls (CML4_47 and CML11_4 both measuring T/S=0.39) (Appendix D7). Boultwood et al. (1999) and Bagheri et al. (2008) found that 22% and 28%, respectively, of CML patients had normal telomere lengths compared to age-matched controls. The lowest T/S ratio

measured for normal individuals in the current study was T/S=0.41 (C12, C14 and C26 being 44, 55 and 30 years of age, respectively).

Using large cohorts and sensitive telomere length assays, the decline of haematopoietic cell telomere length with age in healthy individuals has been shown to be non-linear (Hewakapuge *et al.*, 2008) Furthermore, the telomere length at any given age is highly variable with the most rapid drop occurring in the early years of life (Lansdorp, 2008). In the current study, the plot of T/S ratios versus age in controls demonstrated a trend of telomere length decreasing with age (Figure 3.8, Appendix D7). Variation between age-matched controls was largest between samples taken from two 38-year olds (C04: T/S=2.22 and C09: T/S=0.61, variation T/S=1.61 (≈ 7.2kb) (Appendix D7) and is presumably a result of variation in inherited telomere length, differing rates of telomere shortening between individuals and experimental error (Chen et al., 2011).

3.5 Semi-quantitation of hTERT mRNA

A literature search and online gene analysis (http://www.ncbi.nlm.nih.gov) was performed to identify gene transcripts that would serve as an internal control for hTERT mRNA quantitation in CML. Two housekeeping genes, Ubiquitin C (UBC) and Single-stranded mitochondrial DNA binding protein 1 (ssBP1) were found to be in a stable genomic location in most leukaemias (Table 3.1) (Tiranti et al., 1995; Warrington et al., 2000; Vandesompele et al., 2002; Johansson et al., 2002). The gene encoding ssBP1 was selected since multiple bands were amplified in UBC reactions (Figure 3.9), which most likely resulted from UBC containing multiple directly repeated coding sequences (Dr J Vandesompele, personal communication).

Table 3.1 Internal control genes for hTERT mRNA quantitation in leukocytes.

Symbol	Accession Number[1]	Name	Function	Localisation	Known Pseudogenes
UBC	M26880	Ubiquitin C	Protein degradation	12q24	No
SSBP1	NM_003143	Single-stranded DNA binding protein 1	Stabilises single-stranded DNA	7q34	No

[1] As given on the Genbank website (http://www.ncbi.nlm.nih.gov)

Since the HL-60 cell line and mitogen-activated lymphocytes have been shown to express hTERT mRNA, optimisation of hTERT RT-PCR was performed using these two sets of cells, with unstimulated lymphocyte aliquots serving as a negative control. Reverse transcription of total mRNA was performed using an oligo$(dT)_{15}$ primer in a conventional thermal cycler. First, separate PCR reactions of hTERT and ssBP1 were optimised, and then a multiplex PCR amplifying both hTERT and ssBP1 mRNA in a single tube was optimised in a conventional thermal cycler. Amplification products were visualised as a 302bp hTERT and 194bp ssBP1 fragment (Figure 3.9). Using real-time PCR, melting curve analysis showed that hTERT amplicons melted at ~85.5^{o}C and ssBP1 amplicons melted at ~81.4^{o}C (Figure 3.10). Peak areas of melting curves were analysed and relative hTERT expression calculated as the hTERT to ssBP1 peak area (Appendix D8). Primer dimer amplification was observed in multiplex reactions using real-time PCR but not conventional PCR and is probably a function of the primer concentration and assay sensitivity.

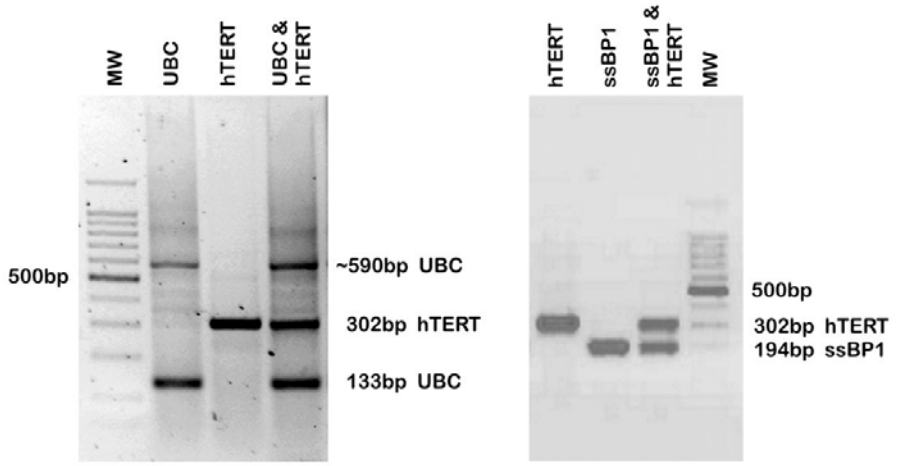

Figure 3.9 Comparison of UBC and ssBP1 as internal controls for hTERT RT-PCR using an HL-60 cell line. Optimisation of hTERT RT-PCR was carried out by first optimising UBC, ssBP1 and hTERT reactions individually (Lanes UBC, ssBP1 and hTERT), then the multiplex reactions (UBC & hTERT and ssBP1 & hTERT). UBC was not considered a suitable internal control as multiple bands were consistently amplified (left); whereas a single product was amplified for ssBP1. MW: 100bp Molecular Size Marker.

As expected, WBCs of control subjects expressed the housekeeping gene ssBP1 but not hTERT (Appendix D8). In CML patients, hTERT expression was detected in 72% of patients analysed (13/18 patients, Appendix D8). Of these, 60% (15 of 25 samples) were taken from patients with blast cells present in peripheral blood (Table 3.2). Of the multiple samplings taken for patients CML3, CML4, CML5 and CML11, not all samplings had detectable hTERT. Overall, the data presented here indicate that hTERT is not ubiquitously expressed in CML.

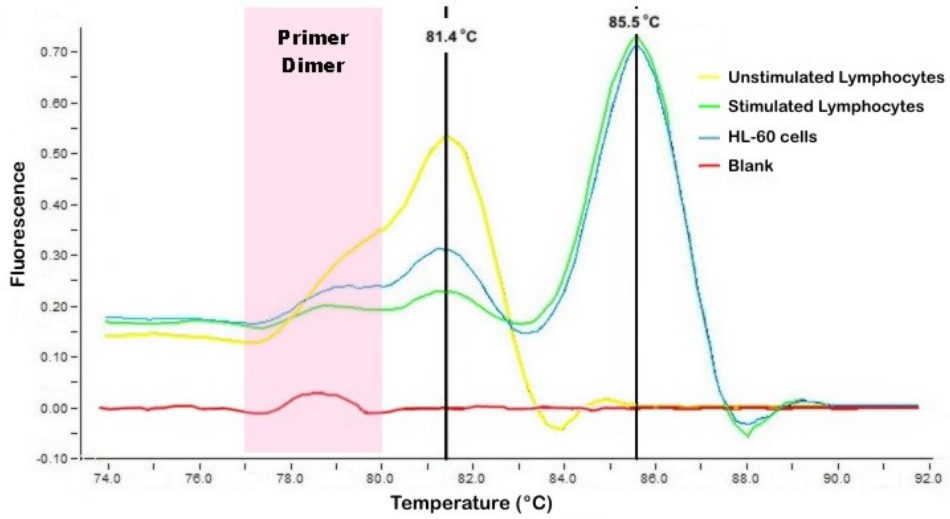

Figure 3.10 Melting curves of hTERT RT-PCR amplicons. Products of real-time PCR were visualised by generation of melting peaks at fragment-specific temperatures. hTERT amplicons generated a peak at 85.5^{o}C and ssBP1 at 81.4^{o}C. Combining results obtained for the blank reaction with reactions containing ssBP1 or hTERT mRNA or both, the LightCycler software determined the melting range for primer dimers (pink shaded region) for subtraction from quantitation calculations.

Chapter 4 Discussion and Conclusions

The finding of this study is that telomere dynamics is altered in CML patients when compared to normal controls. A summary of telomerase activity, telomere length and hTERT mRNA expression for the CML samples assayed in the current study is given in Table 4.1.

Table 4.1 Telomerase activity, telomere length and hTERT mRNA expression in CML.

Sample	Phase Classification	Relative Telomerase Activity[1]	T/S Ratio	Telomere Length (kb)[2]	Relative hTERT mRNA Expression	Blasts in peripheral blood (%)	Age/Sex
CML1_67	Chronic	High	0.29	4 711	0.46	6	53 Female
CML1_68	Accelerated	High	0.04	4 233	0.33	14	
CML1_69	Accelerated	High	0.07	4 290	1.24	16	
CML1_70	Accelerated	High	0.04	4 233	1.23	15	
CML1_71	Accelerated	High	0.13	4 405	1.10	7	
CML1_72	Accelerated	High	0.23	4 596	1.04	11	
CML2_5	Chronic	Absent	0.08	4 309	0	0	35 Male
CML2_9	Chronic	Absent	0.13	4 405	0	0	
CML2_17	Chronic	Absent	0.05	4 252	0	0	
CML3_56	Chronic	Absent	0.08	4 309	0	0	52 Female
CML3_60	Chronic	Moderate	0.07	4 290	0.72	0	
CML3_66	Chronic	Moderate	0.07	4 290	0.74	0	
CML4_43	Chronic	Absent	0.23	4 596	0	0	31 Male
CML4_45	Chronic	Absent	0.30	4 730	0	0	
CML4_46	Chronic	Absent	0.22	4 577	0	0	
CML4_47	Chronic	Absent	0.39	4 902	0	0	
CML4_48	Chronic	Absent	0.13	4 405	0	0	
CML4_49	Chronic	Absent	0.18	4 500	0	0	
CML4_50	Chronic	Absent	0.11	4 367	0	0	
CML4_51	Chronic	Absent	0.06	4 271	0.19	0	
CML4_52	Chronic	Absent	0.24	4 615	0.35	7	

[1] Since the threshold value for telomerase activity varies between the TeloTAGGG Telomerase PCR ELISA^{PLUS} and the TRAPeze ELISA (Equations 4a and 4b, Section 2.6); results were expressed as a "Relative telomerase activity" (RTA). RTA is defined as absent if below the threshold for the respective assay, moderate if up to 10x the negative control for that sample and high if greater than 10x.

[2] Using the equation relating TRF length to T/S ratios published in Cawthon (2002) (y=1910.5x + 4157).

Table 4.1 Telomerase activity, telomere length and hTERT mRNA expression in CML (continued).

Sample	Phase Classification	Relative Telomerase Activity[1]	T/S Ratio	Telomere Length (kb)[4]	Relative hTERT mRNA Expression	Blasts in peripheral blood (%)	Age/Sex
CML5_80	Chronic	Absent	0.21	4 558	No result[2]	0	60 Female
CML5_83	Chronic	Absent	0.30	4 730	No result[2]	0	
CML5_85	Chronic	Absent	0.16	4 463	0	0	
CML5_86	Chronic	Absent	0.26	4 654	0.87	2	
CML5_90	Chronic	Absent	0.23	4 596	0	0	
CML5_91	Chronic	High	0.13	4 405	0.83	2	
CML5_96	Chronic	Absent	0.22	4 577	0.86	2	
CML6_34	Chronic	Absent	0.18	4 501	0.37	0	46 Male
CML6_37	Chronic	Absent	0.16	4 463	0.38	0	
CML6_38	Chronic	No result[2]	0.17	4 482	0.33	0	
CML6_39	Chronic	No result[2]	0.29	4 711	0.26	0	
CML7_49	Chronic	Absent	0.12	4 386	No result[2]	0	39 Male
CML8_27	Chronic	Absent	0.22	4 577	0	0	39 Male
CML9_51	Chronic	Absent	0.11	4 367	0	0	34 Female
CML10_0[3]	Chronic	Absent	0.09	4 328	0	1	61 Female
CML11_3	Chronic	Absent	0.17	4 481	0.72	0.4	44 Male
CML11_4	Chronic	Absent	0.39	4 902	0	0	
CML12_0[3]	Chronic	High	0.15	4 443	0.72	2	47 Male
CML13_0[3]	Chronic	High	0.15	4 443	0.69	1	21 Female
CML14_0[3]	Chronic	High	0.16	4 462	0.89	1	50 Female
CML15_0[3]	Chronic	High	0.26	4 653	0.55	0	50 Female
CML16_0[3]	Chronic	Absent	0.17	4 481	0.13	0	36 Male
CML17_0[3]	Chronic	Absent	0.20	4 539	0.53	9	53 Male
CML18_0[3]	Chronic	Absent	0.17	4 481	0.48	0	61 Female

[1] Since the threshold value for telomerase activity varies between the TeloTAGGG Telomerase PCR ELISA^{PLUS} and the TRAPeze ELISA (Equations 4a and 4b, Section 2.6); results were expressed as a "Relative telomerase activity" (RTA). RTA is defined as absent if below the threshold for the respective assay, moderate if up to 10x the negative control for that sample and high if greater than 10x.

[2] No result reported for samples where the internal control failed for the original sample assay and when the assay was repeated.

[3] Sample taken at time of diagnosis.

[4] Using the equation relating TRF length to T/S ratios published in Cawthon (2002) (y=1910.5x + 4157).

4.1 The TRF and T/S ratio assays

The development of new molecular techniques and their subsequent implementation in laboratories requires validation against accepted standard techniques (Peruski and Peruski, 2003). Validation is performed by comparison of quantitative and qualitative assessments made for each assay.

In the current study, the exact length of telomeres in a sample is not required but rather the detection of significant differences between CML patients and controls. Both the TRF and T/S ratio assays are semi-quantitative, yielding an approximation of telomere length of a sample. Telomere length measurement by real-time PCR is easier than TRF measurement using Southern hybridization. Since the TRF assay includes the sub-telomere which can vary from 2-6kb, results reported are less accurate than those of the T/S ratio assay. The T/S ratio assay reduces handling time and complexity of procedure with a concomitant increase in throughput when compared to the TRF assay. Although Shen *et al.* (2007) demonstrated considerable variability between batches using the T/S ratio assay; this variability was decreased significantly when samples were assayed in triplicate or quadruplicate. In the current study there were often outliers in triplicate reactions, so quadruplicate reactions may be necessary for the T/S ratio assay.

Cawthon (2002) demonstrated a linear relationship between the TRF and T/S ratio assays. The current study however, did not demonstrate a linear relationship between these assays although, as expected, CML patients had low TRF and T/S ratio values whereas controls had high TRF and T/S ratio values (Figure 3.7). The absence of this linear relationship in the current study is most likely a result of the small number of samples assayed using both assays (n=12); whereas Cawthon (2002) assayed 95 individuals and obtained a correlation coefficient of 0.67.

4.2 Considerations when analysing telomere dynamics in CML

Analysis of telomere dynamics in haematological malignancies is complicated by the ability of normal haematopoietic cells to regulate telomerase expression and activity in response to proliferation, differentiation and activation. For example, naïve T-cells

have longer telomeres than memory T-cells, primitive $CD34^+$ cells and mature myeloid cells; however the rate of telomere shortening in lymphocytes is faster than that of myeloid cells (Kimura *et al.*, 2010). Also, telomere shortening in hematopoietic progenitor cells cannot be completely compensated for by telomerase, irrespective of activity level (Zimmermann *et al.*, 2004). These factors were demonstrated by Campbell *et al.* (2006) who by analysing $CD34^+$ stem cells showed a decrease in telomerase expression with CML progression and not the increase observed in studies analysing bone marrow or peripheral blood (Broccoli *et al.*, 1995; Ohyashiki *et al.*, 1997; Bitisik *et al.*, 2000; Verstovsek *et al.*, 2003).

Tumour populations are phenotypically and genotypically heterogeneous, even when originating from a single clone (Dick, 2008). In haematological malignancies, a hierarchical model is followed which mirrors that of normal haematopoiesis, and is apparent in CML. The t(9;22) translocation is detectable in cells of all stages of myeloid differentiation, with the "tumour mass" consisting of t(9;22) expressing stem, progenitor and differentiated cells. Therefore in CML, will the stage of differentiation play a role in telomere dynamics in malignant cells as it does in normal cells; and if so, can results be skewed by the "malignant mass" comprising different proportions of myeloid lineage cells?

To circumvent the potential problem mentioned above, separation of cells using cluster domain (CD) cell surface markers is required and assumes that their expression mirrors normal counterparts. Caution must be taken; however, in assuming a pure population of cells has been selected from a malignant mass. Long-term culture studies in AML have shown CD-selection of cells to be unreliable in isolation of pure populations and the authors speculate that this phenomenon will be mirrored in other leukeamias and even malignancies which are not of haematological origin (Dick, 2008).

The effect of different treatment regimens on telomere dynamics must be considered. Current treatment regimens include kinase inhibitors which target signalling pathways known to play a role in telomere dynamics (Tauchi *et al.*, 2002). Patients in the

current study were not receiving kinase inhibitors, but this effect will play a role if telomere assays are implemented in routine laboratories.

Finally, recent computational analysis showed that tumours are only minimally tissue-specific, with microarray data showing that cancers fall into one of three expression groups. Each group expresses a distinct transcriptional pattern with all groups upregulating a set of core embryonic development genes (Naxerova *et al.*, 2008). Surprisingly tumours of different tissues were found to fall into the same profile, such as bladder cancers and T-cell lymphomas. Therefore, telomere dynamics in CML may not be disease-specific and may parallel other haematological or even non-haematological malignancies.

Several observations indicate that telomere dynamics is dependent on genetic and physiological characteristics of each individual. These include; (1) heterogeneity of telomere length within and between individuals, inherited and acquired (Londoño-Vallejo *et al.*, 2001); (2) non-telomeric functions of telomerase; (3) the contribution of telomere- and telomerase-associated proteins to telomere maintenance and telomerase activity; (4) variations in the number of cells which are differentiated or proliferating in a given sample (Deville *et al.*, 2006); (5) influence of hTERT and hTR haplotypes on telomere length (Atzmon *et al.*, 2010, Codd *et al.*, 2010).

It is not known if CML is driven by a substantial proportion of malignant cells, by a rare subpopulation or by tumour driving sub-clones derived from an original cancer stem cell (Adams and Strasser, 2008; Jamieson, 2008; Shay and Wright, 2010). Each scenario has a variable number of subpopulations with variable renewal potential, hence differing telomere dynamics. This potential variability could account for the initial decrease in hTERT expression between CML1_67 and CML1_68 upon acceleration from chronic phase. Overall hTERT expression and telomerase activity appeared to correlate inversely. Additionally, myeloid cell precursors and blasts increased from 1.79 to 32.36 x 10^9 cells/l (CML1_68 and CML1_72, respectively), so the increase in telomere length could be a function of a greater proportion of primitive haematopoietic cells with long telomeres in circulation.

Gessener *et al.* (2010) showed a mechanistic link between translocations involving AML1 and HOX genes and telomerase. NUP98/HOXA9 and AML1/EV11 are occasionally observed in accelerated and blast phase of CML and could induce sustained TERT expression. Characterising genetic variation in leukaemic cells, Anderson *et al.* (2011) showed clonal architecture to be dynamic with treatment selecting out subclones which themselves evolve dynamic genetic landscapes. It could be inferred that the leukaemic cells analysed in chronic phase CML are not comparable to those of blast phase. Likewise, cells analysed before and after treatment might be functionally distinct.

Telomere dynamics has been shown to affect response to treatment in CML. Iwama *et al.* (1997) demonstrated that patients with normal telomere lengths at time of diagnosis tended to respond favourably to α-IFN therapy, possibly due to a significantly larger number of normal stem cells. Brassat *et al.* (2011) showed that critically short telomeres in BCR-ABL positive cells induce apoptosis via a p53-dependent signalling pathway. They argued that telomerase inhibition would therefore only be effective in the treatment of CML patients with functional p53. Further studies are required in this regard. Imatinib has become the first line treatment for CML and interestingly, telomerase inhibition enhances the effect of Imatinib (Tauchi *et al.*, 2002; Bakalova *et al.*, 2003). These studies suggest that the telomere dynamics of CML patients contribute to variations in treatment response.

4.3 Telomeres are shortened in CML patients

In agreement with Iwama *et al.* (1997), Ohyashiki *et al.* (1997), Engelhardt *et al.* (2000), Drummond *et al.* (2007) and Samassekou *et al.* (2009), the current study demonstrated that telomeres in CML are on average shorter than normal controls (Figure 3.8). Although gender did not appear to influence telomere length in controls or CML patients, the small sample size does not allow for statistical testing. Possible causes of the observed reduction in telomere length in CML patients are: (1) accelerated telomere shortening due to increased cellular turnover, (2) inhibition of

apoptosis of cells with critical telomere lengths, (3) prevention of telomerase access to telomeres and (4) distribution of hTERT variants.

Telomere length in CML patients was limited to a narrow range irrespective of patient age, whereas normal controls demonstrated heterogeneity with a trend of decreasing telomere length with increasing age. Mechanisms must exist which stabilise telomeres in CML. Rodriguez-Brenes and Peskin (2010) used mathematical models to show that telomere state (protected or unprotected), rather than telomere length, determines whether a cell enters senescence. So, proteins involved in telomere capping may play a significant role in telomere stability in CML. Additionally, telomere stabilisation in the majority of cancer cells and transformed cell lines occurs as a consequence of cell crisis and is the mechanism by which these cells bypass crisis into malignancy (Shay and Wright, 2010).

The standard deviation for T/S ratios in the current study was calculated as 0.41. Taking this value into account, several CML samples (such as CML4_47 and CML11_4) could fall within the lower range of values determined for normal controls. Samassekou et al (2009) made a similar observation where the average telomere length in CML samples was lower than normal controls, but TRF smears often extended into the range shown for normal controls. Since long telomeres were measured on specific chromosome arms, Samassekou et al (2009) speculated that chromosomal differences in epigenetic factors within subtelomere regions maintained or elongated those telomeres, since demethylation of subtelomeric regions facilitates telomere elongation by recombination (Vera *et al.*, 2008). An increase in telomere end protection could also be a factor. The result which must be taken into account, however, is that telomere length in CML samples may fall within the range of normal individuals.

In the case of CML4_51 and CML4_52, what appears to be a sharp increase in telomere length as per the T/S ratio (0.06 and 0.24, respectively), translates to an increase of only 344bp (Table 4.1). Interpreting changes in telomere length using the T/S ratio, therefore, must be made with caution and the ratios should be converted to

kb to assess the actual difference in length. These results suggest that the T/S ratio is sensitive to small changes in telomere length. Furthermore, taking the standard deviation calculated for the assay of 0.14, the difference between CML4_51 and CML4_52 becomes T/S = 0.04. Similarly the increase in T/S ratio of 0.22 between CML11_3 and CML11_4 might be a modest increase of 0.08.

4.4 Telomerase activity in CML

The presence of telomerase in a cell does not necessarily translate to telomere lengthening. For example, telomerase which cannot access a telomere end will not lengthen that telomere; however, when the sample is assayed the TS primer is readably available, yielding a positive result (Drummond *et al.*, 2007). This conundrum has been shown in telomerase-positive CML patients who can display shortening, stabilisation or lengthening of telomeres (Harrington, 2003).

No correlation was found between relative telomerase activity and platelet count, leucocyte count, percentage of peripheral blood blasts or myeloid precursors. Additionally no correlation between telomerase activity and disease stage was found since (1) only 50% (4/8) of samples taken at time of diagnosis have high relative telomerase activity; (2) telomerase activity was high in CML1 accelerated phase samples and the chronic phase sample (3) absent, moderate and high levels of telomerase activity were measured in chronic phase samples (for example, CML2, CML3_60 and CML5_91).

Telomerase activity did not correlate with telomere length in the current study, since similar T/S ratios were measured in samples with high, moderate or undetectable relative telomerase activity. In samples where telomerase was present without lengthening of telomeres being observed, the capping function of telomerase might be at play. Experiments have shown that telomerase can cap telomeres and prevent telomere fusions without net telomere lengthening (Chan and Blackburn, 2002). Xu and Blackburn (2007) have shown that increased hTERT alone can also protect telomere ends without increased telomerase activity.

hTERT expression was detected in several samples which did not have detectable telomerase activity (Table 4.1). Furthermore, there was no correlation between the level of hTERT expression and level of telomerase activity. This observation was also made by Kyo *et al.* (2003) who concluded that telomerase activity is regulated by mechanisms unrelated to hTERT expression. Subsequently, mechanisms such as compartmentalization of telomerase components, telomere end availability and telomerase phosphorylation have been suggested. This is illustrated by samples CML1_67 and CML1_68 displaying high telomerase activity with low hTERT expression, samples CML3_60 and CML3_66 displaying moderate telomerase activity with high hTERT expression and sample CML5_96 which had no detectable telomerase activity in the presence of high hTERT expression.

In CML1, relative hTERT mRNA expression over a 5 month period varied by as much as 3.8-fold, with an increase in hTERT expression in response to short telomeres (CML1_68 to CML1_69) followed by a decrease in hTERT expression when telomeres lengthen (CML1_71 to CML1_72). A lagging effect is observed since telomeres are only extended during S-phase and by a length that does not fully compensate for the number of repeats lost during replication, so telomere extension is only observed following several rounds of division.

Although the number of samplings was relatively small and the same trend was not observed in other patients, the stage of disease must be taken into account.

4.5 Telomerase, hTERT and the proportion of cell types in peripheral blood

The current study is in agreement with Rasoul et al. (2004) and other studies (Ju and Rudolph, 2006) in that telomerase activity does not correlate with clinical laboratory findings. The number or proportion of blast cells present in peripheral blood did not appear to play a role in the level of telomerase activity since six samples with blast cells in the peripheral blood did not have detectable telomerase activity. Also, the level of telomerase activity appeared independent of blast cell numbers since high telomerase activity was detected in samples with peripheral blood blast cell counts as low as 0.2 x 10^9/l (2%) (CML5_91) and as high as 13.7 x 10^9/l (11%) (CML1_72)

(Table D1 and D2). Contrary to Boultwood *et al.* (1999) and Li *et al.* (2000) who only detected telomerase activity in CML patients with blast cells in their peripheral blood, the current study detected moderate telomerase activity in two peripheral blood samples which did not contain blast cells (CML3_60 and CML3_66; Table 3.2). However, all blood cell numbers steadily increased from CML3_56 to CML3_66, so measured telomerase activity could be a result of an increased proportion of cycling cells and not disease progression. Drummond *et al.* (2007) found no significant difference in the level of telomerase activity between $CD34^+$ cells isolated from CML and normal individuals after correcting for the proportion of cycling cells in each sample.

Both lymphocytes and monocytes can significantly increase or decrease telomerase activity upon stimulation by specific antigens and cytokines, without an increase in cell number (Anand *et al.*, 1998; Gabriele *et al.*, 2004; Deville *et al.*, 2006; Hiyama and Hiyama, 2003). The current study cannot validate the influence of cell lineage or cell cycle status since cell sorting according to these two parameters did not fall within the scope of the study. An added control could take the form of normal individuals with acute infections causing an immune response (e.g. common cold), which would control for cell proliferation and differentiation versus malignancy.

The expression of hTERT was detected in 76% (19/25) of samples with primitive haematopoietic cells in peripheral blood (Table D1 and D8). Only CML1 transformed during the study period so no definitive conclusions can be made on the association between hTERT mRNA expression and transformation. Expression of hTERT, however, remained low in samples CML1_67 (CP) and CML1_68 (AP), then increased in samples CML1_69 through CML1_72 (all AP). It could therefore be suggested that the increase in hTERT expression observed in CML1 was a consequence of transformation. This is in agreement with Campbell *et al.* (2006) who observed hTERT expression increase with CML progression.

The expression of hTERT is not limited to primitive haematopoietic cells, as evidenced by six samples in the current study (Table D1 and D8). Campbell *et al.*

(2006) demonstrated that the expression of c-Myc correlated with hTERT expression and Chai *et al.* (2011) showed that BCR-ABL can up-regulate hTERT transcription. Furthermore, p53 mediates telomere-driven growth arrest and hTERT transcription (Melo and Barnes, 2007), so repression of hTERT transcription in CML may be limited to patients with cytogenetic changes in p53. Investigations into the effect of additional cytogenetic changes on telomere biology in CML will be valuable in defining the relationship between transformation to blast crisis and telomere biology. These experiments did not fall within the scope of the current study and were not performed.

4.6 Telomere biology and CML prognosis

Telomere lengthening in CML has been attributed to disease remission, whereas telomere shortening and telomerase activation have been associated with disease progression (Boultwood *et al.*, 1999; Brümmendorf *et al.*, 2000; Verstovsek *et al.*, 2003). In the current study, only CML1 progressed from chronic to accelerated phase, however, high relative telomerase activity was measured in both the chronic phase sample and subsequent accelerated phase samples. A conclusion cannot, however, be drawn from a single patient. Whether the increase in telomerase activity in CML3 preceded disease progression could not be determined as further sampling was stopped due to the introduction of Imatinib trials at the Charlotte Maxeke Johannesburg Hospital. Similarly, it would have been of great interest to determine if telomere lengthening accompanied disease remission following Imatinib introduction.

Two recent studies have added a level of complexity to the use of telomere biology in CML prognosis. Nordfjäll *et al.* (2009) observed that telomere attrition rates vary between individuals (n=959; age range 20 - 100 years) and in a third of individuals, telomeres stabilised or increased in length over the study duration (10 years). Samassekou *et al.* (2009) detected unusually long telomeres (16-25kb) on certain chromosomes in some CML cells despite average telomere shortening – a trait observed in ALT-dependent tumours.

Finally, the recent discovery of telomeric repeat containing RNA (TERRA) and its ability to inhibit telomerase activity adds another level of regulation of telomere dynamics (Redon *et al.*, 2010). The exact mechanisms involved in transcribing telomeres are not known so the possibility exists that various cytogenetic changes may affect TERRA production. It will be interesting to investigate whether TERRA production changes as CML progresses, and whether any such changes are related to specific cytogenetic changes.

4.8 Conclusions

Refinement of assays is usually to achieve greater sensitivity, a decrease in time required to obtain results and a decrease in complexity. The use of real-time PCR in the current study to determine telomere length satisfies these requirements in comparison to Southern hybridisation. With many techniques available for telomere length determination, the decision on which is appropriate for a specific study is made on the basis of whether the exact length, average length or trend in length changes over time are required.

Telomere dynamics adds to the pool of knowledge available on mechanisms involved in CML evolution and progression. The current study points to a complex system governing telomere dynamics in CML. Telomerase activity is not necessarily translated into lengthening of telomeres and hTERT expression is not necessarily indicative of telomerase activity. Differences observed between patients suggest that inherent factors, present prior to and possibly independently of malignancy, may play a significant role. Characterisation of these factors will lead to a better understanding of the dynamics of telomere biology in CML.

APPENDIX A Participant Consent Forms

Telomere Dynamics in Leukaemia - Participant Information Sheet

Hi, my name is Marcel Gil and I work at WITS University on Telomere Dynamics in cancer of the blood (leukaemia).

Telomere Dynamics?
Telomeres are the pieces of DNA we all have that make sure our cells age normally. Telomere Dynamics is the name given to the study of telomeres.

What happens in leukaemia?
Telomeres don't work normally in leukaemia cells and may be making those cells worse. Studying telomeres in leukaemia may help us understand the disease.

So what am I trying to do?
No one knows why telomeres change in leukaemia; I want to get to that answer. I am hoping that my work will help in the understanding of leukaemia, and maybe help in better treatment for the disease.

How do I plan to do that?
I plan to invite volunteers who have leukaemia to enrol in my study, and perform telomere studies on samples of their blood. I will also invite healthy volunteers to enrol, so I can compare their normal telomeres to those of leukaemia patients.

What will be needed of you if you choose to enrol?
If you choose to join this study, 15ml of blood (3 teaspoons) will be collected from you by trained medical personnel of Johannesburg General Hospital. There are no additional appointments or tests.

What if you don't want to be part of the study?
Only volunteers are part of this study, if you don't want to participate you don't have to. There are no negative consequences for you if you do not join the study. If you do join the study, you can withdraw from it any time you want to.

If I do join, who will have information about me?
The study carries a guarantee of confidentiality. You will remain anonymous in any published results and to all people working on the study apart from me.

Who supports my work?
WITS University, National Health Laboratory Service (NHLS), Medical Research Council and the Cancer Association of South Africa (CANSA) support my work.

Thank you for taking the time to read this information sheet, if you wish to participate in this study please complete the consent form on page two.

You may contact me at **011-717-2418** for any questions related to this study.

Telomere Dynamics in Leukaemia

Participant Information Sheet - Consent Form

I (Full name)

……..…….. wish to participate in the study TELOMERE DYNAMICS IN LEUKAEMIA cleared at the UNIVERSITY OF THE WITWATERSRAND under ethics clearance number ……..

I (Full name)

……

give consent for the collection of 15ml peripheral blood by venopuncture from myself as stipulated in the above mentioned study.

……..

Signature of study participant

…….

Witness

APPENDIX B Reagent and Equipment Suppliers

AB GeneAmp2 PCR System 2400	Perkin-Elmer, USA
Acrylamide	Merck, Germany
Agarose, SeaKem LE	FMC, USA
Ammonium Chloride	Sigma, USA
Ammonium Persulphate	Promega, USA
Automatic Developer and Replenisher	Axim, RSA
Automatic Fixer and Replenisher	Axim, RSA
Beckman Φ70 pH meter	Beckman, USA
Beckman DU-65 Spectrophotometer	Beckman, USA
Beckman J2-21 centrifuge	Beckman, USA
Benchtop centrifuge HM2	Hägar designs, RSA
Bis-acrylamide	Merck, Germany
Bromophenol blue	Fluka, Switzerland
4-Chloro-1-naphthol	Sigma, USA
Chloroform	Merck, Germany
Chromato-VUE® transilluminator	UVP inc., USA
Conical centrifuge tubes	Nunc, Denmark
Culture flasks ($25cm^2$ and $70cm^2$)	Nunc, Denmark
100bp DNA Size marker	Promega, USA
1,4 Dithiothreitol (DTT)	Roche, Germany
DIG Detection ELISA (TMB)	Roche, Germany
DIG Easy Hyb	Roche, Germany
DIG Wash and Block buffer set	Roche, Germany
Dimethyl Sulphoxide (DMSO)	Merck, Germany
DNA marker XIV	Roche, Germany
DNA Master SYBR® Green	Roche, Germany
DU-65 Spectrophotometer	Beckman, USA
1,2-Di(2-aminoethoxy)ethane-N,N,N',N'-tetra-acetic acid (EGTA)	Merck, Germany
(Ethylenedinitrilo) tetraacetic acid (EDTA)	Roche, Germany
EDTA-BD vacutainer tubes	BD Biosciences, UK
ELx808 microtitre plate reader	Bio-Tek Instruments,USA
Eppendorf centrifuge 5415R	Eppendorf, Germany
Eppendorf centrifuge 5702R	Eppendorf, Germany
Eppendorf Gradient Mastercycler	Eppendorf, Germany
Eppendorf tubes	Eppendorf, Germany
EPS301 Power Supply	Amersham Biotech, Sweden

Ethanol	Merck, Germany
Ethidium bromide	Sigma, USA
Extra thick filter paper 15x20cm	Bio-Rad, USA
Extran	Merck, Germany
FastStart DNA Master SYBR® Green	Roche, Germany
FastStart Taq DNA Polymerase	Roche, Germany
Ficoll Paque PLUS	Amersham Biotech, Sweden
Filter paper	Whatman Ltd,UK
Filtertips	QSP, USA
First Strand cDNA synthesis kit	Roche, Germany
Foetal calf serum, deactivated	Gibco BRL, Germany
Formaldehyde	Merck, Germany
Gel Doc System	Syngene, England
Gene-Amp tubes	Perkin Elmer, USA
Gene Genius Gel Doc system	Syngene, UK
Glacial acetic acid	Merck, Germany
Glycine	Merck, Germany
Glycerol	Merck, Germany
GraphPad Prism v5.00 for Windows	www.graphpad.com
2-[4-(2-Hydroxyethyl)-1-piprazinyl]-ethansulphoric acid (HEPES)	Merck, Germany
8-hydroxyquinoline	Sigma, USA
*Hinf*I	Roche, Germany
Heating block	Hägar designs, RSA
Heparin-BD vacutainer tubes	BD Biosciences, UK
Hoefer PS500X power supply	Hoefer, USA
Hoefer SE400 vertical gel unit	Hoefer, USA
Hoefer SX250 power supply	Hoefer, USA
Hoefer TE42 transblot chamber	Hoefer, USA
Hydrochloric acid	Merck, Germany
Hydrogen peroxide	Merck, Germany
Improved Neubauer Haemocytometer	Electron Microscopy Sciences, USA
Interleukin-2	Sigma, USA
Isopropanol	Merck, Germany
Jouan BR3.11 Centrifuge	Jouan, France
L-glutamine	Fluka, Switzerland
Lab Disc Magnetic Stirrer	IKA-Werke, Germany

LightCycler 1	Roche, Germany
LightCycler Capillary tubes	Roche, Germany
Lumi-Film Chemiluminescent film	Roche, Germany
2-mercaptoethanol	Merck, Germany
Magnetic stirrer hotplate	Stuart Scientific, UK
Mettler AE163 balance	Mettler, Switzerland
Mettler PJ6000 balance	Mettler, Switzerland
Methanol	Merck, Germany
Mineral oil	Sigma, USA
NBT/BCIP solution	Roche, Germany
Nylon Membrane (+ve charge)	Roche, Germany
oligo-dT_{15} reverse transcription primer	Roche, Germany
PCR Core Kit	Roche, Germany
PCR Master Mix	Promega, USa
Penicillin-Streptomycin Solution	Sigma, USA
pH meter 70	Beckman, USA
Phenol crystals	Holpro Lovasz, RSA
Phytohaemagglutinin-M	Becton Dickinson, USA
Phenylmethylsulfonyl fluoride (PMSF)	Roche, Germany
Polycarbonate centrifuge tubes	Beckman, USA
Polypropylene tubes (50ml)	Nunc, Denmark
Polystyrene tubes (15ml)	Nunc, Denmark
Ponceau S	Sigma, USA
Potassium ferricyanide	Merck, Germany
Restriction Buffer L	Roche, Germany
RNase	Roche, Germany
rRNasin RNase Inhibitor	Promega, USA
RNase-free water	Qiagen, UK
RNeasy® Mini Kit	Qiagen, UK
RPMI-1640	Invitrogen, USA
*Rsa*I	Roche, Germany
Silver nitrate	Merck, Germany
Sodium acetate	Merck, Germany
Sodium bicarbonate	Merck, Germany
Sodium citrate	Merck, Germany
Sodium chloride	Merck, Germany
Sodium dodecyl sulphate	Merck, Germany

Sodium hydroxide	Merck, Germany
Sodium thiosulphate	Merck, Germany
Sorvall RMC 14 centrifuge	Sorvall, USA
Sucrose	Merck, Germany
SYBR® Green Taq ReadyMix™	Sigma, USA
TeloTAGGG PCR Telomerase ELISA^{PLUS}	Roche, Germany
TeloTAGGG Telomere Length Assay	Roche, Germany
TEMED	Promega, USA
Thermal cycler Model 480	Perkin Elmer, USA
Thermal cycler Gradient	Eppendorf, Germany
Thermo-Hybaid Shake ‘n Stack	Hybaid, UK
Thermostar Microtitre plate shaker	BMG, Germany
TRAPeze ELISA kit	Serologicals Corp.,USA
TriPure® Isolation Reagent	Roche, Germany
TRI-Reagent®	Sigma, USA
Tris-base	Roche, Germany
Urea	BRL, USA
Zeiss Axiostar Inverted Microscope	Carl Zeiss, Germany

APPENDIX C Buffers and Solutions

Lymphocyte *in vitro* activation

PBS (1x)

137mM NaCl; 2.7mM KCl; 4.3mM Na_2HPO_4; 1.4mM KH_2PO_4

Made to volume using MilliQ water

The pH of the solution is 7.4 and requires no adjustment

Nucleic Acid Extraction

Phenol solution

100g Crystallised phenol was heated at 65^{o}C to dissolve

0.1% (w/v) 8-hydroxyquinoline was added and the solution was extracted five times using 200ml 0.1M Tris-HCl (pH 8.0) until the aqueous phase reached pH 7.6 – 8.0

TE buffer

10mM Tris-HCl (pH 8.0); 1.0mM EDTA (pH 8.0)

TAE buffer (50x Stock solution)

2M Tris; 57.1ml Glacial Acetic acid; 0.1M EDTA (pH 8.0)

Made to 1l using MilliQ water

The pH of the solution is 8.0 and requires no adjustment

DNA/RNA loading dye

25% (w/v) sucrose; 0.5% (w/v) bromophenol blue

Made to volume using MilliQ water

Telomerase Activity

TBE buffer (10x Stock solution)

0.89M Tris; 0.89M Boric acid; 2ml 0.5M EDTA (pH 8.0)

Made to 1l using MilliQ water

The pH of the solution is 8.3 and requires no adjustment

12% Non-denaturing polyacrylamide gel

16ml 30% Acrylamide was gently mixed with 4ml 1% Bis-acrylamide, 4ml 10xTBE and 15.7ml MilliQ water. While swirling, 300µl 10% ammonium persulphate (freshly prepared) and 20µl TEMED were added and the solution poured into the casting chamber for a 15cm vertical gel (Hoefer SE400).

Telomere Length Assays

20x SSC

3.0 M NaCl; 0.3 M Na-citrate, pH 7-8 (pH not adjusted)

Made to volume using MilliQ water

APPENDIX D Data and Calculations

Table D.1 Blood cell count, treatment and disease phase of CML patients

	Units	Normal	CML1					
			Months from Diagnosis					
			67	68	69	70	71	72
White Cells	**x 10^9/ l**	**4 - 10**	37.0	7.1	11.4	16.8	16.6	116.2
Platelets	**x 10^9/ l**	**178 - 400**	236	272	315	288	311	166
Neutrophils	**x 10^9/ l**	**2.0 - 7.5**	17.7	2.3	3.3	5.5	5.2	46.0
Monocytes	**x 10^9/ l**	**0.18 - 0.8**	4.6	0.7	0.9	1.4	2.8	19.9
Lymphocytes	**x 10^9/ l**	**1.0 - 4.0**	0.8	1.2	1.3	1.9	2.3	13.7
Eosinophils	**x 10^9/ l**	**0.0 – 0.5**	0.4	0.06	0	0.2	0.2	0.8
Basophils	**x 10^9/ l**	**0.0 – 0.2**	5.39	0.8	1.49	2.05	1.33	12.44
Myeloid Precursors[1]	**x 10^9/ l**	**0**	7.7	0.79	2.64	3.76	2.50	18.66
Blasts	**x 10^9/ l**	**0**	2.3	1.0	1.8	2.6	1.2	13.7
	%	**0**	6	14	16	15	7	11
Treatment at time of sampling[2]			INF	INF	INF	INF	INF	HU, VIN
Phase Classification[3]			CP	AP	AP	AP	AP[4]	AP

[1] Includes metamyelocytes, myelocytes and promyelocytes

[2] INF = interferon-α; HU = Hydroxyurea; VIN = Vinblastine

[3] Chronic Phase (CP) if blasts < 10%; Accelerated Phase (AP) if blasts 10% - 19%

[4] Although blasts dropped below 10%, relevant clinicians did not classify sample CML1_71 as being indicative of remission.

Table D.1 Blood cell count, treatment and disease phase of CML patients (continued)

	Units	Normal	CML2			CML3		
			Months from Diagnosis			Months from Diagnosis		
			5	9	17	56	60	66
White Cells	**x 10^9/ l**	**4 - 10**	5	7	8.6	4.8	8.0	75.69
Platelets	**x 10^9/ l**	**178 - 400**	158	184	211	589	802	1212
Neutrophils	**x 10^9/ l**	**2.0 - 7.5**	2.43	3.43	5.35	2.0	4.98	49.2
Monocytes	**x 10^9/ l**	**0.18 - 0.8**	0.8	1.23	0.89	0.31	0.38	4.54
Lymphocytes	**x 10^9/ l**	**1.0 - 4.0**	1.67	2.18	2.07	2.33	2.54	3.78
Eosinophils	**x 10^9/ l**	**0.0 – 0.5**	0.03	0.1	3	0.03	0.1	3.03
Basophils	**x 10^9/ l**	**0.0 – 0.2**	0.04	0.1	0.15	0.13	0.01	1.51
Myeloid Precursors[1]	**x 10^9/ l**	**0**	0	0	0	0	0	12.86
Blasts	**x 10^9/ l**	**0**	0	0	0	0	0	0
	%	**0**	0	0	0	0	0	0
Treatment at time of sampling[2]			INF	INF	HU	HU	HU	HU
Phase Classification[3]			CP	CP	CP	CP	CP	CP

[1] Includes metamyelocytes, myelocytes and promyelocytes

[2] INF = interferon-α; HU = Hydroxyurea

[3] Chronic Phase (CP) if blasts < 10% ; Accelerated Phase (AP) if blasts 10% - 19%

Table D.1 Blood cell count, treatment and disease phase of CML patients (continued)

	Units	Normal	CML4								
			Months from Diagnosis								
			43	45	46	47	48	49	50	51	52
White Cells	**x 10^9/ l**	**4 - 10**	5.5	6.4	48.4	9.2	7.4	7.5	7.1	3.5	1.9
Platelets	**x 10^9/ l**	**178 - 400**	179	202	218	203	297	397	147	136	42
Neutrophils	**x 10^9/ l**	**2.0 - 7.5**	3.3	3.7	35.8	5.7	4.2	4.0	4.8	2.0	1.03
Monocytes	**x 10^9/ l**	**0.18 - 0.8**	0.1	0.6	3.9	0.8	0.5	1.0	0.4	0.2	0.15
Lymphocytes	**x 10^9/ l**	**1.0 - 4.0**	1.9	2.0	5.8	2.5	2.4	2.2	1.8	1.3	0.49
Eosinophils	**x 10^9/ l**	**0.0 – 0.5**	0.1	0	0.2	0.2	0.1	0	0.1	0	0
Basophils	**x 10^9/ l**	**0.0 – 0.2**	0.1	0.1	0.3	0.1	0.3	0.2	0.1	0	0
Myeloid Precursors[1]	**x 10^9/ l**	**0**	0	0	3.9	0	0	0	0	0	0.1
Blasts	**x 10^9/ l**	**0**	0	0	0	0	0	0	0	0	0.13
	%	**0**									7
Treatment at time of sampling[2]			HU	HU	HU	HU	HU	HU	HU	HU	HU
Phase Classification[3]			CP	CP	CP	CP	CP	CP	CP	CP	CP

[1] Includes metamyelocytes, myelocytes and promyelocytes

[2] HU = Hydroxyurea

[3] Chronic Phase (CP) if blasts < 10% ; Accelerated Phase (AP) if blasts 10% - 19%

Table D.1 Blood cell count, treatment and disease phase of CML patients (continued)

	Units	Normal	CML5						
			Months from Diagnosis						
			80	83	85	86	90	91	96
White Cells	**x 10^9/ l**	**4 - 10**	33.4	6.6	24.1	11.5	2.5	10.6	8.3
Platelets	**x 10^9/ l**	**178 - 400**	780	579	430	1019	548	380	1499
Neutrophils	**x 10^9/ l**	**2.0 - 7.5**	23.8	1.5	16.1	1.8	1.1	2.9	0.8
Monocytes	**x 10^9/ l**	**0.18 - 0.8**	3.3	0.5	2.3	1.5	0.2	2.9	0.3
Lymphocytes	**x 10^9/ l**	**1.0 - 4.0**	2.3	1.9	2.6	2.4	0.8	1.7	1.7
Eosinophils	**x 10^9/ l**	**0.0 – 0.5**	0.6	0.3	0.8	0.2	0.1	0.3	0.3
Basophils	**x 10^9/ l**	**0.0 – 0.2**	3.4	2.2	2.4	4.5	0.3	2.7	5.2
Myeloid Precursors[1]	**x 10^9/ l**	**0**	0	0.1	0	0.6	0	0	0
Blasts	**x 10^9/ l**	**0**	0	0	0	0.2	0	0.2	0.2
	%	**0**				2		2	2
Treatment at time of sampling[2]			INF	INF	INF	INF	INF	INF	INF
Phase Classification[3]			CP	CP	CP	CP	CP	CP	CP

[1] Includes metamyelocytes, myelocytes and promyelocytes

[2] INF = interferon-α

[3] Chronic Phase (CP) if blasts < 10% ; Accelerated Phase (AP) if blasts 10% - 19%

Table D.1 Blood cell count, treatment and disease phase of CML patients (continued)

	Units	Normal	CML6				CML7	CML8	CML9
			Months from Diagnosis				Months from Diagnosis	Months from Diagnosis	Months from Diagnosis
			34	37	38	39	49	27	51
White Cells	**x 10^9/l**	**4 - 10**	16.1	2.3	5.7	3.9	3.9	4.2	8.0
Platelets	**x 10^9/l**	**178 - 400**	112	164	80	39	215	210	209
Neutrophils	**x 10^9/l**	**2.0 - 7.5**	12.1	0.7	3.6	2.1	2.0	2.1	5.7
Monocytes	**x 10^9/l**	**0.18 - 0.8**	1.6	0.7	0.5	0.5	0.8	0.4	0.6
Lymphocytes	**x 10^9/l**	**1.0 - 4.0**	1.5	0.8	1.6	1.3	1.0	1.6	1.8
Eosinophils	**x 10^9/l**	**0.0 – 0.5**	0	0	0.1	0.1	0.1	0.2	0
Basophils	**x 10^9/l**	**0.0 – 0.2**	0	0	0	0	0	0	0
Myeloid Precursors[1]	**x 10^9/l**	**0**	1.0	0.1	0	0	0	0	0
Blasts	**x 10^9/l**	**0**	0	0	0	0	0	0	0
	%	**0**							
Treatment at time of sampling[2]			HU	HU	HU, INF	HU, INF	HU	HU, INF	NONE
Phase Classification[3]			CP	CP	CP	CP	CP	CP	CP

[1] Includes metamyelocytes, myelocytes and promyelocytes

[2] INF = interferon-α; HU = Hydroxyurea; NONE = No treatment at time of sampling

[3] Chronic Phase (CP) if blasts < 10% ; Accelerated Phase (AP) if blasts 10% - 19%

Table D.1 Blood cell count, treatment and disease phase of CML patients (continued)

	Units	Normal	CML10	CML11		CML12	CML13	CML14
			Diagnosis	Months from Diagnosis		Diagnosis	Diagnosis	Diagnosis
				3	4			
White Cells	**x 10^9/l**	**4 - 10**	69.2	123.0	12.6	413.7	119.6	60.6
Platelets	**x 10^9/l**	**178 - 400**	915	353	418	350	55	714
Neutrophils	**x 10^9/l**	**2.0 - 7.5**	44.3	68.4	8.4	281.3	87.3	47.8
Monocytes	**x 10^9/l**	**0.18 - 0.8**	7.6	17.2	1.6	0	11.9	3.0
Lymphocytes	**x 10^9/l**	**1.0 - 4.0**	4.8	17.2	1.9	4.1	9.5	1.8
Eosinophils	**x 10^9/l**	**0.0 – 0.5**	3.5	0	0	0	0	0
Basophils	**x 10^9/l**	**0.0 – 0.2**	0.7	6.2	0.6	8.28	2.39	1.82
Myeloid Precursors[1]	**x 10^9/l**	**0**	7.6	13.5	0	101.72	7.17	5.46
Blasts	**x 10^9/l**	**0**	0.7	0.5	0	8.28	1.2	0.61
	%	**0**	1	0.4		2.0	1.0	1.0
Treatment at time of sampling[2]			None	HU	INF	None	None	None
Phase Classification[3]			CP	CP	CP	CP	CP	CP

[1] Includes metamyelocytes, myelocytes and promyelocytes

[2] INF = interferon-α; HU = Hydroxyurea; NONE = No treatment at time of sampling

[3] Chronic Phase (CP) if blasts < 10% ; Accelerated Phase (AP) if blasts 10% - 19%

Table D.1 Blood cell count, treatment and disease phase of CML patients (continued)

	Units	Normal	CML15	CML16	CML17	CML18
			Diagnosis	Diagnosis	Diagnosis	Diagnosis
White Cells	**x 10^9/ l**	**4 - 10**	35.3	1.8	146.5	105.6
Platelets	**x 10^9/ l**	**178 - 400**	242	145	490	455
Neutrophils	**x 10^9/ l**	**2.0 - 7.5**	28.9	0.46	112.8	98.24
Monocytes	**x 10^9/ l**	**0.18 - 0.8**	2.05	0.12	2.93	0[4]
Lymphocytes	**x 10^9/ l**	**1.0 - 4.0**	3.11	1.2	8.79	2.11
Eosinophils	**x 10^9/ l**	**0.0 – 0.5**	1.16	0.01	1.46	0
Basophils	**x 10^9/ l**	**0.0 – 0.2**	0	0.01	1.46	1.06
Myeloid Precursors[1]	**x 10^9/ l**	**0**	0	0	2.85	1.06
Blasts	**x 10^9/ l**	**0**	0	0	13.18	0
	%	**0**			9	
Treatment at time of sampling[2]			None	None	None	None
Phase Classification[3]			CP	CP	CP	CP

[1] Includes metamyelocytes, myelocytes and promyelocytes

[2] INF = interferon-α; HU = Hydroxyurea; None = No treatment at time of sampling

[3] Chronic Phase (CP) if blasts < 10% ; Accelerated Phase (AP) if blasts 10% - 19%

[4] Result was checked in duplicate

Table D.2 Telomerase activity of CML patients and controls

Sample	A_{450}	A_{690}	$\Delta A_{sample} = (A_{450} - A_{690})$	$\Delta A_{negative} = (A_{450} - A_{690})$	$\Delta As - \Delta An$[1]	Relative Telomerase Activity (RTA)[2]	Blasts (%)[5]	CML Phase[6]
HL-60	3.542	0.037	3.505	0.038	3.467	High[4]		
Stimulated lymphocytes	0.267	0.035	0.232	0.050	0.182	Moderate[4]		
Unstimulated lymphocytes	0.067	0.038	0.029	0.019	0.010	Absent[4]		
C01	0.067	0.036	0.031	0.019	0.012	Absent[4]		
C02	0.063	0.034	0.029	0.019	0.010	Absent[4]		
C03	0.096	0.044	0.052	0.019	0.033	Absent[4]		
C04	0.056	0.038	0.018	0.019	-0.001	Absent[4]		
C05	0.132	0.036	0.096	0.019	0.077	Absent[4]		
C06	0.052	0.034	0.018	0.019	0.000	Absent[4]		
C07	0.061	0.036	0.025	0.019	0.006	Absent[4]		
C08	0.056	0.034	0.022	0.019	0.003	Absent[4]		
C09	0.086	0.034	0.052	0.035	0.017	Absent[4]		
C10	0.091	0.035	0.056	0.035	0.021	Absent[4]		
CML1_67	1.646	0.037	1.609	0.039	1.570	High[4]	6	CP
CML1_68	1.359	0.033	1.326	0.033	1.293	High[4]	14	AP
CML1_69	0.753	0.033	0.720	0.033	0.687	High[4]	16	AP
CML1_70	0.850	0.035	0.815	0.032	0.783	High[4]	15	AP
CML1_71	0.816	0.034	0.782	0.032	0.750	High[4]	7	AP
CML1_72	1.201	0.042	1.159	0.037	1.122	High[4]	11	AP
CML2_5	0.062	0.032	0.030	0.035	0.030	Absent[3]	0	CP
CML2_9	0.082	0.040	0.042	0.032	0.010	Absent[3]	0	CP
CML2_17	0.068	0.033	0.035	0.035	0.000	Absent[3]	0	CP
CML3_56	0.080	0.036	0.044	0.035	0.009	Absent[3]	0	CP
CML3_60	0.144	0.032	0.112	0.032	0.080	Moderate[3]	0	CP
CML3_66	0.187	0.033	0.154	0.033	0.121	Moderate[3]	0	CP

[1] For simplicity, absorbance values for the internal standard reactions are not given. Also, the value given for each sample is the average of duplicates.

[2] Since the threshold value for telomerase activity varies between the TeloTAGGG Telomerase PCR ELISA^{PLUS} kit and the TRAPeze ELISA kit (Equations 4a and 4b, Section 2.6); results were expressed as a standardised "Relative Telomerase Activity" (RTA). RTA is defined as absent if below the threshold for the respective assay, moderate if up to 10x the negative control for that sample and high if greater than 10x.

[3] Telomerase activity assayed using the TeloTAGGG Telomerase PCR ELISA^{PLUS} kit (Activity threshold = 2 x negative control).

[4] Telomerase activity assayed using the TRAPeze ELISA kit (Activity threshold = 0.150).

[5] Percentage blasts in peripheral blood.

[6] CP = Chronic Phase; AP = Accelerated Phase

Table D.2 Telomerase activity of CML patients and controls (continued)

Sample	A_{450}	A_{690}	$\Delta A_{sample} = (A_{450} - A_{690})$	$\Delta A_{negative} = (A_{450} - A_{690})$	$\Delta As - \Delta An$[1]	Relative Telomerase Activity (RTA)[2]	Blasts (%)[4]	CML Phase[5]
CML4_43	0.075	0.040	0.035	0.032	0.003	Absent[3]	0	CP
CML4_45	0.067	0.034	0.033	0.033	0.000	Absent[3]	0	CP
CML4_46	0.075	0.033	0.042	0.033	0.009	Absent[3]	0	CP
CML4_47	0.077	0.037	0.040	0.037	0.003	Absent[3]	0	CP
CML4_48	0.085	0.036	0.049	0.037	0.012	Absent[3]	0	CP
CML4_49	0.077	0.034	0.043	0.037	0.006	Absent[3]	0	CP
CML4_50	0.070	0.034	0.036	0.037	-0.001	Absent[3]	0	CP
CML4_51	0.067	0.031	0.036	0.035	0.001	Absent[3]	0	CP
CML4_52	0.070	0.034	0.036	0.035	0.001	Absent[3]	7	CP
CML5_80	0.068	0.034	0.034	0.035	-0.001	Absent[3]	0	CP
CML5_83	0.063	0.031	0.032	0.035	-0.003	Absent[3]	0	CP
CML5_85	0.076	0.035	0.041	0.035	0.006	Absent[3]	0	CP
CML5_86	0.064	0.032	0.032	0.035	-0.003	Absent[3]	2	CP
CML5_90	0.066	0.034	0.032	0.035	-0.003	Absent[3]	0	CP
CML5_91	0.574	0.033	0.541	0.035	0.506	High[3]	2	CP
CML5_96	0.092	0.031	0.061	0.032	0.029	Absent[3]	2	CP
CML6_34	0.067	0.034	0.033	0.032	0.001	Absent[3]	0	CP
CML6_37	0.072	0.032	0.032	0.032	0.000	Absent[3]	0	CP
CML6_38	No result[6]						0	CP
CML6_39	No result[6]						0	CP
CML7_49	0.066	0.031	0.035	0.032	0.003	Absent[3]	0	CP
CML8_27	0.064	0.030	0.034	0.032	0.002	Absent[3]	0	CP
CML9_51	0.103	0.031	0.072	0.032	0.040	Absent[3]	0	CP
CML10_0	0.065	0.032	0.033	0.032	0.001	Absent[3]	1	CP
CML11_3	0.053	0.031	0.022	0.032	-0.010	Absent[3]	0.4	CP
CML11_4	0.058	0.032	0.026	0.033	-0.007	Absent[3]	0	CP

[1] For simplicity, absorbance values for the internal standard reactions are not given. Also, the value given for each sample is the average of duplicates.

[2] Since the threshold value for telomerase activity varies between the TeloTAGGG Telomerase PCR ELISA^{PLUS} and the TRAPeze ELISA (Equations 4a and 4b, Section 2.6.); results were expressed as a "Relative telomerase activity" (RTA). RTA is defined as absent if below the threshold for the respective assay, moderate if up to 10x the negative control for that sample and high if greater than 10x.

[3] Telomerase activity assayed using the TeloTAGGG Telomerase PCR ELISAPLUS kit (Activity threshold = 2 x negative control).

[4] Percentage blasts in peripheral blood.

[5] CP = Chronic Phase; AP = Accelerated Phase.

[6] No result reported since the internal control failed for the original sample assay and when the assay was repeated.

Table D.2 Telomerase activity of CML patients and controls (continued)

Sample	A_{450}	A_{690}	$\Delta A_{sample} = (A_{450} - A_{690})$	$\Delta A_{negative} = (A_{450} - A_{690})$	$\Delta As - \Delta An$[1]	Relative Telomerase Activity (RTA)[2]	Blasts (%)[4]	CML Phase[5]
CML12_0	1.958	0.036	1.922	0.038	1.884	High[3]	2	CP
CML13_0	1.085	0.035	1.050	0.044	1.006	High[3]	1	CP
CML14_0	0.837	0.033	0.804	0.043	0.761	High[3]	1	CP
CML15_0	0.969	0.043	0.922	0.024	0.898	High[3]	0	CP
CML16_0	0.186	0.028	0.156	0.084	0.072	Absent[3]	0	CP
CML17_0	0.110	0.028	0.073	0.057	0.016	Absent[3]	9	CP
CML18_0	0.087	0.030	0.054	0.056	-0.002	Absent[3]	0	CP

[1] For simplicity, absorbance values for the internal standard reactions are not given. Also, the value given for each sample is the average of duplicates.

[2] Since the threshold value for telomerase activity varies between the TeloTAGGG Telomerase PCR ELISA^{PLUS} and the TRAPeze ELISA (Equations 4a and 4b, Section 2.6); results were expressed as a "Relative telomerase activity" (RTA). RTA is defined as absent if below the threshold for the respective assay, moderate if up to 10x the negative control for that sample and high if greater than 10x.

[3] Telomerase activity assayed using the TRAPeze ELISA kit (Activity threshold = 0.150).

[4] Percentage blasts in peripheral blood.

[5] CP = Chronic Phase; AP = Accelerated Phase.

Table D.3 Densitometric analysis results for the TRF assay

Sample	Sum of the optical densities[1]	Sum of the optical density to molecular size ratio[2]	TRF Length (kb)	Age (Years)
C31	2887.618	389.982	7.40	30
C32	5431.880	717.327	7.57	39
C33	2839.682	401.181	7.08	26
C34	8509.205	1120.144	7.60	24
C35	4151.336	604.650	6.87	40
C36	3953.451	521.087	7.59	24
C37	5862.693	794.110	7.38	26
C38	7968.608	1036.279	7.69	26
CML2_17	1209.224	487.525	2.48	30
CML3_56	514.735	159.806	3.22	34
CML3_60	774.567	224.242	3.45	34
CML3_66	799.959	233.724	3.42	34

[1] TRF smears are divided into 100 points and the optical density for each point is determined. For simplicity, only the sum of the values for each sample smear is given (Figure 2.1, Section 2.7.1).

[2] The optical density of a point along the smear is divided by the molecular size for that point (Equation 5, Section 2.7.1).

Table D.4 Cycles to threshold for real-time telomere length PCR standard curve

DNA (ng)[1]	Log DNA Amount	Telomere Repeats (C_t)	Single Copy Gene, 36B4 (C_t)
100	2.0	11.84	18.86
		11.88	18.71
		11.97	18.98
59.2	1.77	12.72	19.54
		12.69	19.55
		12.77	19.62
35.4	1.55	13.57	20.62
		13.40	20.54
		13.50	20.49
21.1	1.32	13.86	20.93
		13.99	20.94
		13.94	20.83
12.6	1.10	14.86	22.31
		14.89	22.32
		14.95	22.27

[1] A 1.6-fold dilution series was used as per Cawthon RM (2002)

Table D.5 Cycles to threshold for real-time telomere length PCR replicates

Tube Number	Telomere Repeats $[C_{t(telomere)}]^1$	Single-copy Gene, 36B4 $[C_{t(36B4)}]^1$	T/S Ratio
1	12.91	20.54	0.98
2	13.13	20.61	0.88
3	13.23	20.74	0.89
4	13.48	20.79	0.77
5	13.41	19.85	0.45
6	13.47	20.67	0.72
7	13.39	20.78	0.81
8	13.43	20.80	0.8
9	13.39	20.73	0.79
10	13.35	20.93	0.92
11	13.46	20.91	0.84
12	13.44	20.80	0.8
13	13.45	21.36	1.13
14	13.46	20.91	0.84
15	13.41	20.91	0.87
16	13.43	20.90	0.85
17	13.47	21.30	1.07
Mean	13.37	20.80	0.85
SD	0.15	0.32	0.15

[1] Initial amount of control DNA used in replicates was 21.1ng

Table D.6 Cycles to threshold of a 50ng DNA calibrator for real-time telomere length PCR experiments.

Experiment	Telomere Repeats $[C_{t(telomere)}]$	Single-copy Gene, 36B4 $[C_{t(36B4)}]$	T/S Ratio
1	12.58	21.77	1.31
	12.71	21.82	1.38
	11.41	21.89	0.54
2	11.85	21.77	0.79
	11.74	21.81	0.71
	11.79	21.67	0.81
3	12.73	22.75	0.74
	12.84	22.83	0.76
	13.07	22.84	0.88
4	12.08	22.18	0.70
	12.04	21.97	0.78
	11.97	22.09	0.69
5	12.87	23.58	0.46
	12.83	23.56	0.46
	12.50	23.56	0.36
6	12.88	21.42	2.04
	12.78	21.91	1.37
	12.79	22.41	1.01
Mean	12.41	22.23	0.88
SD	0.50	0.70	0.41

Table D.7 Determination of T/S Ratios of control and CML samples

Sample	Telomere PCR		36B4 PCR		$\Delta C_{t\,(sample)}$ [1]	$\Delta\Delta C_t$ [2]	T/S Ratio [3]	Age/Sex
	C_t [1]	$C_{t\,(telomere)}$	C_t [1]	$C_{t\,(36B4)}$				
C01	11.01	10.95	19.58	19.60	-8.65	-1.55	2.94	25 Male
	10.99		19.59					
	10.86		19.62					
C02	11.46	11.48	18.48	18.49	-7.01	-0.96	1.96	43 Male
	11.44		18.52					
	11.53		18.46					
C03	11.12	11.17	18.99	18.95	-7.78	-1.22	2.32	25 Male
	11.16		18.95					
	11.24		18.91					
C04	11.26	11.23	18.88	18.75	-7.52	-1.14	2.22	38 Female
	11.33		18.76					
	11.08		18.62					
C05	11.24	11.26	18.94	18.93	-7.67	-1.20	2.27	24 Female
	11.28		18.92					
	11.26		18.92					
C06	11.85	11.88	18.90	19.33	-7.45	-1.18	2.27	24 Female
	11.95		19.66					
	11.84		19.44					
C07	11.19	11.22	19.14	18.99	-7.77	-1.22	2.32	25 Male
	11.29		18.98					
	11.19		18.84					
C08	11.23	11.23	18.39	18.42	-7.19	-0.97	1.96	43 Male
	11.24		18.42					
	11.22		18.45					
C09	9.58	9.64	20.50	20.50	-10.86	0.70	0.62	38 Female
	9.53		20.48					
	9.81		20.52					
C10	9.18	9.11	20.14	20.20	-11.09	0.73	0.60	29 Male
	9.21		20.23					
	8.95		20.23					

[1] C_t = Cycles to threshold ; $\Delta C_{t\,(sample)} = C_{t\,(telomere)} - C_{t\,(36B4)}$

[2] $\Delta\Delta C_t = \Delta C_{t\,(sample)} - \Delta C_{t\,(standard)}$, where $\Delta C_{t\,(standard)}$ is calculated from the standard curve for 50ng DNA.

[3] T/S Ratio = $2^{-\Delta\Delta Ct}$

Table D.7 Determination of T/S Ratios of control and CML samples

Sample	Telomere PCR		36B4 PCR		$\Delta C_{t\,(sample)}$ [1]	$\Delta\Delta C_t$ [2]	T/S Ratio [3]	Age
	C_t [1]	$C_{t\,(telomere)}$	C_t [1]	$C_{t\,(36B4)}$				
C11	9.70	9.86	19.28	19.41	-9.55	1.22	0.43	44 Female
	9.92		--[4]					
	9.95		19.55					
C12	9.16	9.39	18.98	18.97	-9.58	1.27	0.41	44 Female
	9.80		19.01					
	9.20		18.94					
C13	10.27	10.17	20.04	20.03	-9.86	1.02	0.49	53 Female
	10.42		20.07					
	9.82		19.98					
C14	9.90	9.89	18.99	18.93	-9.04	1.28	0.41	55 Male
	9.95		--[4]					
	9.83		18.88					
C15	8.60	8.61	19.00	18.96	-10.35	1.17	0.44	27 Male
	8.67		18.94					
	8.57		18.94					
C16	9.34	9.37	20.31	20.13	-10.76	0.85	0.55	32 Female
	9.38		19.86					
	9.41		20.21					
C17	10.07	10.02	21.33	21.41	-11.39	0.17	0.88	32 Male
	9.93		21.29					
	10.04		21.60					
C18	9.11	9.10	20.94	20.98	-11.88	0.17	0.88	40 Male
	9.11		21.00					
	9.07		21.01					
C19	9.18	9.25	21.67	21.60	-12.35	-0.07	1.05	30 Female
	9.26		21.52					
	9.33		21.61					
C20	9.83	9.85	21.80	21.77	-11.92	-0.02	1.01	21
	9.88		21.72					
	9.83		21.79					

[1] C_t = Cycles to threshold ; $\Delta C_{t\,(sample)} = C_{t\,(telomere)} - C_{t\,(36B4)}$

[2] $\Delta\Delta C_t = \Delta C_{t\,(sample)} - \Delta C_{t\,(standard)}$, where $\Delta C_{t\,(standard)}$ is calculated from the standard curve for 50ng DNA.

[3] T/S Ratio = $2^{-\Delta\Delta Ct}$

[4] Failed PCR

Table D.7 Determination of T/S Ratios of control and CML samples

Sample	Telomere PCR		36B4 PCR		$\Delta C_{t\,(sample)}$ [1]	$\Delta\Delta C_t$ [2]	T/S Ratio [3]	Age
	C_t [1]	$C_{t\,(telomere)}$	C_t [1]	$C_{t\,(36B4)}$				
C21	9.93	9.92	20.34	20.50	-10.58	0.87	0.54	26 Male
	9.89		20.61					
	9.94		20.56					
C22	9.14	9.09	19.63	19.61	-10.52	1.11	0.90	26 Male
	8.94		19.60					
	9.14		19.60					
C23	8.83	8.78	19.90	19.93	-11.15	0.93	0.52	48 Female
	8.75		19.94					
	8.75		19.94					
C24	8.64	8.62	21.12	20.15	-11.53	0.79	0.57	47 Male
	8.56		20.20					
	8.62		20.14					
C25	9.79	9.61	21.06	21.04	-11.43	0.56	0.68	30 Female
	9.28		21.05					
	9.75		21.03					
C26	9.11	9.10	19.27	19.23	-10.13	1.28	0.41	30 Female
	9.10		19.24					
	9.10		19.17					
C27	10.28	10.30	19.81	19.88	-9.58	0.78	0.58	51 Female
	10.31		19.86					
	10.30		19.97					
C28	10.92	10.89	20.95	21.00	-10.11	0.39	0.76	49 Male
	10.91		20.99					
	10.84		21.06					
C29	11.01	11.02	20.42	20.45	-9.43	0.63	0.66	53 Male
	11.05		20.40					
	11.00		20.53					
C30	10.48	10.62	19.44	19.55	-8.93	1.03	0.49	55 Female
	10.68		19.39					
	10.70		19.82					

[1] C_t = Cycles to threshold ; $\Delta C_{t\,(sample)} = C_{t\,(telomere)} - C_{t\,(36B4)}$

[2] $\Delta\Delta C_t = \Delta C_{t\,(sample)} - \Delta C_{t\,(standard)}$, where $\Delta C_{t\,(standard)}$ is calculated from the standard curve for 50ng DNA.

[3] T/S Ratio = $2^{-\Delta\Delta Ct}$

Table D.7 Determination of T/S Ratios of control and CML samples

Sample	Telomere PCR		36B4 PCR		$\Delta C_{t\ (sample)}$ [1]	$\Delta\Delta C_t$ [2]	T/S Ratio [3]	Age
	C_t [2]	$C_{t\ (telomere)}$	C_t [2]	$C_{t\ (36B4)}$				
C31	12.70	12.72	24.00	23.97	-11.3	-0.90	1.85	30 Female
	12.67		23.93					
	12.79		23.98					
C32	12.80	12.81	24.17	24.3	-11.37	-1.00	2.00	39 Male
	12.79		24.24					
	12.85		24.48					
C33	12.78	12.81	23.79	23.62	-11.01	-0.66	1.58	26 Male
	12.80		23.41					
	12.85		23.65					
C34	11.65	11.66	22.62	22.72	-10.97	-0.55	1.47	24 Male
	11.63		22.63					
	11.70		22.91					
C35	11.89	12.06	22.62	22.65	-10.73	-0.46	1.36	40 Male
	12.23		22.71					
	12.07		22.63					
C36	11.55	11.72	22.44	22.73	-10.89	-0.51	1.42	24 Female
	11.79		22.95					
	11.80		22.81					
C37	11.46	11.46	22.40	22.59	-10.94	-0.51	1.42	26 Female
	11.42		22.67					
	11.50		22.71					
C38	12.76	12.63	23.4	23.77	-10.64	-0.77	1.69	26 Male
	12.73		23.96					
	12.42		23.94					

[1] C_t = Cycles to threshold ; $\Delta C_{t\ (sample)} = C_{t\ (telomere)} - C_{t\ (36B4)}$

[2] $\Delta\Delta C_t = \Delta C_{t\ (sample)} - \Delta C_{t\ (standard)}$, where $\Delta C_{t\ (standard)}$ is calculated from the standard curve for 50ng DNA.

[3] T/S Ratio = $2^{-\Delta\Delta Ct}$

Table D.7 Determination of T/S Ratios of CML and control samples (continued)

Sample	Telomere PCR		36B4 PCR		$\Delta C_{t\,(sample)}$ [1]	$\Delta\Delta C_t$ [2]	T/S Ratio [3]	Blasts in peripheral blood (%)	Age
	C_t [1]	$C_{t\,(telomere)}$	C_t [1]	$C_{t\,(36B4)}$					
CML1_67	13.78[4] 12.76 12.57	12.67	22.78 22.64 22.64	22.64	-9.97	1.75	0.29	6	53
CML1_68	11.11 11.28 11.13	11.17	22.68 21.42 21.90	22.00	-10.83	4.84	0.04	14	
CML1_69	9.88 9.86 9.72	9.82	18.85 18.36 18.93	18.71	-8.89	3.92	0.07	16	
CML1_70	11.54 12.03 11.84	11.8	21.96 22.87 22.66	22.50	-10.70	4.68	0.04	15	
CML1_71	13.58 13.05 13.13	13.25	21.29 21.18 21.66	21.38	-8.13	2.90	0.13	7	
CML1_72	13.95 13.88 13.99	13.94	22.48 22.33 22.68	22.50	-8.56	2.10	0.23	11	
CML2_5	14.23 14.22 12.64[4]	14.22	20.17 19.69 19.56	19.93	-5.71	3.57	0.08	0	35
CML2_9	12.22 12.40 12.45	12.36	22.14 22.18 22.37	22.23	-9.87	2.89	0.13	0	
CML2_17	8.78 8.84 8.80	8.81	17.68 17.64 17.59	17.64	-8.833	4.18	0.05	0	
CML3_56	11.86 12.19 12.46	12.17	20.42 20.35 20.28	20.35	-8.18	3.65	0.08	0	52
CML3_60	11.50 11.56 11.64	11.57	19.42 19.46 19.46	19.45	-7.88	3.94	0.07	0	
CML3_66	11.56 11.74 11.56	11.62	19.96 19.79 19.94	19.9	-8.28	3.77	0.07	0	

[1] C_t = Cycles to threshold ; $\Delta C_{t\,(sample)} = C_{t\,(telomere)} - C_{t\,(36B4)}$

[2] $\Delta\Delta C_t = \Delta C_{t\,(sample)} - \Delta C_{t\,(standard)}$, where $\Delta C_{t\,(standard)}$ is calculated from the standard curve for 50ng DNA.

[3] T/S Ratio = $2^{-\Delta\Delta Ct}$

[4] Reactions were excluded if their C_t value had a difference >1 compared to the other two values for that sample.

Table D.7 Determination of T/S Ratios of CML and control samples (continued)

Sample	Telomere PCR		36B4 PCR		$\Delta C_{t\,(sample)}$ [1]	$\Delta\Delta C_t$ [2]	T/S Ratio [3]	Blasts in peripheral blood (%)	Age
	C_t [1]	C_t	C_t [1]	$C_{t\,(36B4)}$					
CML4_43	10.44	10.56	21.07	21.20	-10.64	2.10	0.23	0	
	10.57		21.39						
	10.65		21.13						
CML4_45	11.04	11.06	20.78	20.76	-9.70	1.75	0.30	0	
	11.07		20.75						
	11.06		20.74						
CML4_46	11.12	11.28	20.44	20.56	-9.28	2.18	0.22	0	
	11.37		20.55						
	11.34		20.70						
CML4_47	11.38	11.39	21.87	21.95	-10.56	1.36	0.39	0	
	11.38		22.03						
	11.42		21.94						
CML4_48	10.94[4]	9.74	20.38	20.59	-10.85	2.92	0.13	0	31
	9.55		20.55						
	9.92		20.62						
CML4_49	10.65	10.78	20.63	20.52	-9.74	2.48	0.18	0	
	10.80		20.43						
	10.90		20.51						
CML4_50	10.34	10.33	19.59	19.56	-9.23	3.19	0.11	0	
	10.26		19.37						
	10.39		19.71						
CML4_51	10.15	10.04	20.80	21.39	-11.35	4.18	0.06	0	
	8.76[4]		19.92						
	9.92		21.97						
CML4_52	10.80	10.52	21.49	22.09	-11.57	2.06	0.24	7	
	9.50[4]		20.56						
	10.23		22.68						

[1] C_t = Cycles to threshold ; $\Delta C_{t\,(sample)} = C_{t\,(telomere)} - C_{t\,(36B4)}$

[2] $\Delta\Delta C_t = \Delta C_{t\,(sample)} - \Delta C_{t\,(standard)}$, where $\Delta C_{t\,(standard)}$ is calculated from the standard curve for 50ng DNA.

[3] T/S Ratio = $2^{-\Delta\Delta Ct}$

[4] Reactions were excluded if their C_t value had a difference >1 compared to the other two values for that sample.

Table D.7 Determination of T/S Ratios of CML and control samples (continued)

Sample	Telomere PCR		36B4 PCR		$\Delta C_{t\,(sample)}$ [1]	$\Delta\Delta C_t$ [2]	T/S Ratio [3]	Blasts in peripheral blood (%)	Age
	C_t [1]	$C_{t\,(telomere)}$	C_t [1]	$C_{t\,(36B4)}$					
CML5_80	12.05 11.80 11.79	11.88	19.95 19.29 20.27	19.84	-7.96	2.22	0.21	0	60
CML5_83	11.44 11.40 11.38	11.41	20.61 20.60 20.92	20.71	-9.30	1.73	0.30	0	
CML5_85	8.83 8.77 8.40	8.67	16.75 16.84 16.71	16.76	-8.09	2.68	0.16	0	
CML5_86	12.64 12.66 12.56	12.62	20.82 20.83 20.84	20.83	-8.21	1.92	0.26	2	
CML5_90	10.60 10.63 10.59	10.61	19.10 19.23 19.18	19.17	-8.56	2.11	0.23	0	61
CML5_91	13.37 13.27 13.28	13.30	23.88 23.91 23.77	23.85	-10.55	2.97	0.13	2	
CML5_96	11.94 11.86 11.87	11.89	19.82 19.79 20.30	19.97	-8.08	2.21	0.22	2	
CML6_34	12.81 12.46 12.26	12.51	20.65 20.74 20.83	20.74	-8.23	2.50	0.18	0	46
CML6_37	13.32 13.06 13.31	13.23	20.62 20.63 20.78	20.67	-7.44	2.69	0.16	0	
CML6_38	12.64 13.19 13.31	12.97	19.89 21.90[4] 19.47	19.68	-6.71	2.58	0.17	0	
CML6_39	10.86 10.81 10.93	10.87	21.84 21.59 21.80	21.75	-10.88	1.75	0.29	0	

[1] C_t = Cycles to threshold ; $\Delta C_{t\,(sample)} = C_{t\,(telomere)} - C_{t\,(36B4)}$

[2] $\Delta\Delta C_t = \Delta C_{t\,(sample)} - \Delta C_{t\,(standard)}$, where $\Delta C_{t\,(standard)}$ is calculated from the standard curve for 50ng DNA.

[3] T/S Ratio = $2^{-\Delta\Delta Ct}$

[4] Reactions were excluded if their C_t value had a difference >1 compared to the other two values for that sample.

Table D.7 Determination of T/S Ratios of CML and control samples (continued)

Sample	Telomere PCR		36B4 PCR		$\Delta C_{t\,(sample)}$ [1]	$\Delta\Delta C_t$ [2]	T/S Ratio [3]	Blasts in peripheral blood (%)	Age
	C_t [1]	$C_{t\,(telomere)}$	C_t [1]	$C_{t\,(36B4)}$					
CML7_49	12.25 12.32 12.43	12.33	21.63 21.70 21.32	21.55	-9.22	3.06	0.12	0	39
CML8_27	11.48 10.29[4] 12.47	11.98	20.62 21.01 20.96	20.79	-8.81	2.18	0.22	0	39
CML9_51	11.92 11.85 11.67	11.81	21.09 21.02 21.11	21.07	-9.26	3.13	0.11	0	34
CML10_0	12.20 12.89 12.85	12.65	20.23 20.94 21.06	20.74	-8.09	3.49	0.09	1	61
CM11_3	13.29 13.56 13.54	13.46	21.11 21.17 21.00	21.09	-7.63	2.53	0.17	0.4	44
CM11_4	12.36 12.37 12.04	12.25	20.74 20.50 20.53	20.59	-8.34	2.54	0.39	0	
CML12_0[4]	12.26 12.50 12.61	12.46	18.85 20.37 20.40	19.87	-7.41	2.71	0.15	2	47
CML13_0[4]	12.22 12.45 12.51	12.34	18.78 19.72 --[5]	19.25	-6.91	2.71	0.15	1	21
CML14_0[4]	13.24 --[5] 12.88	13.06	20.81 19.25 20.84	20.3	-7.24	2.64	0.16	1	50

[1] C_t = Cycles to threshold ; $\Delta C_{t\,(sample)} = C_{t\,(telomere)} - C_{t\,(36B4)}$

[2] $\Delta\Delta C_t = \Delta C_{t\,(sample)} - \Delta C_{t\,(standard)}$, where $\Delta C_{t\,(standard)}$ is calculated from the standard curve for 50ng DNA.

[3] T/S Ratio = $2^{-\Delta\Delta Ct}$

[4] Reactions were excluded if their C_t value had a difference >1 compared to the other two values for that sample.

[5] Failed PCR

Table D.7 Determination of T/S Ratios of CML and control samples (continued)

Sample	Telomere PCR		36B4 PCR		$\Delta C_{t\,(sample)}$ [1]	$\Delta\Delta C_t$ [2]	T/S Ratio [3]	Blasts in peripheral blood (%)	Age
	C_t [1]	$C_{t\,(telomere)}$	C_t [1]	$C_{t\,(36B4)}$					
CML15_0	11.69 12.09 11.88	11.89	20.65 20.89 20.54	20.69	-8.8	1.92	0.26	0	50
CML16_0	11.69 11.74 11.87	11.77	21.52 21.44 20.77	21.24	-9.47	2.55	0.17	0	36
CML17_0	11.81 11.98 11.01	11.6	20.73 20.78 20.87	20.79	-9.19	2.30	0.20	9	53
CML18_0	11.53 11.66 - [4]	11.6	20.77 21.39 20.87	21.08	-9.48	2.57	0.17	0	61

[1] C_t = Cycles to threshold ; $\Delta C_{t\,(sample)} = C_{t\,(telomere)} - C_{t\,(36B4)}$

[2] $\Delta\Delta C_t = \Delta C_{t\,(sample)} - \Delta C_{t\,(standard)}$, where $\Delta C_{t\,(standard)}$ is calculated from the standard curve for 50ng DNA.

[3] T/S Ratio = $2^{-\Delta\Delta Ct}$

[4] Failed PCR

Table D.8 Melting curve analysis for hTERT mRNA real-time RT-PCR

Sample	hTERT Area Under Peak	ssBP1 Area Under Peak	hTERT/ ssBP1 Ratio	Average Ratio	Blasts in peripheral blood (%)
HL-60	10.76	7.85	1.37	1.43	
	11.12	7.49	1.48		
Stimulated Lymphocytes	9.57	7.27	1.31	1.32	
	10.14	7.62	1.33		
Unstimulated lymphocytes	0	9.66	0	0	
	0	9.57	0		
Normal Individuals[1]	0	10.15 ± 0.44	0	0	
CML1_67	3.30	9.27	0.36	0.46	6
	5.15	9.19	0.56		
CML1_68	2.94	9.38	0.31	0.33	14
	3.01	8.88	0.34		
CML1_69	12.87	10.01	1.29	1.24	16
	11.83	10.06	1.18		
CML1_70	11.76	9.58	1.23	1.23	15
	-	-	-[2]		
CML1_71	12.41	11.23	1.10	1.10	7
	-	-	-[2]		
CML1_72	10.76	10.27	1.04	1.04	11
	-	-	-[2]		
CML2_5	0	8.98	0	0	0
	0	9.62	0		
CML2_9	0	7.26	0	0	0
	0	8.69	0		
CML2_17	0	10.83	0	0	0
	0	10.42	0		
CML3_56	0	8.29	0	0	0
	0	8.52	0		
CML3_60	6.09	8.88	0.69	0.72	0
	6.84	9.02	0.76		
CML3_66	2.26	3.24	0.69	0.74	0
	2.25	2.83	0.79		

[1] Ten normal individuals were analysed

[2] Internal control failed

Table D.8 Melting curve analysis for hTERT mRNA real-time RT-PCR (continued)

Sample	hTERT Peak Area	ssBP1 Peak Area	hTERT/ ssBP1 Ratio	Average Ratio	Blasts present in peripheral blood (%)
CML4_43	0	3.60	0	0	0
	0	4.59	0		
CML4_45	0	9.39	0	0	0
	0	3.65	0		
CML4_46	0	10.76	0	0	0
	0	7.17	0		
CML4_47	0	3.07	0	0	0
	0	8.45	0		
CML4_48	0	8.97	0	0	0
	0	8.91	0		
CML4_49	0	2.9	0	0	0
	0	-	0		
CML4_50[1]	0	10.17	0	0	0
	0	5.87	0		
CML4_51[1]	1.03	11.82	0.09	0.19	0
	2.19	7.53	0.29		
CML4_52	3.54	10.08	0.35	0.35	7
	3.83	10.92	0.35		
CML5_85[2]	0	7.14	0	0	0
	0	7.11	0		
CML5_86	5.21	6.06	0.86	0.87	2
	6.19	6.99	0.88		
CML5_90	0	6.97	0	0	0
	0	7.27	0		
CML5_91	6.81	7.70	0.88	0.83	2
	4.96	6.40	0.77		
CML5_96[1]	5.82	5.66	1.03	0.86	2
	4.96	7.32	0.68		
CML6_34	3.62	9.75	0.37	0.37	0
	3.34	8.99	0.37		
CML6_37	3.52	8.16	0.43	0.38	0
	3.26	9.34	0.34		
CML6_38	3.36	10.30	0.32	0.33	0
	3.64	10.18	0.35		
CML6_39	3.36	10.65	0.31	0.26	0
	2.26	10.95	0.20		

[1] Poor duplicate

[2] For samples CML5_80 and CMl5_83, neither ssBP1 nor hTERT could be successfully amplified

Table D.8 Melting curve analysis for hTERT mRNA real-time RT-PCR (continued)

Sample[1]	hTERT Peak Area	ssBP1 Peak Area	hTERT/ ssBP1 Ratio	Average Ratio	Blasts present in peripheral blood (%)
CML8_27	0	9.93	0	0	0
	0	9.87	0		
CML9_51	0	10.40	0	0	0
	0	10.09	0		
CML10_0	0	10.41	0	0	1
	0	10.66	0		
CML11_3	6.09	8.88	0.69	0.72	0.4
	6.85	9.03	0.76		
CML11_4	0	11.0	0	0	0
	0	10.88	0		
CML12_0	7.3	9.2	0.79	0.72	2
	6.46	10.03	0.64		
CML13_0	7.38	10.54	0.70	0.69	1
	6.9	9.93	0.69		
CML14_0	10	10.7	0.93	0.89	1
	8.93	10.42	0.85		
CML15_0	7.77	13.8	0.56	0.55	0
	9.35	17.2	0.54		
CML16_0	2.98	20	0.15	0.13	0
	1.88	17.9	0.11		
CML17_0	6.43	13	0.49	0.53	9
	7.28	14.5	0.50		
CML18_0	6.62	16.6	0.39	0.48	0
	8.39	14.3	0.58		

[1] No result could be reported for sample CML7_49 where the internal control failed for the original sample assay and when the assay was repeated.

APPENDIX E Assays for the measurement of telomere length

Telomere Length Measurement Method	Reference	Advantages	Disadvantages
Telomere restriction fragment (TRF) ● Non-telomeric DNA digestion ● Southern hybridisation of telomere restriction fragments ● Estimation of telomere length using densitometry	Allshire *et al.*, 1989	● Visualisation of telomere length variability within a sample ● Visual identification of trends between samples	● Sub-telomeric regions of varying size included ● Not sensitive, ~10µg DNA required ● Sheared DNA reduces observed telomere length ● Analysis of smear not accurate ● Time consuming ● Cannot measure telomere length of individual cells or chromosomes
Quantitative Fluorescence *in situ* Hybridisation ● Telomere staining of metaphase spreads using a peptide nucleic acid ● Digital image analysis of fluorescence signal using fluorescence microscopy	Hultdin *et al.*, 1998	● Determine individual and intra-chromosomal telomere length ● Sub-telomeric regions excluded ● Accurate to ~200bp ● High hybridisation efficiency and reproducibility	● Complicated technique requiring specialised costly equipment ● Microscope fluorescence lamp intensity can vary ● Difficult to examine a large number of cells ● Difficult to analyse tissue sample
Flow-Fluorescence *in situ* hybridisation (Flow-FISH) ● Cells hybridized in solution with a telomere specific peptide nucleic acid ● Digital image analysis of fluorescence signal using a flow cytometer	Rufer *et al.*, 1998	● Sub-telomeric regions excluded ● Large number of cells can be processed rapidly ● Interphase cells hybridized in suspension ● High hybridisation efficiency and reproducibility	● Specialised costly equipment ● Not suitable for tissue sample ● Average telomere length of a sample is determined ● Microscope fluorescence lamp intensity can vary ● Time consuming and technically demanding

APPENDIX E Assays for the measurement of telomere length (continued)

Telomere Length Measurement Method	Reference	Advantages	Disadvantages
Double-strand primed *in situ* labelling ● Hybridisation to telomeres in metaphase spreads ● *Taq*-polymerase extension of labelled oligonucleotides ● Digital image analysis of fluorescence signal using fluorescence microscopy	Therkelsen *et al.*, 1995	● Detect individual telomeres ● Detection of interstitial telomere sequence ● Probes commercially available and cost effective ● Rapid : turnaround time ~2 hours	● Specialised costly equipment ● Difficult to examine a large number of cells ● Microscope fluorescence lamp intensity can vary ● At least 100 intact nuclei must be counted
Quantitative polymerase chain reaction ● Real-time PCR of telomere repeats and single-gene copy in separate tube ● Determination of telomere:single-gene copy ratio	Cawthon, 2002	● 50ng DNA required ● Rapid with high throughput ● Sub-telomeric regions excluded ● Normalise to a single gene copy, not centromeres or Alu repeats	● No information on population variability ● Specialised equipment required ● DNA must be intact ● Sensitive to small differences in sample preparation
Single telomere length analysis (STELA) ● Oligonucleotide ligated to 5' end of C-rich telomere strand ● Oligonucleotide primer and chromosome-specific primer used in PCR of telomere	Baird *et al.*, 2003	● Detect telomeres 406bp to 20kb ● Determine individual and intra-chromosomal telomere length ● Probes commercially available and cost effective ● Rapid and simple	● Require chromosome-specific sequences close to telomeres ● Small stretches of sub-telomere regions included

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