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1 INTRODUCTION

1.1 Telomeres, protective structures at the ends of the chromosome

Telomeres, nucleoprotein caps, located at the ends of chromosomes (Sanders and Newman, 2013), consist of G-rich TTAGGG repeats (Samassekou et al, 2010), (Giraud-Panis et al., 2010) which are bound to specific proteins, termed the shelterin complex (Armanios, 2009). Telomeres as well as a proper functioning shelterin complex (Armanios and Blackburn, 2012) are protective structures, mainly protecting from genomic instability (Blackburn and Collins, 2011), such as degradation and chromosomal end-to-end fusions (Blackburn, 2000), (Blackburn, 2005). End-to-end fusions result in dramatic consequences, such as chromosome segregation due to development of cancer cells (Blackburn, 2005). In addition, telomeres control DNA replication and modulate gene expression (Giraud-Panis et al., 2010).

Since DNA has a double helix structure consisting of 2 anti-parallel strands, during each DNA replication only the leading strand can replicate consecutively. In contrast, the replication of the lagging strand needs Okazaki fragments (Proctor and Kirkwood, 2002). After DNA synthesis, elimination of the terminal primer at the lagging strand leaves a small gap (de Lange, 2004), which ends in a G-rich 3' overhang as a result of its inability to replicate the lagging strand continuously (Proctor and Kirkwood, 2002). The 3' overhang can bend on itself within telomeric DNA, thereby forming a lasso-like structure, called t-loop (Samassekou et al., 2010), (Giraud-Panis et al., 2010), which is stabilised by the shelterin complex (Passos et al., 2007).

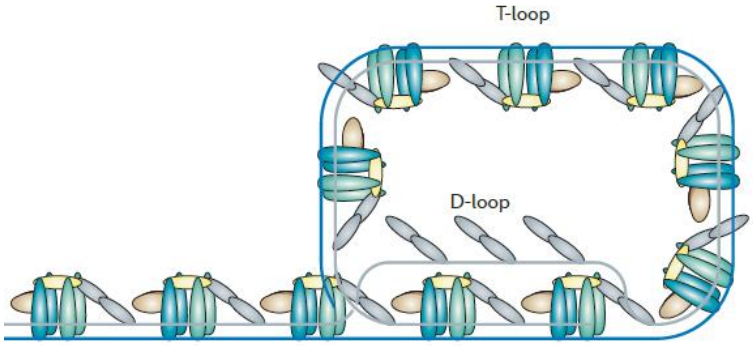

Figure 1.1: The t-loop structure (Martinez and Blasco, 2011)

This formation acts as a first layer for protection of telomeres from being misleadingly recognized as a double stranded break (Bull and Fenech, 2008)). The phenomenon of this inability to copy the ends of DNA (Sanders and Newman, 2013), called "end-replication problem", causes telomere shortening after each cell division (Sanders and Newman, 2013), (Proctor and Kirkwood, 2002). If a certain number of telomeres in a cell become critically short, a DNA damage response (DDR) is induced, which can lead to chromosomal end-to-end fusions or cell cycle arrest and apoptosis (Bull and Fenech, 2008). Therefore eukaryotic chromosomal DNA is in need of other replication mechanisms to lengthen the ends of chromosomes. This ribonucleoprotein (Blackburn and Collins, 2011) whose role is telomere elongation is called telomerase, a DNA polymerase (Blackburn, 2000) (Armanios and Blackburn, 2012), (Giraud-Panis et al., 2013) which synthesises DNA *de novo* at chromosomal ends (Blackburn and Collins, 2011). Telomerase is down regulated, but still present in many adult cells, such as epithelial, fibroblast and endothelial cells (Blackburn, 2000). Telomerase consists of a catalytic telomerase reverse transcriptase protein named TERT and an RNA template called TERC (Armanios and Blackburn, 2012). Telomerase copies the RNA template sequence into DNA, which is the major task of a reverse transcriptase (Blackburn, 2005). TERT is only low or not at all expressed in normal somatic cells. In contrast, it is highly expressed by cancer cells and germ cells (Campisi and d'Adda di Fagagna, 2007), thereby increasing telomerase activity to continue cell proliferation (Sekaran et al., 2014). In an experiment performed on yeast *Saccharomyces cerevisae*, cells without active telomerase stopped dividing after several cell divisions.

In contrast, cells with active telomerase continued to proliferate, even when their telomeres were shorter than the ones in telomerase knock out cells, which already stopped proliferating, after reaching a critical shortness. This experiment highlights that telomere shortness is not associated to loss of function and that the presence of telomerase is the only decisive determinant for telomere elongation (Blackburn, 2000). However, some tumour cells lack telomerase activity, in this case telomere lengthening is maintained by a pathway called Alternative lengthening of telomeres (ALT) (de Cian et al., 2008), (Sekaran et al., 2014). Tumour cells using ALT lengthening seem to maintain telomere length with the help of DNA repair proteins and telomere recombination events (Gocha et al., 2013). These ALT cells show an extremely high heterogeneity of their telomeres length, aberrant telomeric DNA sequences and seem to get activated by an alteration or loss of p53 status (Conomos et al., 2013), investigated *in vitro* and *in vivo* (Gocha et al., 2013).

Two physical states of telomeres exist, the capped state and the uncapped state (see figure 1.2.) (Blackburn, 2000). Telomeres are usually in the capped state with a proper telomere structure (Martinez and Blasco, 2011), meaning they are unrecognizable to a DDR (Passos et al., 2007) and proceeding of cell division can therefore sustain (Blackburn, 2000). Uncapping of telomeres is absolutely normal, as it occurs in dividing cells. The main task of the functional telomere is that it has to switch very fast into the capped state. If the uncapped state remains too long, due to loss of telomeric sequences or changes in telomere proteins (Martinez and Blasco, 2011), then cell cycle arrest is initiated and the telomere gets recognizable to DDR (Blackburn, 2000).

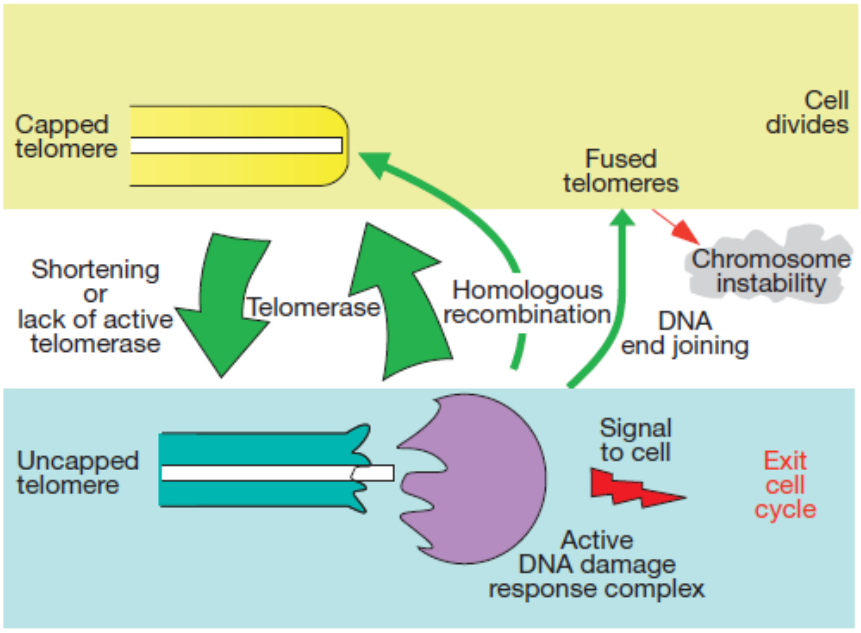

Figure 1.2: The capped-and the uncapped state of telomeres (Blackburn, 2000)

To maintain a balance between telomere loss and telomere lengthening, a telomere homeostasis system, regulated by protein composition of shelterin (de Lange, 2004), telomere sequences and telomerase activity exists (Serrano and Andres, 2004), (Grandin and Charbonneau, 2007). This system initiates telomere lengthening, when telomeres are short, but at the same time, inhibits telomerase activity of non-proliferating cells, to keep the balance in a proper range (Blackburn, 2005). Telomere attrition destabilizes the t-loops which causes a higher probability of getting into the uncapped state (Passos et al., 2007). Therefore, short telomeres, which are more susceptible to fusions (Blackburn, 2000), induce telomerase activity, thus increasing the probability to get into the functioning capped state. Telomere uncapping probability increases as a cause of several components and is not only influenced by telomere length. Longer telomeres can switch faster in the capped state, compared to short telomeres as they have more repeats and therefore more binding sites (Blackburn, 2000). More important, it was shown that telomeres in an uncapped state activate the same DDR as double strand breaks (Passos et al., 2007).

1.2 The shelterin complex and extra telomeric roles

The shelterin complex, formed by six telomeric binding proteins, connects the telomeric DNA with the G rich 3' single stranded overhang (de Cian et al., 2008), (Houben et al., 2007). Shelterin consists of telomeric repeat binding factors 1 and 2 (TRF1 and TRF2), TRF1-interacting protein 2 (TIN2), repressor activator protein 1 (RAP1), tripeptidyl peptidase 1 (TPP1), and protection of telomeres 1 (POT1) (Houben et al., 2007), (Lin and Yan, 2005), (Martinez and Blasco, 2011). TRF1 and TRF2 bind to telomeric DNA, whereas POT1 binds to the single stranded 3'overhang. The regulatory protein TIN2 binds TRF1 and TRF2 and forms the TPP1-POT1 complex, thus representing the bridge for many shelterin components (see figure 1.3.) (Martinez and Blasco, 2011).

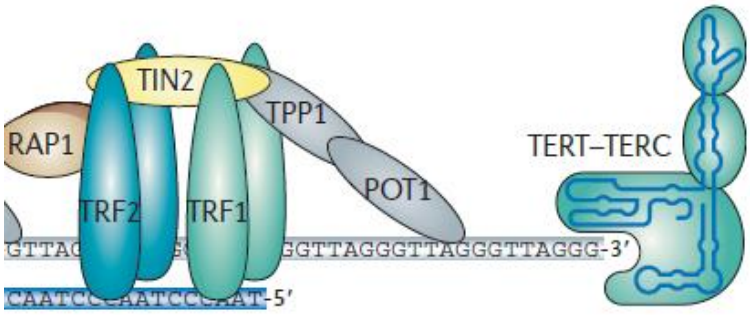

Figure 1.3: The shelterin complex (Martinez and Blasco, 2011)

TRF1 and TRF2 protect the chromosomal ends by formation of t-loops. RAP1 can bind to telomere sequences, prevent telomere recombination (Martinez and Blasco, 2011) and protect telomeres from non-homologous DNA chromosome end-to-end fusion (Houben et al., 2007). Main tasks of POT1 are capping of telomeres, binding, protection of the 3' overhang and regulation of telomere length with TPP1 being a regulator of POT1 itself (Gomez et al., 2012), (Houben et al., 2007), (Martinez and Blasco, 2011). TIN2 has many regulatory functions, as it is a negative regulator of telomere lengthening. It was even demonstrated that enhanced TIN2 protein levels inhibit telomere elongation (Houben et al., 2007).

Of strong importance is TPP1, which is tethered on TIN2 (Abreu et al., 2010) and its task in telomerase recruitment. It was shown that in human cells with down regulated TPP1 concentrations, telomerase could not even get recruited. In addition, TPP1 knockout in mice ended in reduced TERT binding and therefore enhanced telomere attrition. Based on these observations, TPP1 is important for protection against chromosomal end-to-end fusions and is essential for telomerase binding and function (Martinez and Blasco, 2011). In shelterin loss of function models, enhanced aging is often the dramatic consequence (Lopez-Otin al., 20013). Moreover, genetic variations in shelterin proteins are directly associated to cancer, assuming that shelterin is essential for telomere function, expecting that mutations of these proteins could lead to genomic instability as well (Martinez and Blasco, 2011), (Lu et al., 2013). Some of these shelterin proteins possess extra telomeric roles. RAP1 for instance acts as a transcription factor in many different pathways and interacts with TRF2 and other genes involved in insulin secretion, peroxisome proliferator-activated receptor (PPAR) signalling and nuclear factor-κβ (NF-κβ) regulated pathways (Gomez et al., 2012), (Martinez and Blasco, 2011). Several shelterin associated proteins exist; one of outstanding importance is called SNM1B/Apollo (Lu et al., 2013). This 5' exonuclease interacts with TRF2 and TPP1 and recruits the 3' single stranded overhang to protect telomeres from inappropriate repair, thus playing an important role in telomere protection (Lam et al., 2010), (Lu et al., 2013) and interstrand crosslink repair (Gomez et al., 2012). In gene knockout studies, performed on mice, Apollo dysfunction resulted in aberrant telomeres and telomere fusion, induced a DDR, consequently resulting in senescence (Lam et al., 2010).

1.3 Aging and other causes of senescence

Several studies showed that telomere length is mainly modulated by two processes. Already mentioned is the process of cellular replication due to the end-replication problem and the second process is oxidation (Sanders and Newman, 2013).

The end-replication problem appears because DNA polymerase is not able to copy the final stretch of the lagging strand, thus, after each cell division telomeric DNA is lost (Bull and Fenech, 2008). It is estimated that about 50 base pairs in human somatic cells get lost with each cell division, due to the end-replication problem (Houben et al., 2008), (Sanders and Newman, 2013). With increasing cell divisions, due to aging, infections or diseases (Ligi, 2011), less cells remain in the actively proliferating cell pool, assuming that there is an amount of cells which constantly drop out of the cell cycle at each replication. In addition, the proportion of these dropouts increases with age of the culture, leading to a senescent phenotype (von Zglinicki, 2002). Nowadays, it is known that the shortest telomeres are critical for various cell responses, thus, it is assumed that critically short telomeres caused by a disruption in their protective shelterin structure (Karlseder et al., 2002), (Kuilman et al., 2010) are the ones which can activate a DDR (Samassekou et al., 2010) and at the same time inhibit DNA repair (Campisi, 2013). Such dysfunctional telomeres are then recognized as a type of DNA damage, generate telomere dysfunction induced focis (TIF) (Sekaran et al., 2014) and activate the major tumour suppressor pathways p53/p21 and $p16^{INK4a,}$ resulting in cellular senescence (see figure 1.4.) (Samassekou et al., 2010), (Campisi and d'Adda di Fagagna, 2007), (Rodier and Campisi, 2011), (Sharpless and dePinho, 2007).

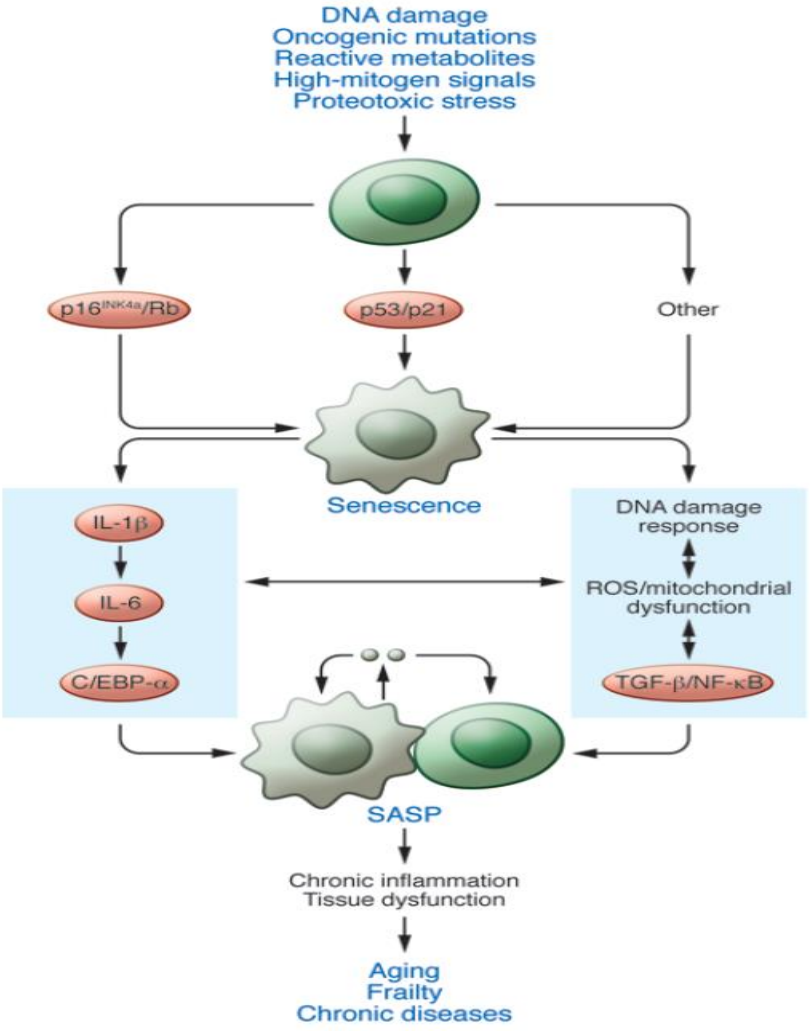

Figure 1.4: The p53/p21 and p16^{INK4a}/RB tumour suppressive pathway
(Tchkonia et al., 2013)

Cellular senescence represents a cellular response, characterized by an irreversible growth arrest and phenotypic modifications (Lopez-Otin et al., 2013). This permanent growth arrest of senescent cells leads to a reduction of tissue renewal and repair, leading to depletion of stem cell-and fibroblast pools, thereby contributing to aging (Rodier and Campisi, 2011). Damaged cells can either undergo cellular senescence which includes affecting the neighbour cells or they can switch to apoptotic cell death without any negative consequences for their neighbour cells. Both fates pose important tumour suppressive mechanisms to protect an organism (Collado et al., 2007), (Vicencio et al., 2008), from experiencing neoplastic transformation (Rodier et al., 2007).

Moreover, whether a cell fate will result in senescence or apoptosis seems to depend strongly on the cell type (Collado et al., 2007). The senescence response, as a consequence of dysfunctional telomeres depends on a faultless p53 pathway (Campisi, 2005). The major tumour suppressor protein p53 ensures longevity and inhibits formation of cancer cells, by decreasing cell mutations and limiting proliferation of malignant cells (Rodier et al., 2007). Senescence is not only restricted to telomere shortening (Rodier and Campisi, 2011), it sometimes even happens at non-telomeric sites, which possibly leads to a persistent DDR signalling and an arrest of cell proliferation (Rodier and Campisi, 2011). Some senescence-causing triggers, besides exhaustive cell proliferation, are DNA damage, mitogenic signals, such as oncogenes (Rodier and Campisi, 2011) and elevated ROS levels (Tchkonia et al., 2013). Similarly, these triggers are able to activate the p53 and $p16^{INK4a}$/pRB pathways as well, which seem to interact and modulate each other (Rodier and Campisi, 2011), (Campisi, 2013). Moreover, an activation of p53 and $p16^{INK4a}$/RB seems to be critically needed for a cell to enter senescence (Kuilman et al., 2010). Senescence can be triggered by cytotoxic stress, one of which is chronic oxidative stress (von Zglinicki, 2002).

Oxidative stress leads to formation of oxidized bases, which might further result in formation of single-stranded or double-stranded breaks in DNA (Passos et al., 2007). Telomeres, as they contain triple guanine (GGG) structures, are very sensitive to UV radiation damage (Houben et al., 2008), and oxidative stress (von Zglinicki, 2002). Furthermore, it has been reported that oxidative damage to bases agglomerates with age. In senescent cells, there is even a 30% higher occurence of oxidised guanines present in DNA (Houben et al., 2008). Oxidative damage of lipids and proteins due to reactive oxygen intermediates, is normally removed by a properly functioning cell turnover, whereas oxidised DNA bases have to be repaired by DNA repair mechanisms, to inhibit accumulation of point mutations, which can affect important sensitive genes. If the damage is too severe or if there are not enough antioxidants to protect the DNA, then the repair system is overburdened and single-stranded breaks can occur (Bull and Fenech, 2008).

Unfortunately, telomeres are less proficient in repairing these single-stranded breaks, mainly because they lack specific base excision repair mechanisms (von Zglinicki, 2002), (Serrano and Andres, 2004), (Richter and von Zglinicki, 2007). Petersen et al. exposed human fibroblasts to hydrogen peroxide and counted the single-stranded breaks in telomeres and minisatellites. This study showed that repair of single-stranded breaks observed in minisatellites was finished completely after one day, whereas repair in telomeres was not only slower, moreover it was simply insufficient (Houben et al., 2008). These unrepaired single-stranded breaks accumulate at telomeres and consequently lead to telomere shortening during DNA replication (von Zglinicki, 2002). In a study, performed by Richter and von Zglinicki, telomere shortening in fibroblasts from humans and sheep under oxidative and non-oxidative conditions was investigated. They could demonstrate that oxidative stress and enhanced telomere attrition correlated exponentially, independent of species and cell strain (Richter and von Zglinicki, 2007). Another study performed by Haendeler et al., could demonstrate that with aging, ROS formation increases, whereas the nuclear TERT activity diminishes in endothelial cells, assuming that this age-related increase in ROS and mitochondrial DNA damage trigger the nuclear export of TERT into the cytoplasm, thus leading to senescence (Haendeler et al., 2004), (Passos et al., 2007). Senescent cells are characterised by enhanced ROS levels, elevated DNA double-strand breaks and dysfunctional mitochondria. Furthermore, ROS found in the mitochondria seem to accelerate the senescence process (Houben et al., 2008), (Kuilman et al., 2010).

1.4 Aging and Cancer

The senescent phenotype leads to irreversible growth inhibition, apoptosis resistance and alterations in gene expression (Campisi and d'Adda di Fagagna, 2007), (Jeyjapalan and Sedivy, 2008), (von Zglinicki, 2002). These transcriptional changes mainly affect genes, important for inflammatory and mitochondrial responses (Lopez-Otin et al., 2013). Senescent cells are characterized by long term cell cycle arrest, (Kuilman et al., 2010), flattened appearance and a high lysosomal content. Thus they can get stained for their senescence-associated-

beta-galactosidase activity (SA-β-Gal) (Chandler and Peters, 2013), which is increased in many senescent cells (Kuilman et al., 2010), (Campisi, 2013) but as well in many non-senescent cells (Kuilman et al., 2010). Of strong importance in senescent cells is the INK4a/ARF locus, which encodes for tumour suppressor protein $p16^{INK4a}$. It was shown that $p^{16INK4a}$ is increased in senescent cells, whereas this locus is low expressed at young age (Collado et al., 2007), (Kuilman et al., 2010). Furthermore, $p16^{INK4a}$ seems to be unexpressed in already terminally differentiated cells, suggesting that this marker is of outstanding importance for further research in senescence (Jeyjapalan and Sedivy, 2008), (Rodier and Campisi, 2011). Genome wide association studies (GWAS) directly linked the INK4a/ARF locus which encodes for $p16^{INK4a}$ and p^{19ARF} to many age-associated pathologies, for example CVD, atherosclerosis, metabolic disease and diabetes (Jeck et al., 2012), (Lopez-Otin et al., 2013). Senescent cells can secrete many pro-inflammatory cytokines, chemokines (Vicencio et al., 2008) and matrix metalloproteinases (MMP), able to change the tissue structure, while affecting their neighbouring cells. This is termed as Senescence Associated Secretory Phenotype (SASP) (Campisi, 2005), (Rodier and Campisi, 2011), (Vicencio et al., 2008), (Chandler and Peters, 2013), (Lopez-Otin et al., 2013), (Tchkonia et al., 2013). Moreover, many proteins linked to SASP, for example IL-6, MMP increase with higher age, and are considered as hallmarks of inflammation (Rodier and Campisi, 2011), (Tchkonia et al., 2013). Since SASP is characterized by a release of many of these inflammatory cytokines, chronic non-microbial inflammation is best documented in age-related diseases (Rodier and Campisi, 2011), such as atherosclerosis, dementia, diabetes and cancer (Tchkonia et al., 2013).

Cellular senescence only affects mitotic cells, since they can proliferate. From these cells, cancer cells can generate, being critically prone to mutations. Therefore, organisms with renewable tissues bear some problems in aging (Campisi and d'Adda di Fagagna, 2007), (Samassekou et al., 2010). As DNA mutations accumulate with aging, cells gain the efficiency to proliferate improperly. With the help of genomic instability, they gain phenotypes with enhanced cell proliferation and migration, such as cancer cells (Campisi and d'Adda di Fagagna, 2007), (Campisi, 2013). Moreover, triggers which are able to induce senescence, can promote carcinogenesis as well (Rodier and Campisi, 2011).

Some type of matrix metalloproteinases can even enhance invasiveness of tumour cells by inhibiting differentiation of for example breast epithelial cells (Rodier and Campisi, 2011). Cells which fail to enter senescence by bypassing the cell cycle checkpoint, due to their loss of DNA repair (Aubert and Lansdorp, 2008), (Rodier et al., 2007), but still hold the ability to proliferate, despite their dysfunctional telomeres can go into a crisis state. This state is often called mortality phase 2 (M2). Then these cells can develop chromosomal abnormalities and aberrant cells can proliferate indefinitely, resulting in transformation, phenotyped as cancer cells (Campisi and d'Adda di Fagagna, 2007), (Samassekou et al., 2010). In addition, mutations affecting tumour suppressor pathways are often associated to cancer cells, due to disruption of these pathways (Collado et al., 2007), (Rodier et al., 2007). Consistent with this observation, loss of tumour suppressors is critically needed for such a transformation, observed in human cells in an *in vitro* study. (Collado et al., 2007). Their released pro-inflammatory molecules seem to be able to change microenvironment, beneficial for cancer cells (Campisi, 2005), resulting in late-life cancer (Campisi, 2013). Therefore it is said that cellular senescence is not only beneficial for an organism, in fact it is harmful in some cases. Senescent cells can fuel cancer progression and increase inflammation by their production of these pro-inflammatory secretes known as SASP (Vicencio et al., 2008), (Rodier and Campisi, 2011), (Lopez-Otin et al., 2013). Studies have shown that SASP can only establish with a persistent DDR signalling (Chandler and Peters, 2013). Still, not every SASP factor is able to promote cancer (Jeyjapalan and Sedivy, 2008), (Rodier and Campisi, 2011). In contrast, some cytokines released by SASP, IL-6 and IL-8 are able to enhance senescence, thus reducing the risk of formation of cancer cells (Tchkonia et al., 2013).

1.5 Telomere length and genetic determinants

Telomere length is very heterogeneous, because it differs between cells and even between chromosomes in the same cell (Samassekou et al., 2010), speaking of a chromosome specific pattern, created already at conception (Graakjaer et al., 2003). In humans, many determinants appear to influence the length of telomeres, such as sex, ethnicity, environmental exposure and genetics (Aviv, 2012). Evidence exists about telomeres and their genetic heritability, seen in animal models such as M. castaneus mice, which have very short telomeres in general. When investigating the telomeres of their offspring, they have short telomeres too, suggesting that there is a strong influence of heritability. In humans, many population based studies evidenced that the influence of heritability ranges between 0.36 and 0.84 (Armanios and Blackburn, 2013) and that a short telomere length at birth seems to persist with age. Notably, individuals with long telomeres in one tissue, have long telomeres in other tissues as well (Houben et al., 2008), (Samassekou et al., 2010).

A very important determinant which affects telomere length is the paternal age of conception (PAC). Kimura et al. found out that at time of conception, older fathers hand down longer telomeres to their offspring. The authors say that this phenomenon appears, because donors, older than 50 years have higher telomere length in sperm, compared to donors, younger than 30 (Aviv, 2012). They also suggested that there are age dependent telomere dynamics in sperm, confirming that telomere length increases with age in spermatozoa (Kimura et al., 2008), (Samassekou et al., 2010). Interestingly, whereas no gender difference exists at time of birth, this difference appears after 3 and 15 months in rats, assuming that sex hormones play a major role (Cherif et al., 2003), and that telomere length decreases at a higher rate in men compared to women (Samassekou et al., 2010).

1.6 Telomere length and dietary factors

Not only do genetic determinants influence telomere length, shorter telomeres are associated with male gender (Ligi, 2011), aging, life stress, chronic diseases and certain lifestyle factors like alcohol consumption, smoking, physical activity, lipid levels (Serrano and Andres, 2004) and consumption of processed meat (Lin et al., 2012). Since DNA repair and metabolism depend on the presence of some specific nutrients, it is very important to pay regard to some of them, as they act as important cofactors in several metabolic pathways. Furthermore, micronutrient deficiency could lead to genomic damage as chemical carcinogens or UV radiation. (Bull and Fenech, 2008).

Folate, for example, plays a major role in DNA integrity as well as in methylation. Having folate deficiency often leads to a replacement of a thymidine base in the telomeric sequence with uracil. This replacement results in chromosome breakage (Bull and Fenech, 2008), DNA damage and therefore an imbalance of nucleotides in the cell (Ligi, 2011). Some studies evidenced that high plasma homocysteine levels are correlated to shorter telomeres, opposed to high plasma folate levels and longer telomeres (Lin et al., 2012).

Worth mentioning in connection to telomeres are the vitamin nicotinamide and the mineral nutrients magnesium and zinc. Nicotinamide used in cell culture studies showed a significant reduction of telomere shortening and lower levels of ROS. Important is the fact that nicotinamide itself is not an anti-oxidative vitamin but it may indirectly influence cellular factors linked to ROS production.

Magnesium is essential for catalytic activity of several enzymes used in DNA replication and DNA repair. Therefore it is assumed that magnesium has a protective effect on telomere length and plays a role in the oxidative stress system. Magnesium deficiency leads to chromosomal abnormalities and reduced DNA repair mechanisms (Ligi, 2011). Shah et al., discovered that mice with short term magnesium deficiency for only 21 days had significantly lower telomerase activity and a severe DNA oxidation (Shah et al., 2014). Since reverse transcriptases, such as telomerase are zinc dependent enzymes, it is not surprising that zinc deficiency can cause greater DNA damage (Ligi, 2011).

Nutrients, like vitamin C often are correlated with long telomeres, because of their reactive oxygen species (ROS) scavenging functions in the oxidative stress system (Lin et al., 2012). One of the first telomere studies showed that age dependent telomere shortening and telomerase attrition in human vascular endothelial cells (HUVEC) can be reduced with the help of the anti-oxidative vitamin C in a culture medium (Ligi, 2011). HUVEC which were treated with Asc2P, which is an oxidation-resistant form of vitamin C (Houben et al., 2008), and had a significantly higher telomerase activity, compared to untreated cells. Furthermore, telomerase activity decreased age-dependently. In Asc2P treated cells, less lesions in telomeric DNA were the consequence of a diminishment of intracellular ROS (Furumoto et al., 1998). The underlying mechanism of how ROS accelerate telomere shortening are not fully understood. It is said that increased telomere shortening is a result of DNA damage, which is caused by several factors, for example ROS. Since aging continues in the presence of antioxidants as well, there must be other mechanisms, underlying telomere shortening and attrition. One reason simply is that telomeres shorten as a result of DNA replication, independent of oxidative damage (Tzanetakou et al., 2012).

Marine omega 3 fatty acids are beneficial for telomere length in context with the anti-oxidative system (Ligi, 2011), as well as with their cardio protective and anti-inflammatory characteristics, including reduction of plasma triglycerides (Kalupahana et al., 2011), (Farzaneh Far et al., 2010). In a study performed on mice, a high consumption of omega-3 fatty acids significantly increased the activity of anti-oxidative enzymes, such as superoxide dismutase and catalase. It is known that consuming a high amount of omega-3 fatty acids can reduce DNA damage, resulting in a deceleration of telomere attrition (Ligi, 2011). Farzaneh Far et al., investigated the effects of omega 3 fatty acids on telomere length in patients suffering from coronary heart disease (CHD). They measured LTL at the baseline and again after 5 years in 608 CHD patients. The results of this study showed after five years, the highest rate of telomere attrition was seen in the lowest quartile of docosahexaenoic-acid (DHA) and eicosapentaenoic-acid (EPA) consumption, compared to the slowest rate of telomere shortening, observed in the highest quartile of DHA and EPA (Farzaneh Far et al., 2010).

1.7 Telomere length and disease risk

Most of human population studies which investigated telomere length, measure the average telomere length per tissue sample, often used are telomere lengths of leukocytes. These studies revealed that short telomeres are associated to a higher risk of many age-related diseases and even cancer (Mather et al., 2011), (Vera and Blasco, 2012). Up to date, there are inconsistent findings about short telomeres and a higher risk of cancer, CVD, diabetes, obesity and overall mortality. Some studies evidenced an association, whereas other studies could not reveal a link between short telomeres and a higher risk of age-related maladies.

Decreased telomerase activity is associated with an increased risk of CVD, but independent of age (Ornish et al., 2008). Therefore Epel et al., studied 62 healthy women at the age of 40, to find out if there is a connection of telomerase activity and telomere length to cardiovascular disease risk (Epel et al., 2006). The study showed low telomerase activity, but telomere length was not directly linked to major CVD risk factors including smoking, high blood pressure, high LDL and total cholesterol, high fasting glucose and obesity (Ornish et al., 2008), (Epel et al., 2006). In the study performed by Epel et al., the reason why there was no correlation of telomere length and the investigated risk factors is explained by the young age of the study participants and by limitations in the applied techniques. The authors assume that mean telomere length of young and healthy people is not sensitive enough to detect subtle telomere changes (Epel et al., 2006).

However, there is a major reason why cardiovascular diseases are observed more often in men than in premenopausal women. Estrogen has a strong protective effect on lipoprotein metabolism, resulting in direct protective effects on vascular endothelial cells. Estrogen activates the phosphoinositol 3-kinase (PI3K)/Akt pathway, resulting in an increased telomerase activity via TERT phosphorylation. (Serrano and Andres, 2004). Further studies showed that inhibition of this PI3K pathway with pro-atherogenic

factors, for example oxidized LDL leads to a decreased telomerase activity and therefore might increase senescence in endothelial cells which could result in the development of age-related diseases (Breitschopf et al., 2001).

In addition, estrogen activates gene expression of Manganese Superoxide Dismutase (MnSOD), an antioxidative enzyme found in mitochondria, thus contributing to an increased telomere attrition observed in men (Houben et al., 2007).

Obesity is considered as a major determinant in the regulation of adipose tissues, paired with altered metabolic factors, leading to insulin resistance, CVD and diabetes mellitus type 2. Several studies could show that excess nutrition enhances cortisol and insulin levels, whereas anabolic hormones are repressed, resulting in a metabolic imbalance state which consequently affects adipose tissue aging (Tzanetakou et al., 2012). Furthermore, since obesity is linked to increased levels of inflammatory cytokines (Lopez-Otin et al., 2013), released by SASP, it may also be the reason of insulin resistance (Tchkonia et al., 2013). New studies demonstrate that in obese people, protein p53 is the main actor in the adipose tissue aging process. Minamino et al., explored that inhibition of p53 activity in adipose tissues lowers plasma insulin levels and ameliorates insulin resistance (Tzanetakou et al., 2012). In this study, they tested Ay mice with ectopic expression of the agouti peptide to induce a disruption of the melanocortin pathway, thus leading to excessive caloric intake, type 2 diabetes like disease and obesity. In these mice, they found elevated oxidative stress levels in adipose tissue and enhanced senescence-like states, for example higher expression of p53 and pro-inflammatory cytokines. This observation is surprising, since differentiated adipocytes as postmitotic cells do not enter senescence. Since fat tissue is characterized by a high turnover rate, high fatty acid levels and mitogens, it is possible that this could be a cause of cellular senescence, spreading in fat tissues, forcing other cells, including differentiated cells to enter senescence (Tchkonia et al., 2010). In contrast, in adipo-p53-deficient mice with type 2 diabetes like disease, inhibition of p53 activity showed a decreased expression of pro-inflammatory cytokines, as well as improvement in insulin resistance, assuming that aging of fat tissues plays a crucial role in the development of diabetes (Minamino et al., 2009).

Other studies reveal that insulin resistance may be a trigger of CAD and diabetes, since it is linked to inflammation and oxidative stress (Adaikalakoteswari et al., 2007). In addition, enhanced concentrations of TNF-α, observed in atherosclerosis (Marin et al., 2013), obesity and diabetes type 2 interfere with insulin action, by inhibiting insulin signal transduction. Consequently, this may inhibit anti-inflammatory effects of insulin, thus promoting pro-inflammatory effects, manifested in telomere shortening (Houben et al., 2007). In mice, Rap1, one of the shelterin proteins was shown to be protective against obesity. In a study performed by Martinez et al., Rap1 knockout mouse cells showed early manifestations of obesity, including agglomeration of abdominal fat, elevated plasma concentrations of insulin, glucose and cholesterol. Furthermore, in Rap1 knockout cells, a decreased Pparα expression and down regulation of some target genes was observed, leading to disturbances in metabolic pathways. PPARs act as ligand activated transcription factors, influence nutrient homeostasis, thereby contributing to the onset of diabetes and obesity. This study showed that Rap1 is needed for Ppar transcription and that the lack of Rap1 leads to critical alterations in various metabolic pathways, resulting in obesity (Martinez et al., 2013).

1.8 Methods to measure telomere length

Up to now, there is a high number of methods available for determination of telomere length. Here, the specific features and functionalities focusing on important advantages and drawbacks of each method are highlighted. Section “Materials & Methods” gives a more detailed explanation on some of the methods, used in the study. There is still a strong need of finding the appropriate and reliable method, depending on the application, whether the aim is to define the proper telomere length or to highlight the appearance of short telomeres.

1.8.1 TRF Analysis

The history of telomere length measurement started in 1988 with the most commonly used Southern blotting of TRF (=Terminal restriction fragments), which still represents the gold standard for telomere length analysis (Aubert et al., 2012). The measurement of telomere lengths with Southern blotting relies on the fact that the telomeric region lacks any restriction enzyme recognition sites. So the DNA can get digested with restriction endonucleases which produce terminal restriction fragments (TRF). Restriction enzymes e.g. *Hinf*I or *Rsa*I act in such a manner that they release a terminal restriction fragment, containing full length telomeric repeats and a short sequence of subtelomeric DNA, including noncanonical parts at the proximal telomeric region (Aviv, 2008), (Lin and Yan, 2005). DNA then gets separated using gel electrophoresis and transferred to a nylon or nitrocellulose membrane, followed by hybridization with a radioactive or a non-radioactive probe against the telomeric repeats (Lin and Yan, 2005). For the transfer of DNA fragments from the electrophoresis gel to the nylon membrane, many possibilities exist, such as upward or downward capillary transfer. Upward capillary transfer is usually combined with a high salt buffer, so that DNA can bind to the membrane but does not get permanently immobilized. Due to many disadvantages with the upward capillary transfer, e.g. the gel can become crushed because of the high weight of the filter papers and towels which are placed on the gel. For the electrophoretic transfer of the proteins, an electric field helps to elute proteins from the agarose gel to the nylon membrane. Using electrophoretic transfer, one can choose between tank transfer and semi-dry transfer.

Hybridization technique is used to detect specific DNA sequences with the help of specific probes which are complementary to the sequence of interest. The basic principle is that denatured DNA dissociates and reanneals with complementary strands below their melting point (T_m). T_m is defined as the temperature at which half of DNA exists in its single-stranded form. Very often, blocking agents are added into the hybridization buffer, such as Denhart's solution. According to Hybridization Guide by Thermo Electron Corporation, Denhart's is used to block sites, where the probe would

otherwise bind to non-specifically. Probes can be double-stranded, single-stranded or of the oligonucleotide type. These probes are directed against the sequence of interest, can be labelled with radioactivity, enzymes or fluorescence and give a signal when they are incubated with an applicable substrate.

Later the membrane gets visualized and analysed, by measuring how far the lanes migrated on the gel. The results are expressed in absolute values in kilo bases. The main objective is measuring the mean telomere length per given sample, mainly to give information on the number of telomeric repeats. However, a big disadvantage of this method appears here: when measuring the terminal restriction fragments, an unknown size of subtelomeric sequence is included in the analysis which results in an overestimation of the actual telomere length (Lin and Yan, 2005), (Vera and Blasco, 2012).

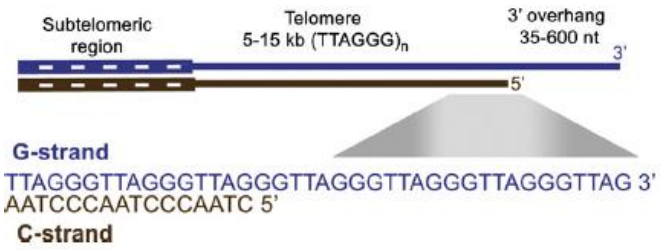

Figure 1.5: The telomeric sequence (Samassekou et al., 2010)

Furthermore, TRF analysis requires a high amount of pure high quality DNA (minimum 1.5 µg) and it is very time-consuming. (Lin and Yan, 2005), (Samassekou et al, 2010). TRF analysis does not give an estimation of the short telomeres and is not able to measure telomere length in individual cells (Kimura and Aviv, 2011), (Vera and Blasco, 2012). However, it is still one of the most used techniques to measure telomere length and acts like a control comparing the results, obtained by other methods (Lin and Yan, 2005).

Several modified versions of Southern blotting have been developed. Two of them are slot blot and currently the dot blot method, trying to circumvent the shortcomings of TRF analysis (Baird, 2005), (Lin and Yan, 2005).

The disadvantages of TRF analysis were addressed by Norwood et al. who established a slot blotting method in which the telomere length estimation is based on a telomere to centromere (T/C) DNA content ratio. Ogami et al., used a modified slot blot method in a study, to measure mean telomere length in coronary endothelial cells of patients suffering from coronary artery disease (CAD). A big advantage of this technique is that slot blotting can be performed with even a minimum amount of DNA. Another simplified tool, based on TRF analysis is called dot blot. Here the relative DNA content of every single dot is analysed by a DNA stain (Dx), hybridised and measured with a digoxigenin (DIG) labelled telomeric probe (T). The ratio T/Dx shows a good correlation with average telomere length obtained by TRF. One main advantage of using dot blot is that only the canonical part is measured. Dot blot analysis is a fast, cheap and easy method of measuring mean telomere length with the advantage of requiring just a small amount of DNA (minimum 5 ng) (Kimura and Aviv, 2011). A key limitation of these techniques is that they have large variability and are not able to detect subtle changes in telomere length (Lin and Yan, 2005). Furthermore, these measurements lack the ability of giving information on telomere length distribution (Baird, 2005).

1.8.2 FISH methods

In the year 1996 a new very sensitive method called FISH (Fluorescence in situ hybridization) had its breakthrough, closing the gap of inability to measure telomere length in an individual single cell (Samassekou et al, 2010). The FISH method first needs preparation of cells, followed by labelling of telomeres with oligonucleotide probes, staining with 4', 6'-diamidino-2-phenylindole (DAPI) or propidium iodine (PI) and visualisation by fluorescence microscopy (Lin and Yan, 2005), (Samassekou et al, 2010).

Derived from FISH analysis, two new methods could be established, namely the Q-FISH method using fluorescence microscopy and the Flow FISH method adapted from flow cytometry (Vera and Blasco, 2012).

The quantitative FISH (Q-FISH) can be performed in metaphasic and interphasic cells. Q-FISH on metaphases relies on labelling of telomeres with fluorochrome labelled peptide nucleic acid (PNA) oligonucleotide (ON) probes and quantification of telomeric signals, paired with digital microscopy in proliferating cells in metaphase. PNA probes act as synthetic petides, which are homologous to DNA, being extremely stable and allowing a specific hybridization of the probe to the DNA. The PNA probe is then able to recognize three telomeric repeats, meaning the intensity of the fluorescent signal from the probe is proportional to the length of the telomeres (Vera and Blasco, 2012). PNA probes are used because they are more stable than usual oligonucleotide probes, reach a better staining, thus achieving an increased sensitivity of the method. The fluorescence intensity expressed as telomere fluorescence intensity (TFI) has a good correlation with the mean TRF (Lin and Yan, 2005).

The main goal using Q-FISH is gaining an estimation of individual telomere length in a single cell and receiving information about the abundance of short telomeres (Vera and Blasco, 2012). It analyses the telomere length on each chromosome arm expressed as mean of fluorescence units correlated to the telomere length in kb (Samassekou et al., 2010).

This method is very sensitive, with an extremely low detection limit of 0.15 kb. This low limit is highly important for detecting signal-free ends with less than 0.15 kb of telomeric repeats. The appearance of signal-free ends is linked to the beginning of a DDR and chromosome end to end fusions. Critically short telomeres with signal-free ends lose their DNA repair mechanisms, then send a DNA damage response which subsequently leads to replicative cell senescence (Vera and Blasco, 2012). A key limitation of q-FISH in metaphase is its need of live, high quality, proliferating metaphasic cells and therefore senescent cells cannot be analysed (Baird, 2005).

Other disadvantages of the q-FISH in metaphases are the high costs, the need of a highly trained laboratory and only a few samples can be measured simultaneously (Samassekou et al., 2010). These weaknesses limit the utility of q-FISH analysis as well.

The q-FISH method in interphasic cells does not need live cells anymore, as this method is able to analyse tissue sections and senescent cells. A big drawback, however, is that it only measures telomere spots emerged from aggregations of telomeres and not the telomere signals. So this method is not able to measure the signal-free ends but it gives a good estimation on the abundance of short telomeres (Vera and Blasco, 2012).

In 1998 the simple and accurate Flow-FISH method was established using labelling of telomeres with a fluorescent PNA probe and Fluorescent activated cell sorting (FACS) device. Flow FISH method needs cell preparation, hybridization with a fluorescent PNA probe, washing steps to get rid of the unbound probe, DNA staining and FACS analysis (Lin and Yan, 2005). This technique only gives information about the mean telomere length in a cell which does not include quantification of short telomeres or individual telomeres (Vera and Blasco, 2012). Furthermore main disadvantages are that this method needs a cytological preparation to get cells in suspension, and a large number of cells (Samassekou et al, 2010). However, several studies using Flow-FISH showed a good correlation of telomere length compared with telomere length results obtained by TRF (Lin and Yan, 2005). A new method which tried to circumvent all those disadvantages is called high throughput q-FISH (HT Q-FISH). It relies on q-FISH in metaphases and analysis in interphasic nuclei. It is a combination of FISH, labelling of telomeres with PNA probes and fluorescence microscopy. The average telomere length in individual nuclei analysed with HT q-FISH correlate very well with those obtained from normal q-FISH (Vera and Blasco, 2012). A big advantage of HT q-FISH is the fact that it can calculate percentage of cells with critically short telomeres by using frequency histograms of telomere spots (Canela and Vera, 2007). These histograms can quantify the frequency of low intensity telomere spots meaning they can calculate the frequency of short telomeres. HT q-FISH can be used for many cell types and just needs a small amount of cells without losing its high accuracy. Moreover HT q-FISH can calculate the frequency of short telomeres in a single cell, even in large cell populations (Vera and Blasco, 2012).

1.8.3 quantitative PCR (qPCR)

In year 2002, quantitative PCR (qPCR) was established as a new method for assessment of telomere length. qPCR relies on amplification of telomeric sequences with the help of specific primers, using a reference gene to normalize the results (Samassekou et al, 2010). qPCR gives the ratio of telomere repeat copy number (T) to a single copy gene copy number (S). This so called (T/S ratio) is directly proportional to the mean telomere length (Lin and Yan, 2005). This method is a simple and fast, high throughput technique as it does not need live cells. Instead it uses genomic DNA, like TRF, but only a small amount (25-50 ng) is required (Samassekou et al, 2010). In the year 2008, Cawthon developed a monochrome multiplex qPCR (MMQPCR) to minimize variance in the T/S ratio and to enhance its efficiency by only needing half as many reactions (Sanders and Newman, 2013). T/S ratios of the multiplex qPCR are better correlated with results obtained by terminal restriction fragment lengths (R^2 = 0.844) compared to the original qPCR method (R^2 = 0.677) (Cawthon, 2008). The main weaknesses of measuring telomere length with qPCR as well as with MMQPCR is that it only measures the mean telomere length and cannot give any data on telomere length distribution (Baird, 2005), (Vera and Blasco, 2012). Moreover they do not give any information on individual short telomeres (Vera and Blasco, 2012). Furthermore, a big shortcoming of the PCR methods is their lack of ability to compare results from different studies and the results are only expressed in relative units, making it difficult to convert them in absolute values, like kilo bases (Samassekou et al, 2010), (Vera and Blasco, 2012). This problem was solved by O'Callaghan et al., by introducing an oligomere standard, being able to measure absolute telomere length (Vera and Blasco, 2012).

1.8.4 STELA

Based on qPCR analysis, a new method called Single telomere analysis, STELA was developed. It is a low throughput, PCR based technique which can quantify the length of individual telomeres and is able to give an estimation on critically short telomeres (Lin and Yan, 2005). STELA works in a way where a single stranded linker called telorette targets the G-rich strand 3' overhang of the telomeric terminus and then gets ligated to the 5' end of the complementary C-rich strand of the telomere (Baird, 2005), (Lin and Yan, 2005). STELA relies on a ligation, PCR related method with universal primers, and a second, chromosome specific primer. STELA technique was developed for the analysis of the telomere at the end of the short arm of human sex chromosomes, XpYp telomere (Baird et al., 2003). Therefore it is only amenable to properly characterised chromosome ends. However, it was already successfully adapted for chromosomes 2p, 11q and 17p (Samassekou et al, 2010). A big advantage of STELA analysis is that it only needs little DNA concentrations, even the assessment of telomere analysis in the picogram range can be performed (Aubert et al., 2012). Another benefit of the STELA approach is its ability to determine accurate sequence composition of telomeric molecules (Baird, 2005). A big limitation of STELA method is that it only measures telomere lengths from 406 bases to a maximum of 20 kilo bases and is therefore not suitable for measuring longer telomeres (Lin and Yan, 2005). Although STELA does not need specialized tools and only little starting material, it is still very labor intensive. A tool called Universal STELA (USTELA) to simultaneously measure short telomeres in all chromosome ends regardless of their characterisation was already developed (Vera and Blasco, 2012). It is a suitable method for measuring absolute numbers of short telomeres and more importantly shorter telomere distribution, but it cannot measure average telomere length, therefore it is not an appropriate method for large scale studies (Aubert et al., 2012), (Bendix et al., 2010).

Method	Short telomeres	Amount of material needed	Unique application
TRF	No	minimum 10^5 cells	Average telomere length of whole cell population
qPCR	No	20 ng DNA	Average telomere length per sample
Q FISH in metaphases	Yes	20 metaphasic live cells	Important for detection of signal-free ends
Q FISH in interphases	Yes	30 non proliferating interphasic cells	Measurement of telomere spots
Flow FISH	No	Minimum $0.5x10^6$ live cells	Comparison of cell subpopulations
HT Q FISH	Yes	$0.01x10^6$ adherent cells	Percentage of cells with critically short telomeres
STELA	Yes	$0.1x10^6$ cells	Critically short telomeres

Table 1: Short overview on different methods and their best application (Baird, 2005)

1.8.5 Choice of appropriate methods

Every method bears some important advantages but also weaknesses (compare table 1), so it is critically essential to know which region is important for the analysis, if the subtelomeric parts are included and whether it is a large scale study or a small study. Furthermore, the right cell preparation for each method has to be kept in mind to reach high accuracy and sensitivity. With its high throughput ability of the highest sample testing, qPCR is perfectly suited for large epidemiological studies.

Therefore, it is not the best choice in detailed studies, or when absolute measurement is required. Flow-FISH is a very accurate method, which is often used in population studies, as well as in long epidemiological studies (Aubert et al., 2012). Moreover, Flow-FISH is the state of the art approach when measuring the complex telomeric sequences of the immune system (Baird, 2005). The best analysis for large studies is therefore reached with qPCR, Flow-FISH and HT Q-FISH whereas in small studies the best approaches are TRF, Q-FISH in metaphasic cells and STELA (Vera and Blasco, 2012). The last two mentioned techniques Q-FISH and STELA are as well perfectly suited for detailed studies reaching a high accuracy and being able to measure short telomeres. When precise measurements are needed, studies have shown that choosing qPCR and TRF are the worst option because of their inability to detect small changes in telomere length (Aubert et al., 2012). Another limitation of TRF analysis is that it is a hybridization based method and therefore not able to estimate the short telomeres because they give the lowest hybridization signal, speaking of a telomere length threshold, which cannot be addressed with this technique (Baird, 2005).

STELA and Q-FISH only need a low amount of cells compared to qPCR and Flow FISH which need a higher amount of cells. TRF even requires much more DNA, so it is absolutely not suited for analysis on rare samples, whereas STELA and Q-FISH are the best approach for those special analysis (Aubert et al., 2012). Due to the fact that telomere length is associated to age-related diseases, further research is needed to find suitable improved tools, able to measure telomere length. Furthermore, methods for the abundance of short telomeres in a cell or in a sample should be assessed, maybe to even predict different maladies, acting as a new biomarker (Lin and Yan, 2005).

2 OBJECTIVES

Telomeres are protective structures at the ends of chromosomes, protecting from chromosome end-to-end fusions and genomic instability. In many studies, it is shown that having shorter telomeres is linked to a higher risk of getting age-related diseases, including cancer. Many methods for measurement of telomere length exist, but no single method fits for screening of telomere length in populations and analysing telomere length at the molecular level at the same time (Aubert et al., 2012). This study tries to develop a method for determination of telomere length which is cheap, simple, does not need a highly equipped lab, only requires a small amount of DNA and at the same time is able to compare results, obtained from different studies. Therefore, the aim of this thesis was to develop and optimize a dot blot method to measure the relative telomeric DNA content in a simple and more time-efficient manner. For this study, DNA from human K562 leukemia cell line was chosen as a control, due to their frequent use of monitoring telomere length in human studies. We aimed to develop this method in order to quantify telomeric DNA content in samples, obtained from the Vienna Active Ageing study. Samples consist of DNA, extracted from whole blood, collected at different time points. Due to the nature of the samples, having a relatively low DNA concentration and variable quality, traditional methods such as qPCR or TRF were not considered as best choice. As dot blot has the advantage of requiring only a small amount of DNA, and does not require high quality material, we chose it as the most appropriate method. Furthermore, we introduced several changes in the original protocol, in order to reach improvements of the technique. Since the lab already conducts qPCR as one of the standard methods, a further aim was to establish TRF, in order to confirm results, obtained with dot blot.

3 MATERIALS & METHODS

Materials:

Acetic acid	Riedel de Haën
Albumine from bovine serum (BSA)	SIGMA-ALDRICH
Boric acid	SIGMA-ALDRICH
Bromophenol blue powder	SIGMA-ALDRICH
Casein	SIGMA-ALDRICH
Clarity Western ECL Substrate	BIO-RAD
CutSmart buffer 10x concentrate	New England Biolabs
DNeasy Blood&tissue kit	QIAGEN
Dulbecco's phosphate buffered saline (PBS)	SIGMA-ALDRICH
EDTA disodium salt dihydrate (Ethylendiamintetraessigsäure Dinatriumsalz-2-hydrat)	Riedel de Haën
Ficoll 400	SIGMA-ALDRICH
GelRed Nucleic Acid Gel Stain (10.000x in water)	BIOTIUM
Glycerol	SIGMA-ALDRICH
HinfI 10.000 U/ml	New England Biolabs
mAb anti-Digoxigenin antibody (21H8) (HRP)	NOVUS BIOLOGICALS
Maleic acid	SIGMA-ALDRICH
Marker Quantitas 200 bp-10 kb	Biozym
Methylene blue	SIGMA-ALDRICH
N – Lauroylsarcosine sodium salt (Sarcosyl)	SIGMA ALDRICH
Polyvinylpyrrolidone	SIGMA-ALDRICH
QuantiFluor ds DNA system	Promega
RNaseA solution	SIGMA-ALDRICH
RsaI 10.000 U/ml	New England Biolabs
Sea Kem LE Agarose	Lonza
Skimmed milk powder (Magermilchpulver)	Fixmilch
Sodium chloride	SIGMA-ALDRICH
Sodium citrate	SIGMA-ALDRICH
Sodium dodecyl sulphate (SDS)	BIO-RAD
Sodium hydroxide	SIGMA-ALDRICH

Sodium phosphate (dibasic)	SIGMA-ALDRICH
Telo DIG	SIGMA-ALDRICH
TRF Telo Alexa 488	SIGMA-ALDRICH
Tris-HCl	SIGMA-ALDRICH
Trizma base	SIGMA-ALDRICH
Tween 20	SIGMA-ALDRICH
Xylene Cyanol FF	SIGMA-ALDRICH

Equipment:

Bio-Dot SF microfiltration apparatus	BIO-RAD
Centrifuge 5417R	eppendorf
Chemi Doc™ XRS+ system Universal Hood II	BIO-RAD
EasyPhor Medi (gel electrophoresis chamber)	Biozym
Incubator model 500	memmert
NanoDrop ND 2000-c	peqlab
PerfectBlue Gel System Mini L	peqlab
pH lab 827	Metrohm
Power Pac HC	BIO-RAD
Shaker Skyline™	ELMI
Trans Blot SD semi-dry transfer cell	BIO-RAD
Vortexer REAX 2000	Heidolph

3.1 Basic procedures for dot blot and TRF analysis

3.1.1 DNA extraction

For method optimization, cancer cells, human K562 leukemia cells were used. Blood was collected from healthy donors, aged 65-98 from the Vienna Active aging study. Peripheral Blood Mononuclear Cells (PBMC's) were separated and viably frozen until further processing. DNA is extracted using DNeasy blood&tissue extraction kit,

according to the manufacturer's instructions with minor modifications. 100 µl of lymphocyte suspension is mixed with 100 µl PBS. Then 20 µl proteinase K is added and the cell pellet is resuspended in 200 µl of AL lysis buffer. The tube gets vortexed and incubated at 56° in a water bath for 40 minutes. 200 µl ethanol (96-100%) are added into the tube and extraction proceeds according to the kit instructions. At the end of the extraction, a freshly prepared AE elution buffer, purged with nitrogen is added into the test tube. DNA samples are stored at -20°C.

3.1.2 Measurement of DNA using Nano drop spectrophotometer

DNA concentration is measured using Nano Drop spectrophotometer and purity is calculated.

- 2 µl of dH_2O are pipetted on the lower measurement pedestal for equilibrating the photometer, 2 µl of AE elution buffer are used as blank.
- 2 µl of each extracted sample are pipetted on the lower measurement pedestal and nucleic acid concentration is measured.
- DNA purity is estimated using the ratio of the absorbance at 260 and 280 nm.

3.1.3 Measurement of dsDNA using QuantiFluor ds DNA system

QuantiFluor dsDNA system for measurement of double-stranded (ds) DNA concentration is used. This kit includes a fluorescent dsDNA-binding dye, which specifically quantifies dsDNA. The kit consists of a 20X TE buffer, a 200X ds DNA dye and a tube of lambda standard with a concentration of 100 µg/ml and a length of 48.5kb.

Using the provided stock solutions, a 1X TE buffer and a standard tube with a dilution of 1:50 in 1X TE buffer are prepared.

Additional standards (B-G) from standard tube A are prepared according to table 3.1. Standard, blank and samples are pipetted into a 96er well plate (200 µl assay) and measured in triplicate. Samples are prepared in a 1:100 dilution in 1X TE buffer. 200X dsDNA dye is diluted to a 1X dsDNA dye in 1X TE buffer. 100 µl of standard, sample or blank are added in each well in triplicate, followed by 100 µl of the freshly prepared dsDNA dye, carefully mixed in the wells, incubated for 5 minutes in the dark.

Standard	Volume standard	1X TE buffer	Final ds DNA concentration after adding dye
Blank	0 µl	1000 µl	0 ng/ml
A	20 µl lambda DNA standard	980 µl	1000 ng/ml
B	250 µl standard A	750 µl	250 ng/ml
C	250 µl standard B	750 µl	62.5 ng/ml
D	250 µl standard C	750 µl	16 ng/ml
E	250 µl standard D	750 µl	3.9 ng/ml
F	250 µl standard E	750 µl	1 ng/ml
G	250 µl standard F	750 µl	0.2 ng/ml

Table 3.1: Pipetting scheme for ds DNA concentration measurement using QuantiFluor ds DNA system

The plate is measured at 504 nm excitation and 531 nm emission. Calculations are made, subtracting the fluorescence of the blank from the raw data. Linear regression calculation of the standard curve is made and concentrations of the unknown samples, based on this standard curve are calculated.

3.1.4 Hybridization technique and detection with chemiluminescent substrates

In this study, a fluorescently synthetic single-stranded oligonucleotide probe is used. At this probe, the fluorescent label Alexa 488 is directly bound to the probe. This probe is used because the Chemi Doc^{TM} XRS+ system can detect the fluorophore of Alexa 488. The TRF Telo Alexa 488 consists of 4 (TTTAGG) oligonucleotide repeats, meaning it has a length of 24 bases and a T_m of 64.8 °C. The total amount of the probe Telo Alexa 488 is 44.8 µg in a dry condition. In order to get a final concentration of 0.1 µg/µl, 448 µl of an AE elution buffer are added and carefully mixed.

A second probe is used, Telo DIG with a sequence of 3 (CCCTAA) oligonucleotide repeats, which are complementary to the canonical telomeric sequence. This oligonucleotide probe is labelled at the 3'end with digoxigenin (DIG). Telo DIG has a length of 18 bases and a T_m of 55.6 °C. The concentration of Telo DIG is 100 ng/µl and the amount is 28.6 µg. 286 µl of the AE elution buffer are added directly into the tube.

After hybridization, the Telo DIG probe is detected with anti-DIG antibody and chemiluminescent substrates. The Telo DIG probe is chosen, because the antibody used in this study reacts with free and bound digoxigenin. The monoclonal antibody is directly coupled to the enzyme horseradish peroxidase (HRP), which functions as a marker enzyme. The concentration of the antibody is 0.2 mg/ml, and dilution should be made, ranging from 1:500 to 1:5000.

The substrate, used in this study is the chemiluminescent type, because of its speed and sensitivity. In the presence of peroxide, HRP enzymes catalyze a reaction, the oxidation of luminol, thereby generating light. Clarity western peroxide reagent and Clarity western luminol/enhancer reagents are mixed 1:1 in a test tube, placed on the membrane at the crosslinker, are incubated for 30 seconds in the dark and images are taken.

3.2 Dot blot study for telomeric content measurement

3.2.1 Principle of the method

The principle of using dot blot for measuring telomeric length is that each dot on the membrane gives information on the relative telomeric DNA content. The DNA content is measured using the DNA stain (Dx) and the telomeric DNA content is measured using the telomeric probe (T). The ratio of T/Dx, the telomeric DNA signal in relation to the total DNA signal represents the amount of repeats per unit DNA (Kimura and Aviv, 2011). In order to find optimal conditions for the application, several different approaches are used. The detailed protocols are listed below.

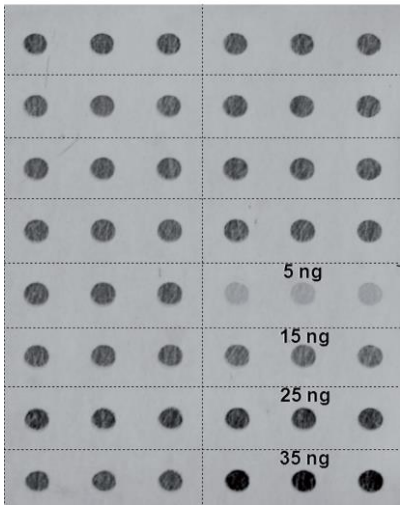

Figure 3.1: A typical dot blot (Kimura and Aviv, 2011)

3.2.2 Choosing the right membrane

There are several types of membranes, such as nylon, nitrocellulose and Polyvinylidene Fluoride (PVDF). According to Transfer Membranes booklet by AppliChem, the right choice of membrane depends on the type of blotting, reprobing, and the type of probe used in the analysis. The appropriate membrane is a critical parameter for the success of nucleic acid analysis. The membrane, used in this study is a positively charged hydrophilic nylon membrane, which belongs to the supported class, according to

Amersham Hybond-N+ product booklet by GE Healthcare, it is characterised with high physical strength and high binding of nucleic acids.

3.2.3 First dot blot preparation using Telo Alexa 488 probe

Buffer	Content
0.5X TAE buffer	25 ml of 10X TAE mixed with 475 ml dH_2O
10X SSC	87.65 g NaCl, 44.1 g Sodium citrate, dissolved in 800 ml with dH_2O, adjusted to pH=7, filled up to 1000 ml with dH_2O
10X TAE buffer	24.2 g Tris base, 5.71 ml Acetic acid, 1.85 g Disodium EDTA are filled up to 500 ml dH_2O
Hybridization buffer	5X SSC, 0.1% Sarcosyl, 0.5% SDS, 0.1 g/L Bovine serum albumin (BSA), dissolved in 100 ml dH_2O
Methylene blue solution	0.1% methylene blue in 0.4M sodium acetate
Wash buffer 1	2X SSC, 0.1% SDS, filled up to 1000 ml dH_2O
Wash buffer 2	2X SSC, filled up to 1000 ml dH_2O
Wash buffer TAE	50ml 10X TAE mixed with 950 ml dH_2O
Wash buffer X	0.2X SSC, 0.1% SDS

Table 3.2: Buffer preparation for 1st dot blot protocol

3.2.4 First dot blot protocol using Telo Alexa 488 probe

- DNA is diluted in an appropriate amount of AE buffer
- 2 µl of each standard are added carefully on the membrane, avoiding touching the membrane with gloves
- The membrane is rinsed in wash buffer 2, dried and UV-cross-linked DNA side down for 5 minutes using Chemi Doc™ XRS+ system, essential for DNA fixation.
- For blot staining, the membrane is washed with dH_2O for 10 minutes, placed in 0.5XTAE (Tris Acetate EDTA) and stained with a 1:10.000 dilution, of GelRed in 0.5X TAE buffer

  Optionally, the membrane is stained with a 1:333 dilution, or 1:5000 dilution of GelRed in 0.5X TAE buffer

- The membrane is washed with wash buffer TAE for 10 minutes, the buffer is discarded and the washing procedure is repeated
- The membrane is placed on the Chemi Doc™ XRS+ system, DNA side down and images of the membrane are taken using Image Lab™ software

  To reduce high background, a 5% BSA solution in 20 ml of 0.5XTAE is used, and the membrane is soaked in 0.5X TAE for 5 minutes. GelRed stain is reused and staining is performed. When using a freshly prepared solution, staining should proceed for 30 minutes (GelRed Biotium). Images are taken, using Chemi Doc™ XRS+ system and Image Lab™ software.

Optionally: a methylene blue solution replaces GelRed staining. Methylene blue is prepared using 0.4M sodium acetate in dH_2O.

The methylene blue solution, which works as a nucleic acid specific stain is used as a control for DNA fixation and poured over the membrane, incubated for 5 minutes.

The membrane is washed off from methylene blue, using dH_2O, the membrane is placed on the shaker again, leaving for another 5-10 minutes.

The methylene blue solution is poured in a tube for reuse and stored at room temperature.

The membrane is placed on the Chemi Doc™ XRS+ system to detect DNA after methylene blue procedure and images are taken.

- The membrane is prehybridized, by soaking in hybridization buffer for 2 hours on a shaker at 55°C
- 8 µl of the probe are added into 10 ml of the hybridization buffer, to contain a total amount of 400 ng of the probe
- After prehybridization, the hybridization buffer is discarded and the hybridization solution is added to the membrane, incubated over night at 55°C
- The next day, the hybridization solution is removed and stored at -20°C for reuse
- The membrane is washed using wash buffer 1 twice, each 15 minutes at room temperature
- After discarding wash buffer 1, the membrane is washed in wash buffer X for 10 minutes at 55°C
- The membrane is placed on Chemi Doc™ XRS+ system and images are taken

3.2.5 Second dot blot preparation using Telo Alexa 488 probe

Buffer	Preparation
100X Denhart's solution	2% BSA, 2% Ficoll 400, 2% Polyvinylpyrrolidone, filled up with 50 ml dH_2O
EDTA solution 200mM	29.224 g 200mM EDTA, filled up with 500 ml dH_2O
Hybridization buffer	5X SSC, 0.1% Sarcosyl, 0.5% SDS, 0.1 g/L BSA, 1 ml of 100X Denhart's solution, filled up to 100ml with dH_2O
Methylene blue solution	0.1% methylene blue in 0.4M sodium acetate
NaOH solution 1M	20 g 1M NaOH filled up with 500 ml dH_2O

Table 3.3: Buffer preparation for 2nd dot blot protocol

3.2.6 Second dot blot protocol using Telo Alexa 488 probe

- A positively charged nylon membrane is pre-wetted in d H_2O.
- The samples are diluted in an appropriate buffer, containing 1M NaOH and 0.2M EDTA at pH=8.2, to reach a final concentration of 6X SSC, 0.4M NaOH and 0.01M EDTA
- A stock of 50 ng/µl in a total volume of 20 µl in each dilution is prepared, using the appropriate amount of AE buffer
- A standard dilution is prepared, containing a concentration, ranging from 2.5 ng/µl to 17.5 ng/µl (see table 2.1)
- DNA is denatured by placing the sample in a 100°C water bath for 10 minutes
- The samples have to be put immediately on ice

- Still on ice, samples are vortexed and 2 µl of each sample are applied on the membrane
- The membrane is placed on the Chemi Doc^{TM} XRS+ system for DNA fixation, DNA side down, exposed for 5 minutes

Optionally: the methylene blue solution is poured over the membrane, incubated for 5 minutes. The membrane is washed off from methylene blue, using dH_2O, the membrane is placed on the shaker, leaving for another 5-10 minutes.

The membrane is placed on the Chemi Doc^{TM} XRS+ system to detect DNA after methylene blue procedure and images are taken.

- The membrane is prehybridized for 2 hours. The content of the hybridization buffer is changed, using 0.5% SDS and 1 ml of Denhart's solution in 100 ml of the hybridization buffer as a mixture of blocking agents
- For preparation of the hybridization solution: 4 µl of the probe is added in 15 ml hybridization buffer and poured on the membrane to incubate over night at 55°C
- Continue with washing steps using wash buffer 1 two times each 10 minutes at and wash buffer X once for 15 minutes at 55°C
- The membrane is dried and images are taken

Concentration	NaOH solution	EDTA solution	Standard	H_2O
17.5 ng/µl	8 µl	1 µl	7 µl	4 µl
15 ng/µl	8 µl	1 µl	6 µl	5 µl
12.5 ng/µl	8 µl	1 µl	5 µl	6 µl
10 ng/µl	8 µl	1 µl	4 µl	7 µl
5 ng/µl	8 µl	1 µl	3 µl	8 µl
2.5 ng/µl	8 µl	1 µl	2 µl	9 µl

Table 3.4: Dilution row for 2nd dot blot protocol

3.2.7 Dot blot protocol using a manifold and Telo Alexa 488 probe

- The membrane is pre-wetted in dH_2O for 10 minutes
- The manifold is prepared, a piece of Whatman 3MM filter paper is soaked in dH_2O and placed on top of the manifold
- The membrane is placed on this filter paper and air bubbles are carefully rolled out
- The unit is closed and the vacuum line is connected
- 1M NaOH and 200mM EDTA are pipetted to the samples, reaching a final concentration of 0.4M NaOH and 10mM EDTA, to get a final volume of 100 µl in each reaction
- Samples are analysed in triplicates
- To reach a final concentration of 5 ng/µl in the diluted samples, the standard tube consists of 5 µl DNA suspension of K562 cells with 826.9 µl elution AE buffer

Concentration	NaOH solution	EDTA solution	Standard	H_2O
2.5 ng/µl	160 µl	20 µl	4 µl	216 µl
5 ng/µl	160 µl	20 µl	8 µl	212 µl
7.5 ng/µl	160 µl	20 µl	12 µl	208 µl
10 ng/µl	160 µl	20 µl	16 µl	204 µl
12.5 ng/µl	160 µl	20 µl	20 µl	200 µl
15 ng/µl	160 µl	20 µl	24 µl	196 µl
17.5 ng/µl	160 µl	20 µl	28 µl	192 µl
Control	160 µl	20 µl		220 µl

Table 3.5: Pipetting scheme for manifold dot blot protocol in triplicate analysis

- The samples are denatured in a 100°C water bath for 10 minutes
- Samples are put on ice
- The suction of the manifold is switched on, 500 µl of dH_2O is applied on each well
- Samples are vortexed and 100 µl of each sample is applied on the membrane in triplicate analysis
- Each well is rinsed with 500 µl 0.4M NaOH and the membrane is dried
- The membrane is placed on the Chemi Doc™ XRS+ system, DNA side down and crosslinked for 5 minutes
- Proceed with hybridization and washing steps using 4 µl of Telo Alexa 488 probe in 8ml hybridization buffer with Denhart's solution
- For washing on the next day: the membrane is washed with wash buffer 1 two times each 15 minutes at room temperature and wash buffer X for 15 minutes at 55 °C in the incubator
- The membrane is dried and images are taken

3.2.8 Dot blot preparation using Telo DIG probe and mAb anti-DIG

Buffer	Preparation
0.4M NaOH	8 g NaOH, filled to 500 ml dH_2O
Antibody solution	4 µl antibody in 8ml PBS
Antibody wash buffer	50 ml PBS and 50 µl Tween 20
Hybridization buffer	5X SSC, 0.1% Sarcosyl, 0.5% SDS, 1% BSA, dissolved in 100 ml dH_2O
Hybridization solution	8 µl Telo DIG in 15 ml hybridization buffer
PBS blocking solution	50 ml PBS, 0.1% Tween 20, 1% BSA

Table 3.6: Buffer preparation for dot blots using mAb anti-DIG

3.2.9 Dot blot protocol using Telo DIG probe and mAb anti-DIG

- A small piece of positively charged nylon membrane is cut
- DNA is denatured in a water bath at 100°C for 10 minutes
- DNA is placed immediately on ice
- Still on ice, 2 µl of DNA are spotted on the membrane in triplicate analysis
- The membrane is placed on a pre-wetted 0.4M NaOH filter paper for 5 minutes
- The membrane is UV crosslinked using Chemi Doc™ XRS+ system, exposed for 5 minutes
- The membrane is placed in a plastic bag and prehybridized with the hybridization buffer for 1 hour at 65°C on the shaker in the incubator
- 8 µl Telo DIG are pipetted into 15 ml of the hybridization buffer, poured over the membrane and incubated overnight on the shaker in the incubator
- Washing procedure starts using wash buffer 1 two times at room temperature for 15 minutes and wash buffer X once for 10 minutes at 65°C in the incubator
- After washing, a PBS blocking solution is prepared consisting of 50 ml PBS, 0.1% Tween, 1% BSA
- The blocking solution is poured on the membrane, incubated for 1 hour at room temperature
- The blocking solution is discarded and the antibody solution is prepared using a 1:2000 dilution in PBS
- The antibody solution consists of 4 µl antibody in 8 ml PBS and is poured over the membrane, incubated for 90 minutes at room temperature
- The antibody-solution is freezed for reuse
- The membrane is washed in antibody wash buffer twice for 15 minutes at room temperature
- 1ml of each chemiluminescent (ECL) reagent is mixed in a tube and pipetted on the membrane
- The membrane is placed on the Chemi Doc™ XRS+ system and DNA is detected

3.3 Southern blotting of TRF

3.3.1 Principle of TRF analysis

For Southern blotting, digestion of genomic DNA has to be prepared one day before, followed by separation of DNA fragments by gel electrophoresis, transfer from the agarose gel to the positively charged nylon membrane, hybridization with the Telo DIG probe and detection with the DIG-specific antibody, using chemiluminescent substrates. The enzymes for DNA digestion, used in this study are frequently used cutters HinfI and RsaI (see figure 3.2 and 3.3) because this is the most used well understood combination, minimizing the subtelomeric regions, thereby increasing the accuracy of the analysis (Lin and Yan, 2005).

A semi-dry transfer cell is chosen for the transfer (Brown, 2004), according to BIO-RAD product sheet, this method guarantees a more rapid transfer without destroying the gel, does not need pre-treatment of the gel and only requires a small amount of transfer buffer. In the semi-dry transfer cell, the membrane and gel are sandwiched between two extra thick Whatman 3MM filter papers which are in direct contact to the electrodes, thus the field strength guarantees a maximized transfer. Of note, it is very important that the filter papers and the membrane should have the same size as the gel, referred to protein blotting book by Millipore, the current can flow through the gel properly without any disturbances. Critical parameters, such as current and transfer time have to be selected accurately, to achieve a complete transfer of the nucleic acids, as well as to avoid too fast migration of nucleic acids.

Figure 3.2: Sites where HinfI cuts the sequence (neb.com)

Figure 3.3: Sites where RsaI cuts the sequence (neb.com)

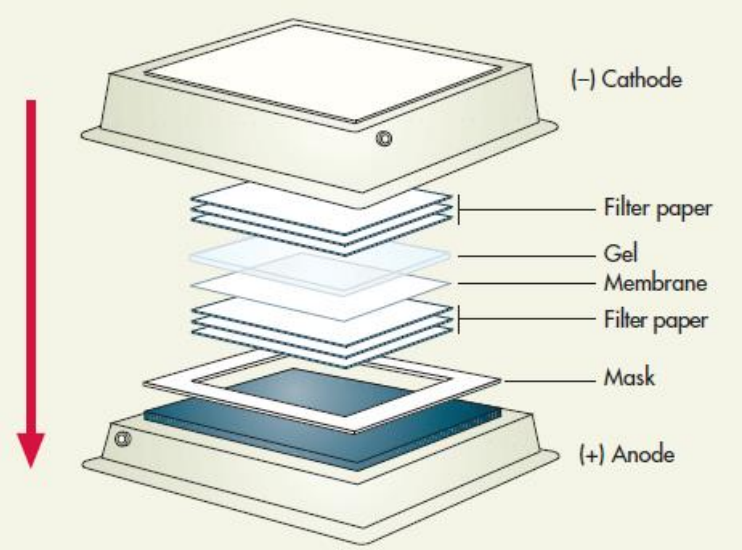

Figure 3.4: Basic principle of a semi-dry transfer cell ("protein blotting handbook" Millipore)

3.3.2 Preparation of TRF analysis

Buffer	Preparation
0.5X TBE buffer	50 ml of 10X TBE buffer, filled with 950 ml dH_2O
10X TBE buffer	121.1 g NaCl, 61.8 g Boric acid, 7.4 g EDTA disodium salt, mixed with 1000 ml dH_2O
1X TAE buffer	100 ml of 10X TAE buffer, filled to 1000 ml with dH_2O
Antibody solution	8 µl antibody in 15 ml PBS
Antibody wash buffer	50 ml PBS and 50 µl Tween 20
PBS blocking solution	50 ml PBS, 0.1% Tween 20, 1% BSA

Table 3.7: Buffers for TRF analysis

3.3.3 Protocol for TRF analysis

- Genomic DNA is digested using equal volumes of restriction enzymes RsaI and HinfI
- 10 µl of isolated cell suspension, 1 µl RsaI (10U/µl), 1 µl HinfI (10U/µl) enzymes (pipetted directly on ice) and 1X CutSmart are needed for each reaction.
- This reaction tube is incubated over night at 37°C in a water bath
- On the next day, samples are taken out of the water bath, stored in the fridge and 0.8 volume Isopropanol is mixed to each sample
- Samples are incubated in the freezer for 50 minutes and centrifuged twice at 14.000 rpm at 4° C, each 10 minutes
- The supernatant is poured away and the pellet is washed in 500 µl of 70% ice cold ethanol
- 10 µl AE buffer is added to the cell pellet in the tube and the samples are boiled for denaturation in a 100°C water bath for 10 minutes
- Samples are put immediately on ice and the DNA marker (200 bp - 10 kb) is prepared
- 10 µl of the marker are added in a new tube, mixed with 4 µl loading dye on a vortexer
- For the samples: 4 µl loading dye are pipetted to each sample and are vortexed
- A 0.7% agarose gel is prepared in 120ml 1X TAE buffer and 3X GelRed is added
- After polymerization of the gel, the electrophoresis chamber is filled with 1X TAE buffer and samples are applied in the gel slots
- The gel voltage is set to 30 V for 3 hours
- After the run is finished, the transfer cell is set
- A piece of a positively charged nylon membrane is cut to the same size as two sheets of Whatman 3MM filter papers
- Before use, the 0.5X TBE buffer is chilled in the freezer for 10 minutes
- The nylon membrane is soaked in 0.5X TBE buffer for 15 minutes
- The filter papers are soaked in 0.5X TBE buffer for 5 minutes

- The semi-dry transfer cell is opened and the mask is placed on the anode
- The first pre-wetted filter paper is placed in the electrophoresis semi-dry transfer cell
- The soaked membrane is placed on the filter paper and edges of the paper and membrane should get aligned
- The gel, soaked in 0.5X TBE for five minutes is placed on top of the membrane
- The other pre-wetted filter paper is placed on the gel, and air bubbles are carefully rolled out
- The cathode plate is placed on the gel and with the safety lid the transfer cell is closed
- The power supply is switched on and settings for a constant current of 0.4 ampere are made for a duration of 11 minutes
- The power supply is switched off, the lid is carefully opened and the membrane and the gel are taken out of the transfer cell
- A filter paper, already pre-wetted in 0.4M NaOH is placed in a plastic box and the membrane is placed on top of this filter paper for 5 minutes
- The membrane is crosslinked for 5 minutes in the Chemi Doc™ XRS+ system and the gel is checked for remaining DNA using Image Lab™ software
- The membrane is placed in the hybridization buffer, using 1% BSA and is incubated for an hour at 66°C
- 8 µl Telo DIG are added into 15 ml of the hybridization buffer, poured over the membrane and incubated overnight on the shaker in the incubator
- The solution is freezed for reuse of the probe and washing procedure starts using wash buffer 1 at room temperature two times each 10 minutes and wash buffer X at 66°C once for 10 minutes
- After washing, a PBS blocking solution is prepared, the membrane is placed in a plastic bag and the PBS solution is poured on the membrane, incubated on a shaker for 1 hour at room temperature
- The PBS solution is discarded and the antibody solution, 8 µl antibody in 15 ml PBS is poured over the membrane and incubated on a shaker for 90 minutes

- The membrane is washed in Antibody wash buffer twice for 15 minutes at room temperature
- The membrane is taken out of the wash buffer and 1ml of each enhanced chemiluminescent (ECL) reagent is mixed in a tube and pipetted on the membrane
- The membrane is placed on the Chemi Doc^{TM} XRS+ system and visualized using chemiluminescence, exposed for different duration, depending on the signal

4 RESULTS & DISCUSSION

Accurate measurement of telomere length critically lies on the chosen specific application. Most methods require high quality starting material, which is not always easy to obtain. Therefore, dot blot was chosen for samples measurement, since it does not require high quality DNA samples.

4.1 DNA concentration

At the beginning of the procedure, Nano drop spectrophotometer was chosen to measure the concentration of DNA, extracted from K562 cancer cells. As a matter of fact, when using Nano drop, the spectrophotometer cannot discriminate between dsDNA, ssDNA and RNA. All concentrations measured at this time point were false positive, because the measured concentration was far too high and DNA from cancer cells is very high in RNA. Since RNase was not used, a DNA concentration measurement system for dsDNA had to be introduced. Therefore, QuantiFluor dsDNA system which specifically measures dsDNA was used and DNA of cancer cells as well as DNA samples were measured using this kit. Since regression coefficient, R^2 in statistical analysis was always higher than 0.95, this system was the method of choice for measuring all DNA samples. Table 4.1 shows raw data after measuring with QuantiFluor dsDNA kit, in a 96er well plate assay to calculate unknown concentrations of DNA samples.

Concentration (ng/ml)	Mean fluorescence
Standard A =1000	57336.5
Standard B = 250	16387
Standard C = 62.5	4108
Standard D = 16	993
Standard E = 3.9	253.5
Standard F = 1	60
Standard G = 0.2	17

Table 4.1: Raw data of fluorescence after measuring with QuantiFluor dsDNA system

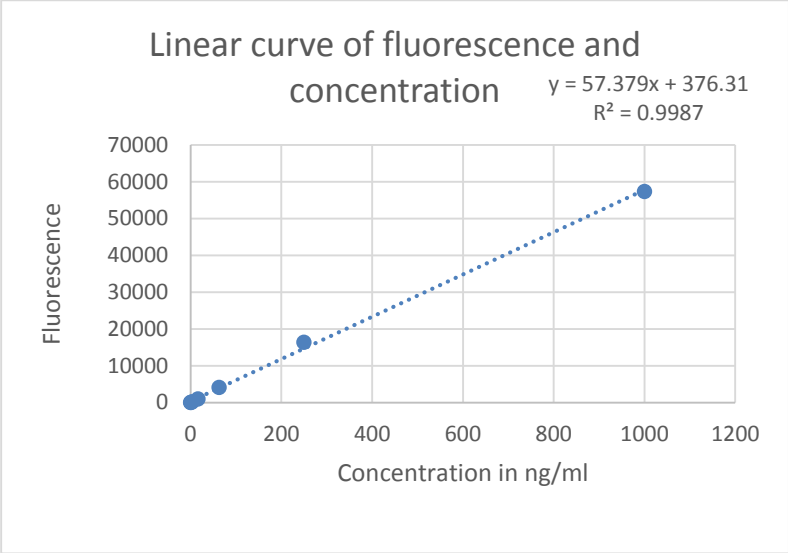

Figure 4.1: Linear curve of fluorescence and concentration based on QuantiFluor dsDNA system

4.2 Dot blot

Using the first dot blot protocol, GelRed was chosen as the appropriate DNA stain, since it is sensitive, non-toxic and has a great affinity to bind dsDNA as well as to recognize ssDNA. At the beginning of the experiments, a hybridization procedure was not performed, therefore staining of the membranes with GelRed was chosen to just check blotting efficiency of the membrane. The samples in figure 4.2, DNA extracted from K562 cancer cells were measured with Nano drop spectrophotometer, had a concentration of 321.8 ng/µl with a purity ratio A260/A280 of 1.94.

The samples were denatured at 100°C in a water bath for 10 minutes prior to blotting. This dot blot shows 4 dots containing 35 ng of DNA in 4 µl, when using only GelRed staining procedure in a concentration of 1:3333 in 0.5X TAE buffer, crosslinked for 5 minutes.

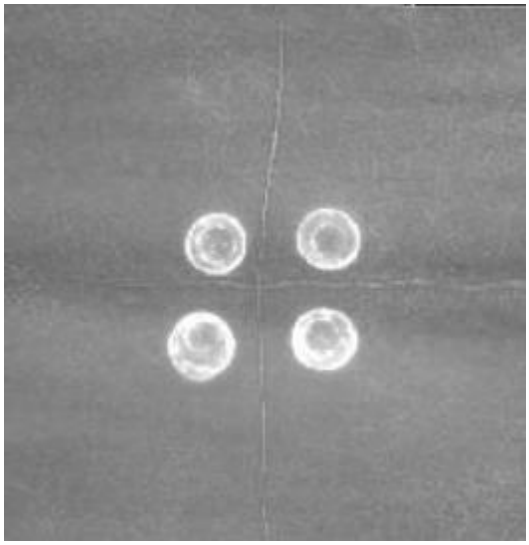

Figure 4.2: Dot blot using first dot blot protocol

In figure 4.3, DNA concentration of K562 cells, measured with Nano drop was 399.8 ng/µl with a purity ratio A260/A280 of 2.02. Samples were diluted in AE elution buffer to reach a concentration of 30 ng/µl. To make sure if the positively charged membrane needs to be pre-wetted prior to hybridization, a control experiment was performed. Picture a) shows a membrane which was pre-wetted in dH2O prior to sample application. Figure b) shows a membrane where samples were applied directly on the dry membrane. The dot blot volume was 4 µl and 2 µl. Both membranes were stained with methylene blue and destained with dH_2O for the same duration. This test experiment evidenced, that it is critical essential, that dots are applied on the dry membrane, otherwise the samples blur on a wet membrane and cannot even get detected.

a) b)

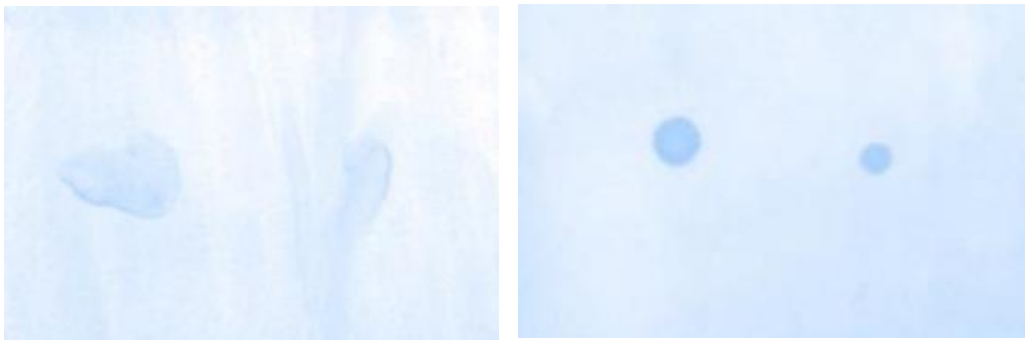

Figure 4.3: Methylene blue detection using first dot blot protocol

Using second dot blot protocol, samples were diluted in 1M NaOH and 0.2M EDTA because NaOH as an alkali solution helps keeping the DNA sample denatured after boiling in the water bath. EDTA is a chelat binder, which binds divalent cations, which otherwise would stabilize DNA duplexes. This sample preparation was chosen to ensure DNA was single-stranded, critical essential for hybridization. The sample, used in figure 4.4, extracted from K562 cancer cells, measured with Nano drop had a concentration of 333.2 ng/µl with a purity ratio A260/A280 of 2.05. This sample was diluted in different concentrations ranging from 17.5 ng/µl in the first row to 2.5 ng/µl in the last row. Different concentrations of DNA, extracted from cancer cells were used to see if the more diluted dots, applied on the membrane had a different content than the less diluted dots. Spots were applied in triplicates and visualized with GelRed, using a concentration of 1:3333 in 0.5X TAE buffer. Figure 4.4 shows a great variability between triplicates but no real differences when analysing the dots with different concentrations. However, it was expected to observe some differences between the different concentrations but the signal did not seem to follow a decline.

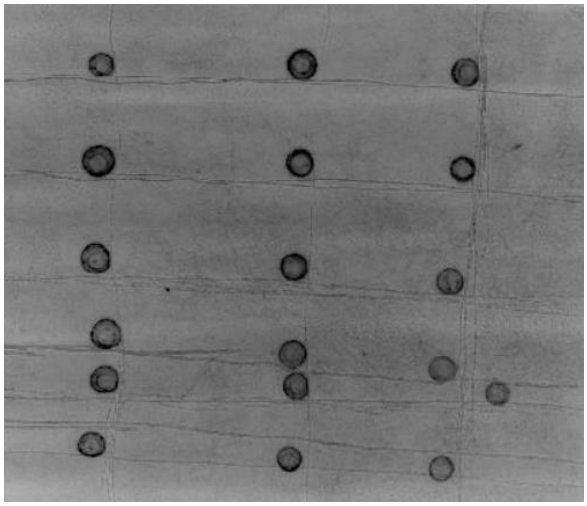

Figure 4.4: Dot blot using second dot plot protocol without hybridization

Figure 4.5 shows the same membrane used in figure 4.4 but already after hybridization with hybridization buffer containing 0.5% SDS and Denhart's solution. The signal did not change significantly after hybridization compared to non-hybridized membranes, most likely due to a carry-over of fluorescence from GelRed. In addition, the signal to background ratio declined, which indicates a non-specific binding of Telo Alexa 488 probe to the membrane. When it was observed that GelRed gives a signal alone, even without binding to DNA, GelRed staining was excluded from the whole experiment.

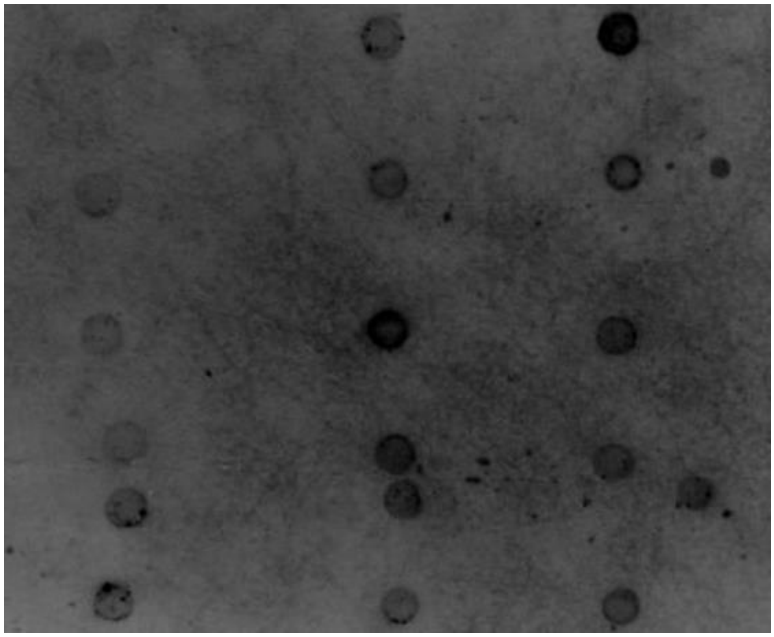

Figure 4.5: Dot blot using second dot blot protocol after hybridization

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As GelRed staining did not seem to work since there was always a carry-over of fluorescence and no differences in concentrations were observed, hybridization with Telo Alexa 488 probe was introduced to detect specific telomeric sequences of interest. Alexa fluor dyes are hydrophilic, fluorescent dyes with a laser excitation wavelength of 488 nm. Telo Alexa has 4(TTTAGG) oligonucleotide repeats, where the fluorescent label Alexa 488 is bound to the probe.

In figure 4.6, DNA concentration of K562 cancer cells, after measuring with QuantiFluor dsDNA system was 185.2 ng/µl. DNA was diluted in an appropriate amount of AE elution buffer. Different concentrations were used ranging from 160 µl in the first row to 2.5 µl in the last row (not shown on this picture). Hybridization was performed using Telo Alexa 488 probe. The dots on this membrane were analysed using Image Lab™ software and linear curves, based on volume and number of pixels in each dot were calculated.

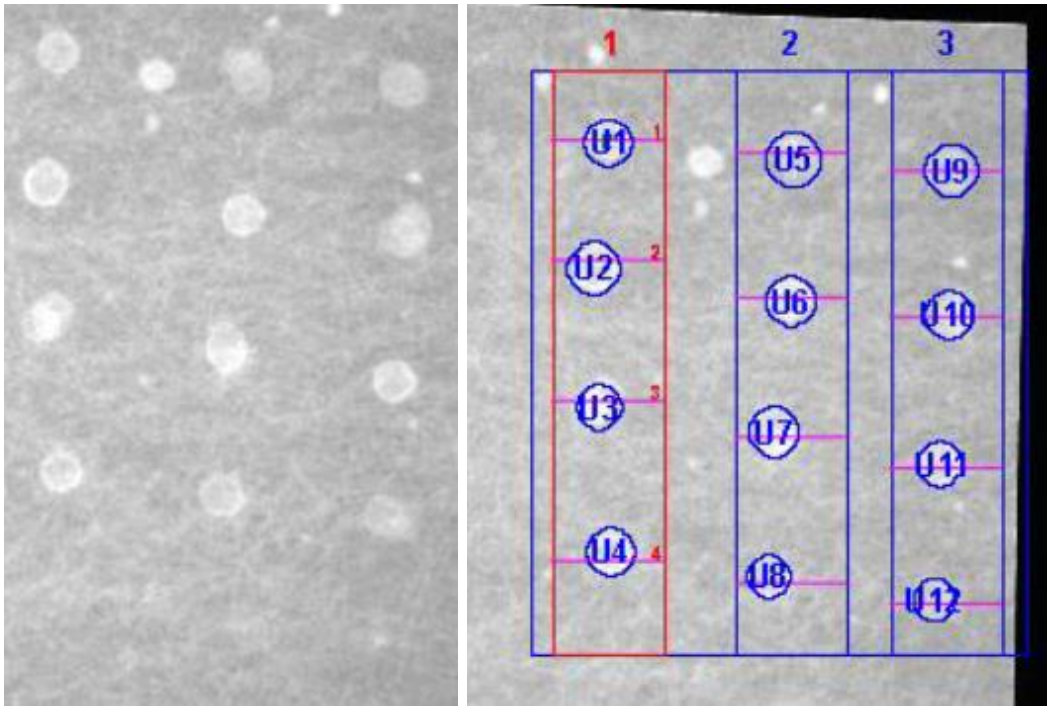

Figure 4.6: Dot blot using first dot blot protocol

This was the first successful blot where the amount of telomeric sequences could be estimated, using a standard curve for volume and concentration, as well as a standard curve for number of pixels and concentration. However, the background noise was still relatively high.

	Volume	Mean	Concentration
U1	9.272.704	11276142	160 ng/µl
U5	11.998.192		
U9	10.554.092		
U2	12.066.748	10000546	80 ng/µl
U6	10.752.804		
U10	9.248.288		
U3	9.206.100	8845300	40 ng/µl
U7	11.060.888		
U11	8.484.500		
U4	9.737.196	7284166	20 ng/µl
U8	7.521.484		
U12	7.046.848		

Table 4.2: Raw data of volume and concentration based on figure 4.6

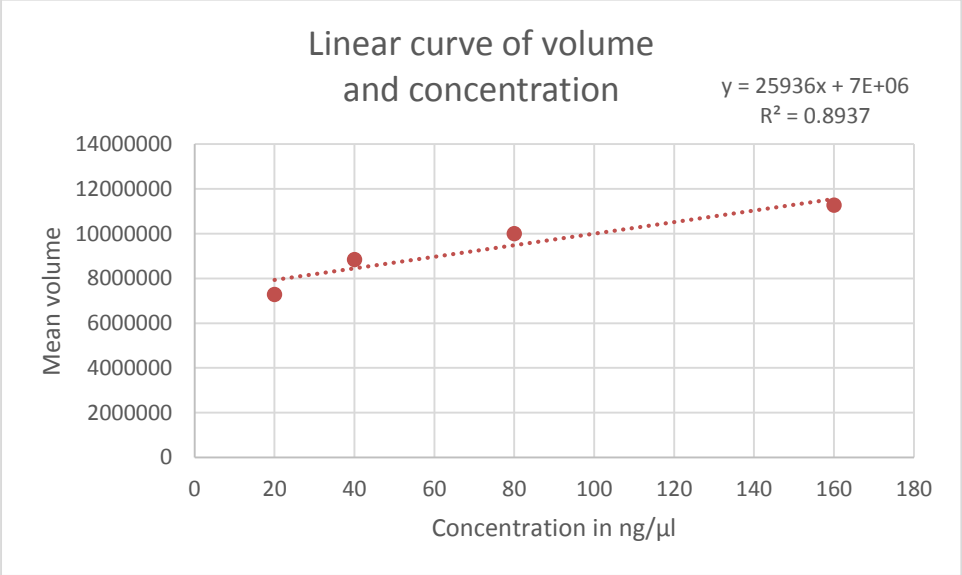

Figure 4.7: Linear curve of volume and concentration based on figure 4.6

	Number of pixels	Mean	Concentration
U1	1.041	1353	160 ng/µl
U5	1.421		
U9	1.285		
U2	1.285	1101	80 ng/µl
U6	1.161		
U10	1.041		
U3	929	929	40 ng/µl
U7	1.161		
U11	929		
U4	1.041	829	20 ng/µl
U8	829		
U12	829		

Table 4.3: Raw data of pixels and concentration based on figure 4.6

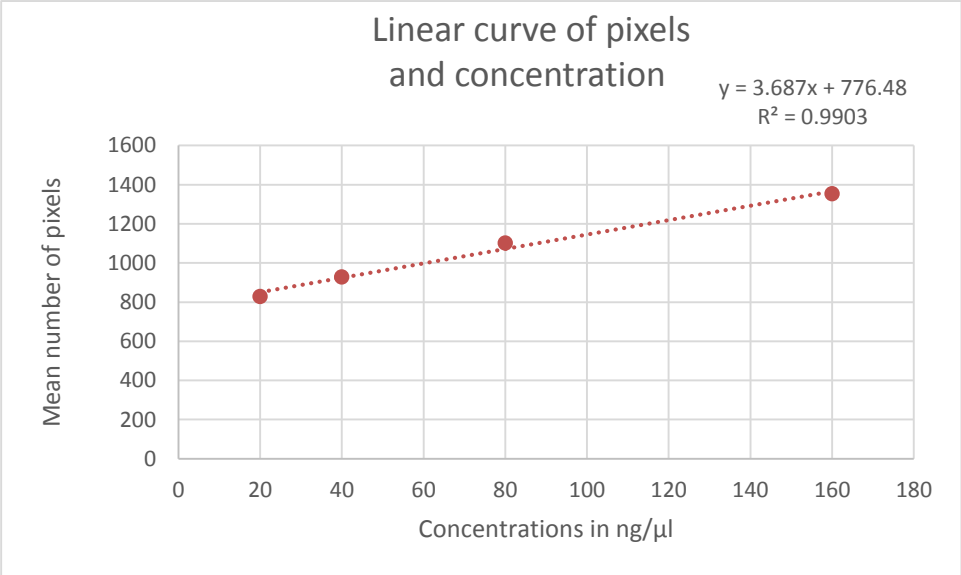

Figure 4.8: Linear curve of pixels and concentration based on figure 4.6

In figure 4.9, DNA concentration of K562 cancer cells, after measuring with QuantiFluor dsDNA system was 124 ng/µl. The sample was diluted in AE buffer, 1 M NaOH and 0.2M EDTA to reach a final concentration of 0.4M NaOH and 0.01M EDTA. Different concentrations ranging from 17.5 ng/µl in the first row to 7.5 ng/µl in the last row were prepared. Figure 4.9 shows the membrane after methylene blue staining, crosslinked for

5 minutes. The data of the dots on this membrane were analysed using Image Lab™ software and linear curves based on volume and number of pixels were calculated.

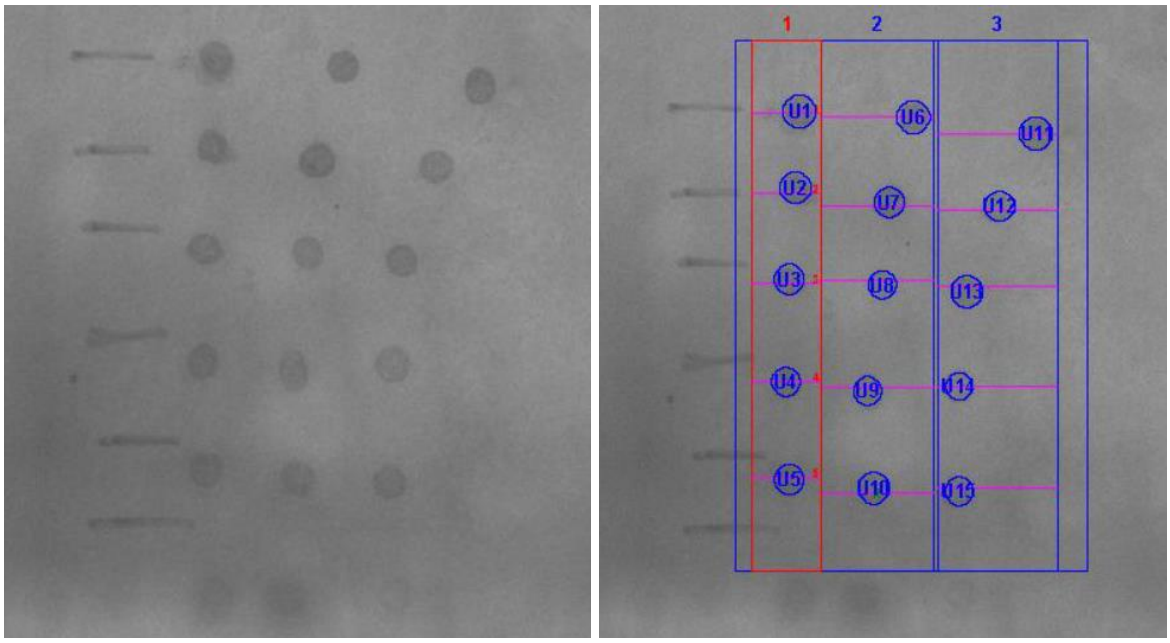

Figure 4.9: Dot blot using second dot blot protocol and methylene blue staining

	Volume	Mean	Concentration
U1	82.170.583	82015009	17.5 ng/µl
U6	81.772.307		
U11	82.102.139		
U2	74.524.691	67168075	15 ng/µl
U7	67.637.731		
U12	66.698.419		
U3	67.403.651	63732373	12.5 ng/µl
U8	60.061.095		
U13	74.336.839		
U4	61.003.531	58266669	10 ng/µl
U9	60.351.171		
U14	53.445.307		

Table 4.4: Raw data of volume and concentration based on figure 4.9

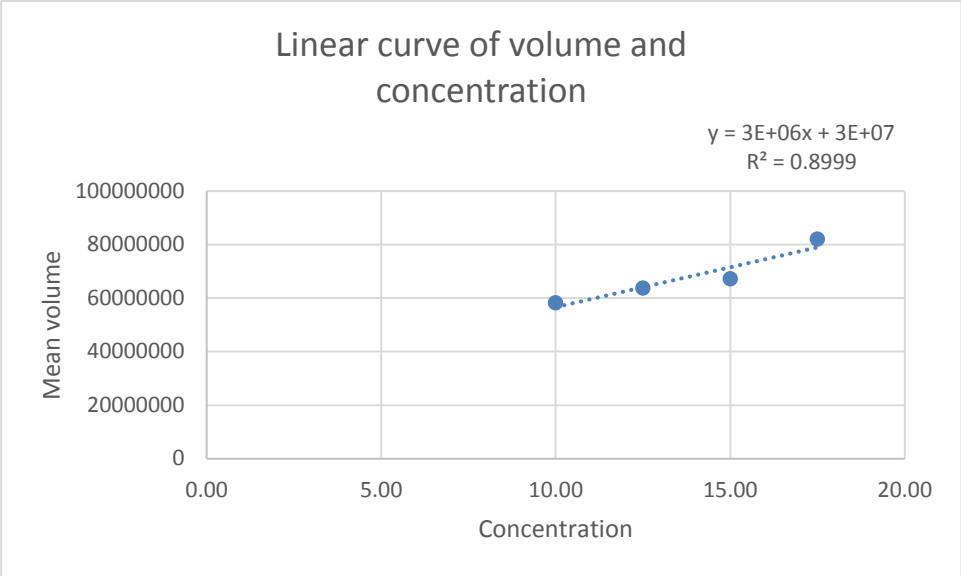

Figure 4.10: Linear curve of volume and concentration based on figure 4.9

	Pixels	Mean	Concentration
U1	1421	1421	17.5 ng/µl
U5	1161		
U9	1041		
U2	1285	1161	15 ng/µl
U6	1421		
U10	1285		
U3	1161	1101	12.5 ng/µl
U7	1161		
U11	1421		
U4	1041	1004	10 ng/µl
U8	1041		
U12	1161		

Table 4.5: Raw data of pixels and concentration based on figure 4.9

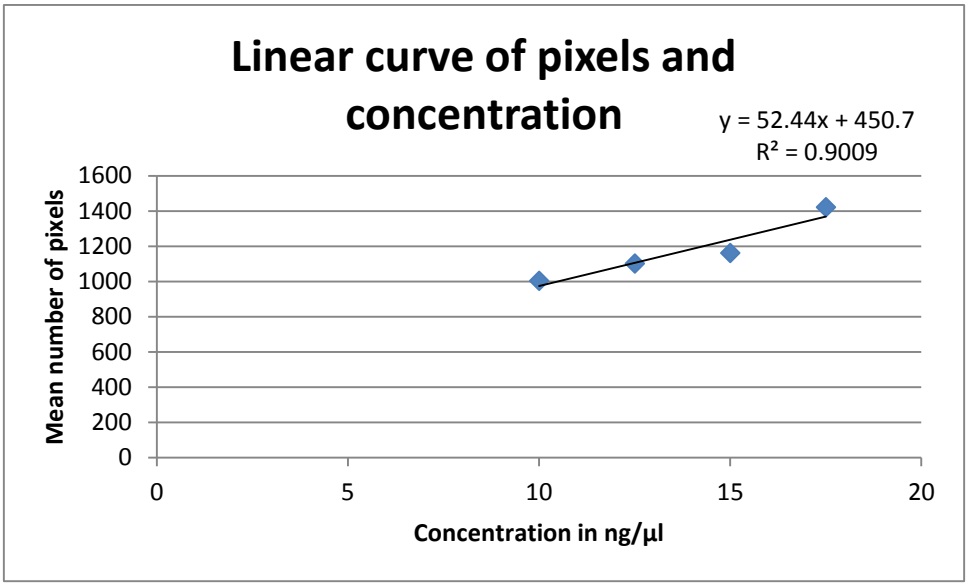

Figure 4.11: Linear curve of pixels and concentration based on figure 4.9

To guarantee a more consistent application of samples and a more reliable analysis compared to manual dot blotting, a slot blot manifold was introduced. In figure 4.12, DNA concentration of K562 cancer cells, after measuring with Nano drop was 831.9 ng/µl with a purity ratio A260/A280 of 2.04. This slot blot was performed using different concentrations ranging from 17.5 ng/µl in the first row to 2.5 ng/µl. The last row was used as a control, where no DNA should be detected. After application of the samples, the membrane was soaked in 2X SSC buffer. The membrane was then hybridized using Telo Alexa 488 probe and the hybridization buffer with 0.5% SDS and 1 ml Denhart's solution. Denhart's solution as a mixture of blocking agents was added to prevent unspecific binding of nucleic acids. No significant differences in background were observed, so Denhart's solution was excluded from the procedure. In figure 4.12, it seemed that there was a carry-over of samples in all wells, especially in the last row, which should not contain any DNA since it was used as the control. This problem possibly occured due to a leak in the vacuum of the manifold.

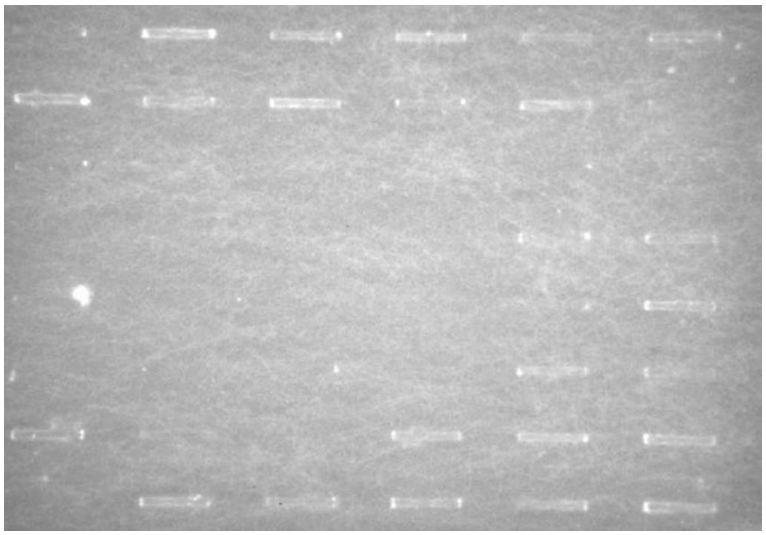

Figure 4.12: Slot blot using manifold dot blot protocol

When comparing hybridization procedure of dot blot protocol 1 with dot blot protocol 2, it was evidenced that dots with sample preparation of dot blot protocol 2 were nearly always detected prior to hybridization. Using methylene blue, DNA detection was more successful and all problems which appeared later had to be due to inconsistent hybridization or washing procedure.

Hybridization procedure seemed to be more inefficient compared to first dot blot protocol, meaning some dots could not even get detected after hybridization, independent of concentration. It was not possible to find out the threshold for the lowest detectable concentration in a sample when using second dot blot protocol. Using first dot blot protocol, minimum concentration of detected DNA with Telo Alexa 488 probe was 10ng/µl.

Since supported positively charged nylon membranes have a high binding capacity and a higher potential for backgrounds (Valker and Rapley, 2008), hybridization procedure seemed to be highly problematic. This finding was consistent with observations made in this study, because nearly every problem appeared after hybridization and washing. Failure to perform appropriate hybridization is manifesting later in very high backgrounds and cannot be thoroughly controlled. In order to achieve good hybridization, it is important to select a hybridization temperature which allows

annealing of the probe, but at the same time prevents non-specific binding. Since the hybridization temperature varies with different probes, hybridisation conditions of synthetic oligonucleotide probes seem to be highly error-prone, according to Hybridization Guide by Thermo Electron Corporation. Using the very short Telo Alexa 488 probe with only 24 bases, it was not really surprising, that hybridization was inconsistent. Besides, all the rules for efficient hybridization and hybridization temperature cannot be extrapolated to hybridization with oligonucleotide probes, according to Nucleic Acid Hybridization manual by Roche. In general, higher specificity is reached when hybridization is performed at high stringency, meaning a high temperature and low salt content, according to Hybridization Guide by Thermo Electron Corporation. It is proposed to avoid washing at room temperature since it will cause higher backgrounds. Washing should start with a high salt content and finish with a lower salt content for background reduction.

In dot blot protocol 1, washing was performed using an initial washing step with wash buffer 1 at room temperature, followed by washing in wash buffer 2 at 55°C which resulted in a much lower background compared to dot blot protocol 2 where washing temperature was constant for both wash buffers at 55°C. Since an initial wash buffer step at room temperature in combination with Telo Alexa 488 probe resulted in a lower background, in contrast to other recommendations, this washing procedure was preferred.

However, the results were still inconsistent and not satisfying. Since positively charged nylon membranes are recommended in combination with a DIG-labelled probe because of their high signal-to noise ratio (Valker and Rapley, 2008), an alternative probe Telo DIG in combination with mAb anti-DIG was introduced in the dot blot study. Telo DIG with a sequence of 3 (CCCTAA) oligonucleotide repeats is labelled at the 3’ with DIG and has a Tm of 55.6°C. Using this probe, hybridization temperature was set to 65°C and the membrane was washed in wash buffer 1 at room temperature, followed by a washing step using wash buffer 2 at 65°C.

In order to find out if the antibody, probe and ECL reagents are even compatible with each other, different control experiments with Telo DIG probe were performed.

Figure 4.13 shows a test experiment of mAb-anti DIG in combination with ECL reagents. Therefore the left dot in the upper position was diluted in a concentration of 1:500 and the spot in the lower position was diluted in a concentration of 1:5000 in PBS. The dots on the right are from another antibody, diluted in the same concentrations. This experiment was used to find out if the ECL reagents work with the HRP enzyme, which is directly coupled to the antibody.

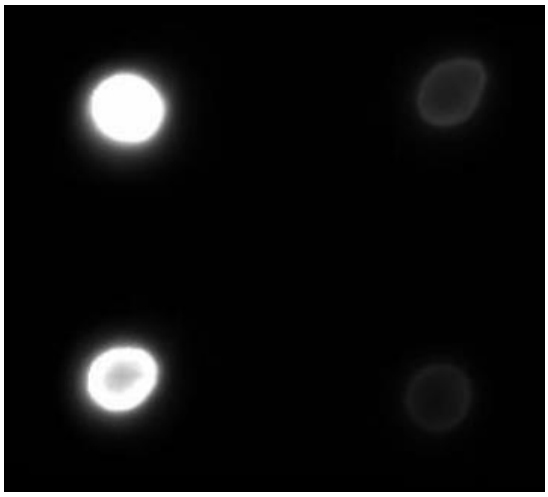

Figure 4.13: Testing of ECL reagents in combination with antibody

In order to find out if the ECL reagents are compatible with the probe, figure 4.14 shows a test experiment of the Telo DIG probe in combination with mAb anti-DIG. 2 µl of the Telo DIG probe were added to 5 ml of the hybridization buffer. 5 µl were applied to each dot and the membrane was crosslinked using Chemi Doc^{TM} XRS+ system for one minute. The membrane was blocked in the blocking solution and an antibody solution was prepared with concentrations of 1:50 for the first dot, 1:500 for the second dot and 1:5000 for the third dot. The membrane was washed in wash buffer antibody twice for 15 minutes at room temperature. ECL reagents were prepared and applied on the membrane. The membrane was visualized using chemiluminescence of the Chemi Doc^{TM} XRS+ system, exposed for 5-30 seconds. This membrane shows that the ECL reagent is compatible with the probe binding to the antibody.

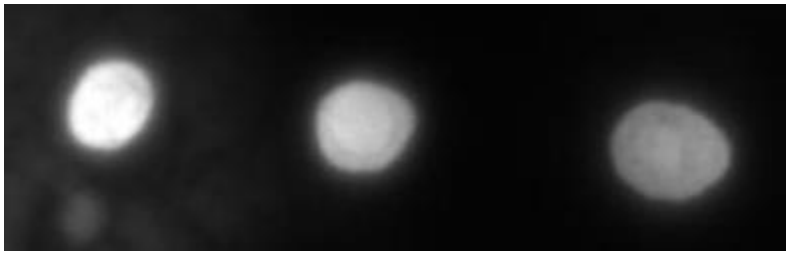

Figure 4.14: Testing of probe binding to antibody using different concentrations

In figure 4.15, DNA concentration, after measuring with QuantiFluor dsDNA system was 35 ng/µl in the first row and 40 ng/µl in the second row. 2 µl of each dot were applied on the membrane using Dot blot protocol with Telo DIG and mAb anti-DIG. The hybridization buffer contained 0.5% SDS, 1% BSA. The dilution of the antibody was 1:2000 in PBS. The membrane was prehybridized for 1 hour at 65°C and hybridization was performed overnight. The decisive determinant of successful dot blots in combination with Telo DIG probe seemed to be an extra-denaturation step. Prior to crosslinking, the membrane was placed on a pre-wetted 0.4M NaOH filter paper. This denaturation step helped to increase equal DNA denaturation stage of all applied dots. Since dot blot is based on hybridization with a probe, due to the high background, the analysis was not reliable. Further experiments are needed in order to try reducing the high background.

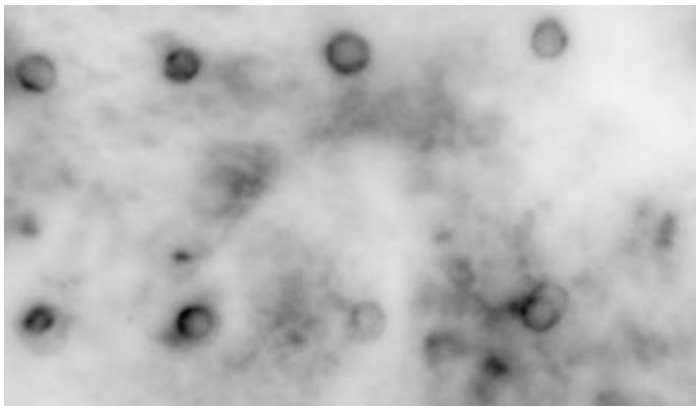

Figure 4.15: Dot blot using Telo DIG and mAb anti-DIG

4.3 TRF analysis

In figure 4.16, DNA concentration of the sample, after measuring with QuantiFluor dsDNA system was 40 ng/µl. 12 µl of GelRed for DNA detection were added to the agarose solution before polymerization of the gel. On the left side, the 200 bp-10 kb marker was loaded, and on the right side, the sample was loaded using 3 µg and 1.5 µg.

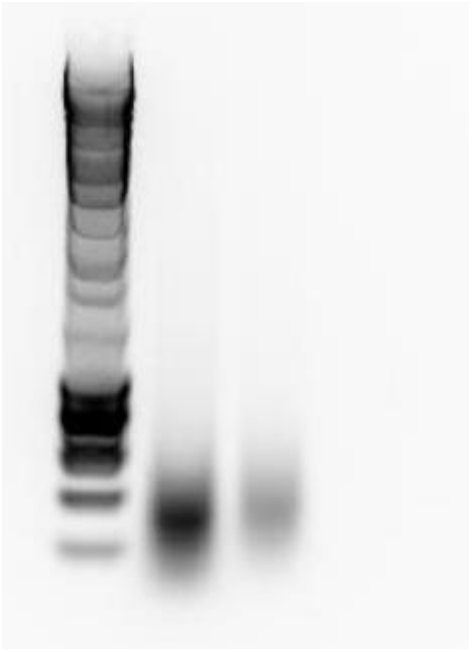

Figure 4.16: Southern blotted DNA on 0.7% agarose gel before transfer

Figure 4.17 a) shows remaining DNA on the same 0.7% agarose gel of the sample with a concentration of 40 ng/µl, seen in figure 4.16. This figure shows that the marker was not transferred completely whereas the samples were successfully transferred, using the semi-dry transfer cell for a duration of 11 minutes with a constant current of 0.4 amper. Figure 4.17 b) shows the southern blotted DNA, successfully transferred onto the membrane. The membrane was soaked in a filter paper, which was pre-wetted in 0.4M NaOH, placed on top of this filter paper for 5 minutes and crosslinked with Chemi DocTM XRS+ system, exposed for 5 minutes.

This figure shows the bald spot of the marker due to an air bubble which was not rolled out properly.

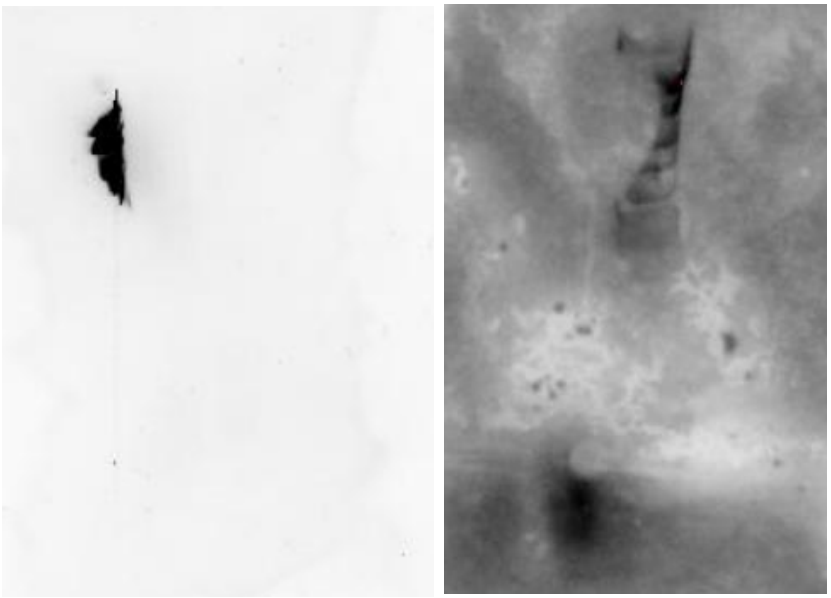

Figure 4.17: Southern blotted DNA after transfer on the a) gel and b) nylon membrane

When performing Southern blotting of TRF analysis, transfer of the samples with the semi-dry transfer cell worked well. However, a big problem was formation of air bubbles between the membrane and the gel. If any air bubbles were trapped, they later appeared as bald spots and led to incomplete transfer of DNA.

Figure 4.18 shows the same membrane, used in figure 4.17 but already after hybridization, antibody blocking and washing. This membrane was hybridized using 8 µl Telo DIG probe in 15 ml hybridization buffer containing 1% BSA and a 1:2000 dilution of mAb anti-DIG in PBS. 1 ml of each ECL reagent was applied on the membrane after washing with wash buffer antibody, exposed for 10 minutes using chemiluminescence of Chemi Doc^{TM} XRS+ system. This membrane shows that no real signal is detected and that there is just background on the membrane.

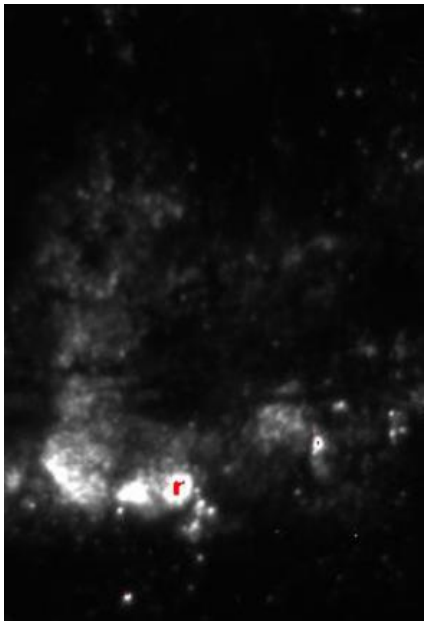

Figure 4.18: Membrane after antibody reaction and ECL detection

Due to high binding capacity of nylon membranes, excessive blocking was required. Since non-fat dry milk was not recommended as it quenches signals, BSA and casein were chosen as blocking agents. Since casein could not even get completely dissolved, BSA was the preferred blocking agent, using a blocking solution of 1% BSA. Hybridization procedure or blocking was inefficient in combination with Telo DIG probe due to high non-specific binding of the probe. According to Hybridization guide by Millipore, antibody blocking agents should consist of BSA with 0.2-0.5% and Tween 20 with 0.05-0.1%. Referring to Nucleic-Acid Hybridization manual by Bio-Rad, BSA is known to increase sensitivity at the expense of higher non-specific binding. Due to the fact that poorly selected blocking reagents obscure the sequence of interest, this could have been the main problem. This high background indicated that different probes and samples require different hybridization and washing procedures. Maybe the differences in the salt content between the wash buffers should not be so harsh since wash buffer 1 consisted of 2X SSC and wash buffer X consisted of 0.2X SSC. Furthermore, it could be possible that hybridization temperature was set too high and that washing should be performed at a lower temperature as well. Oligonucleotide probes have a high affinity for non-specific binding. Normally, DNA extracted from salmon sperm is used to reduce unspecific binding. In case of a telomeric probe, this was simply not possible. One possibility to reduce high non-specific binding is to use plasmic DNA. To alleviate high non-specific binding, further experiments are needed to optimize blocking reagents, as well to decrease high background noise to achieve reliable analysis.

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„Ich habe mich bemüht, sämtliche Inhaber der Bildrechte ausfindig zu machen und ihre Zustimmung zur Verwendung der Bilder in dieser Arbeit eingeholt. Sollte dennoch eine Urheberrechtsverletzung bekannt werden, ersuche ich um Meldung bei mir."

6 SUMMARY

Telomeres are protective structures at the ends of chromosomes which shorten after every cell division. In order to keep a balance, they have to be replenished by an RNA dependent DNA polymerase, called telomerase. Since the enzyme telomerase is not able to restore the whole length of the telomere, telomeres shorten, which is one reason for aging. If there is a critical length of telomeres in a cell of a healthy organism, a DNA damage response is introduced, which reduces cell proliferation, thus cells are excluded from cell cycle or are undergoing apoptosis, to kill themselves.

Many age-related diseases are associated with shorter telomeres, such as diabetes type 2, cardiovascular diseases or cancer. Up to date, there are many methods available to measure telomere length in an organism such as TRF, qPCR, FISH and Stela. All these methods have some advantages as well as some drawbacks and no single measurement is able to measure telomere length at the molecular level and in large human population studies at the same time.

In this master thesis, it was aimed to optimize a dot blot method to measure the relative telomeric DNA content. Therefore DNA was extracted from leukemia K 562 cancer cells. Furthermore, DNA from PBMC's, obtained from donors of the Vienna Active Aging study was isolated. This work tried to detect specific telomeric sequences, applied on dot blots and Southern blots, using hybridization and different telomeric probes. Results of the study showed that hybridization with oligonucleotide probes is quite difficult which seems to underlie different conditions, dependent on type of sample and probe and cannot be generalized. Further experiments are needed in order to optimize this time-saving method which does not require high quality starting material and does not need a highly equipped lab, in order to establish a high throughput method which one day may help to reveal some links to age-associated diseases.

6 ZUSAMMENFASSUNG

Telomere sind schützende Strukturen an den Enden von Chromosomen, die sich nach jeder Zellteilung verkürzen und von einer RNA abhängigen DNA Polymerase, dem Enzym Telomerase, aufgefüllt werden müssen. Da das Enzym Telomerase nicht die volle Länge wiederherstellen kann, werden die Telomere im Laufe der Zeit immer kürzer, der Grund für das Altern eines Organismus. Hat eine bestimmte Anzahl von Telomeren in einer Zelle eine kritische Größe erreicht, so wird in einem gesunden Organismus der Zellzyklus herabgesetzt und die Zellen werden aus dem Zellzyklus ausgeschlossen oder leiten eine Apoptose ein. Viele altersabhängige Erkrankungen werden mit kurzen Telomeren assoziiert, so zum Beispiel Diabetes mellitus II, Kardiovaskuläre Erkrankungen und Krebs. Es gibt bereits sehr viele Methoden, mit welchen man die Länge von Telomeren messen kann, so zum Beispiel die TRF, qPCR, FISH-Methoden oder STELA. All diese Methoden haben ihre Stärken und Schwächen, doch keine ist in der Lage, Messungen auf molekularer Ebene durchzuführen, als auch in großen Humanstudien einsetzbar zu sein. In der vorliegenden Arbeit wurde versucht eine Methode namens Dot blot zu optimieren, um den relativen Gehalt von Telomeren zu messen. Dafür wurde DNA von humanen K562 Leukämie K562 Zellen isoliert. Weiters wurde DNA von Peripheren mononukleären Zellen von Probanden der Active Aging Studie isoliert. Diese Studie hatte das Ziel, spezifische Telomersequenzen mit Hilfe von Hybridisierung und verschiedenen Oligonukleotid Sonden in Dot blots als auch Southern blots zu detektieren. Die Resultate zeigten, dass die Hybridisierung mit Oligonukleotiden ein sehr komplizierter Vorgang ist, der mit jeder Sonde und Art von Probe andere Bedingungen erfordert. Weitere Forschungen sind nötig, um diese zeitsparende Methode zu etablieren, da sie nur eine geringe Qualität an DNA erfordert, schnell durchführbar ist und keine besondere Laborausstattung erfordert.

7 CURRICULUM VITAE

Name	Sandra Arz
Date of Birth	June 22, 1988
Place of Birth	Vienna

Education:

september 1994 to 1998	elementary school Dietmayrgasse 3
1998 to 2006	secondary school "Erich – Fried Realgymnasium" (with distinction)
october 2006 to december 2010	Bachelor program Nutrition science at university of vienna. First experience with GC, DC, HPLC and NMR – spectroscopy
since march 2011	Master program Nutrition science at university of vienna, specialization: Molecular nutrition Immunology courses, microbiological and molecularbiological courses

Job experience:

december 2010, january 2011	internship at University of Vienna: Department "Emerging Focus Nutrigenomics" at professor Jürgen König's group tasks: LC/MS work experience, preparing of serial dilutions and buffer solutions, preparing of agar for C.elegans, experiments with C. elegans, SPE sample preparation
march 2011 to september 2012	production assistant at "Less is More cosmetics" production of natural cosmetic shampoo and hairstyling products under GMP conditions, preparing and analysis of germ tests, preparing of SOPs for shampoo production, assistant in creation of shampoos
october 2012 to february 2013	internship at chemical university of Vienna. “Half quantitative identification of higher oligosaccharides in honey with LC/ESI-MS“ at professor Eberhard Lorbeer's group

may 2013 to august 2013 internship at AKH Wien.

"Anticancer activity of selected N'' – substituted hydroxyguanidines" at professor Georg Krupitza's group

tasks:

performing of growth inhibition assays, cytidine assays, Hoechst/propidium iodide double staining method, Western blotting, determination of dNTPs via HPLC

Interests:
immunology, biochemistry, toxicology and microbiology, HPLC spectroscopy, cell culture experiments, epigenetics

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