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DISSERTATION

Comparison of Different DNA Extraction Methods and their Impact on Telomere Length Measurement

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Katharina Sonja Kim

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List of abbreviations

CV	Coefficient of variation
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FISH	Fluorescence in situ hybridization
RNA	Ribonucleic acid
STELA	Single telomere length analysis
TAE	TRIS-Acetate-EDTA
TLM	Telomere length measurement
TRF	Terminal restriction fragmentation
T/S	Telomere/ Single-copy gene
qPCR	Quantitative polymerase chain reaction

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Abstract

English

Background

Telomere length is associated with biological age and cellular stress. For example, various studies suggest that chronic diseases, such as atherosclerosis, diabetes mellitus and arterial hypertension, are associated with shorter telomeres in affected patients than in healthy individuals. Yet, it remains unclear how the presumed shortening of telomeres under conditions of stress compares to technical variations, and particularly in terms of the choice of DNA extraction method, which can introduce considerable variation in the resulting measurement of telomere length. This thesis presents a systematic analysis of variation in the measured telomere length within and among five different methods of DNA extraction, as well as among an age-stratified patient cohort.

Methods

Blood samples were collected from fifteen individuals and stratified in three age groups of five individuals each. Samples were processed individually as well as pooled by age group, and DNA was extracted using five different methods. All resulting DNA samples were tested for concentration, purity, as well as fragmentation, and telomere length was determined using qPCR, with three technical repeats taken on three consecutive days. Mean values of telomere length and their coefficients of variation were compared both within one extraction method and across extraction methods. Finally, for each method, samples were ranked by mean telomere length.

Results

Among all DNA extraction methods, the Invisorb Blood Universal Kit achieved best results for highly concentrated, pure, and non-fragmented DNA. Technical repeats produced consistent measurements of telomere length with low variation across repeats. The different extraction methods, however, yielded high inter-assay variation of up to 14% on average for individual samples, and an inter-assay variation of up to 11% on average for age-pooled samples. As a consequence of this variation, ranking of the samples by telomer length was inconsistent across different extraction methods.

Conclusion

All considered methods of DNA extraction yielded consistent measurements of telomere length across technical repeats. Comparing measurements of different extraction methods derived from the same blood samples, however, produced significant variations. The choice of DNA extraction method can therefore critically influence the results of a study. In particular, considering that different extraction methods resulted in inconsistent rankings of telomere length across samples, it was shown that samples extracted with different methods cannot be compared with one another. Differences in telomere length observed in studies, such as those in chronically ill patients, may in part be due to the choice of DNA extraction method.

German

Hintergrund

Telomerlänge ist mit dem biologischen Alter und zellulärem Stress assoziiert. Mehrere Studien deuten darauf hin, dass Patienten mit chronischen Krankheiten wie Atherosklerose, Diabetes mellitus und arterieller Hypertonie kürzere Telomere aufweisen als gesunde Individuen. Es bleibt jedoch unklar, wie sich die vermutete Verkürzung der Telomere unter Stressbedingungen verglichen zur technischen Variation verhält. Insbesondere die Wahl der DNA-Extraktionsmethode kann zu erheblichen Variationen bei der Messung von Telomerlängen führen. Diese Arbeit umfasst eine systematische Analyse der Variation der gemessenen Telomerlänge innerhalb und zwischen fünf verschiedenen DNA-Extraktionsmethoden, sowie innerhalb drei verschiedener Altersgruppen.

Methoden

Von fünfzehn Personen wurden Blutproben entnommen und drei Altersgruppen a fünf Personen zugeteilt. Die Proben wurden sowohl einzeln als auch gepoolt nach Altersgruppen verarbeitet und die DNA mit fünf verschiedenen Methoden extrahiert. Alle DNA-Proben wurden auf Konzentration, Reinheit und Fragmentierung getestet und daraufhin die Telomerlänge mittels qPCR bestimmt. Drei technische Wiederholungen wurden an drei aufeinanderfolgenden Tagen durchgeführt. Die Mittelwerte der Telomerlänge und ihre Variationskoeffizienten wurden sowohl innerhalb einer Extraktionsmethode als auch zwischen den Extraktionsmethoden verglichen. Zuletzt wurden die Proben nach mittlerer Telomerlänge in eine Rangfolge geordnet.

Ergebnisse

Von den fünf DNA-Extraktionsmethoden erzielte das Invisorb Blood Universal Kit die besten Ergebnisse für hochkonzentrierte, reine und nicht-fragmentierte DNA. Die technischen Wiederholungen innerhalb einer Methode ergaben konsistente Messungen der Telomerlänge mit geringer Variation zwischen den Wiederholungen. Zwischen verschiedenen Extraktionsmethoden ergab sich dagegen eine hohe Inter-Assay-Variation von bis zu 14 % im Durchschnitt für individuelle Proben und von bis zu 11 % im Durchschnitt für nach Altersgruppe gepoolte Proben. Als Folge dieser Variation resultierte eine inkonsistente Rangfolge der Telomerlänge zwischen den verschiedenen Extraktionsmethoden.

Schlussfolgerung

Alle berücksichtigten Methoden der DNA-Extraktion ergaben konsistente Messungen der Telomerlänge in Bezug auf technische Wiederholungen. Jedoch ergab der Vergleich von Telomerlängen verschiedener Extraktionsmethoden aus denselben Blutproben eine signifikante Variation der Telomerlänge. Die Wahl der DNA-Extraktionsmethode beeinflusst somit die Ergebnisse einer Studie. Insbesondere die Tatsache, dass unterschiedliche Extraktionsmethoden zu inkonsistenten Rangfolgen der mittleren Telomerlänge führten, zeigt, dass mit verschiedenen Methoden extrahierte Proben nicht miteinander verglichen werden können. In Studien beobachtete Unterschiede in der Telomerlänge, zum Beispiel bei chronisch kranken Patienten, könnten teilweise auf die Wahl der DNA-Extraktionsmethode zurückzuführen sein.

1. Introduction

1.1 Telomeres

Telomeres are structures at the end of each chromosome (1, 2), which consist of non-coding repetitive sequences of TTAGGG (3). These structures have an important role in protecting the chromosomes from deterioration, fusion, and recombination with neighboring chromosomes (6, 7). This protective function is called telomere capping (6). Telomere length is shortened during each cell division (2). Due to the so-called end replication problem, a small fragment of DNA is lost during each cell cycle at the 3´-end of the DNA since DNA polymerase cannot perform a complete replication of strands (4). After about 40 to 60 cell divisions, a critical telomere length is reached, and the cell will undergo senescence or apoptosis (5).

Later research discovered the existence of telomerase, a DNA polymerase specialized in the elongation of telomere ends to enable cells to maintain the length of their telomeres (2). This function is diminished during embryonic development in most somatic cells; however, it stays active in germline cells, activated lymphocytes and stem cell population (6). In the absence of telomerase, a quick shortening of telomere length occurs, leading to cell death (5). Conversely, enabled telomerase is known to be activated in cancer cells, allowing cells to become immortal by avoiding cell death through an infinite number of cell divisions (7, 8).

In recent years, scientists have discovered that telomere length is already established in childhood and that even newborns already display variation in telomere length, depending on a variety of factors that influence intrauterine development (9). It can be assumed that two key determinants influence telomere biology over the lifetime of an individual: the baseline telomere length (10), determined by genetics; and telomere attrition (11), which is enhanced by environmental factors such as lifestyle, oxidative stress, and inflammation, and which could start as early, as in pregnancies through external factors such as smoking (12). Telomere length is therefore longest at the time of birth and decreases with increasing age (13).

1.2 Association of telomere length with disease

Telomeres and telomere length have received increasing attention during the past few decades. A group of degenerative illnesses known as telomere syndromes originate from a short telomere defect (14). The rare genetic disorder dyskeratosis congenita was the first to be associated with a mutant telomere gene (15-17). Affected individuals bear three main features: hyperpigmentation, skin leukoplakia and nail dystrophy (18). The main causes of death are idiopathic pulmonary fibrosis, bone marrow failure and cancer (18), which patients with dyskeratosis congenita are particularly prone to (19). Further telomere syndromes include idiopathic pulmonary fibrosis (20), aplastic anemia (21, 22) and liver cirrhosis (23).

Several studies have also revealed a connection between shorter telomeres and the progression of age-related diseases (24-26) such as arterial hypertension (13), cardiovascular diseases (27-30) and diabetes mellitus (31, 32). Shortened telomeres can lead to telomere end fusions and chromosome instabilities (33, 34), and possibly cause diseases (35). Smoking, diabetes, obesity, dyslipidemia and shift work are known risk factors for cardiovascular diseases and have been linked to short telomere length (36). In 2003 a study showed that individuals over the age of 60 with shorter telomere lengths had a threefold higher chance to die from heart diseases than the control group (37). Patients suffering from chronic heart failure and myocardial infarctions, moreover, displayed significantly shorter telomere length (27, 38).

In contrast, cancer cells display elevated telomerase levels, in fact approximately 90% of all tumors express telomerase to elongate telomeres (8, 39), including various cancer types such as cervical cancers, hepatocellular cancers, lung tumors, breast carcinomas and neuroblastoma, as well as pediatric carcinomas (40-43). Telomerase may therefore serve as a target in anti-cancer treatment by means of telomerase inhibition, but to date no treatment of this kind has been established (35, 39).

1.3 Challenges in measuring telomere length

A wide range of techniques for telomere length measurement (TLM) exist. These methods can be divided into direct (absolute telomere length) and indirect (relative telomere length) methods (44-48).

A direct method to measure absolute telomere length, and the gold standard so far, is Terminal Restriction Fragmentation (TRF) (49, 50). A DNA digest with restriction enzymes is performed, and since restriction enzymes do not contain sites for telomeric and sub-telomeric structures, these remain intact. The digested DNA is separated by size through agarose gel electrophoresis and visualized by either Southern blotting or in-gel hybridization using a telomere-specific probe. The smear on the gel is then compared to a DNA ladder with known fragment sizes, which makes it possible to size the fragments and therefore telomere lengths, giving the parameters of telomere length distribution (49, 50). A limitation of this method is that sub-telomeric regions are included into telomere length measurement, essentially leading to overestimation of telomere length (47). A higher quantity of DNA is therefore needed, and the method is comparably work- and cost-intensive (47, 51). In addition, telomeres that are relatively short are difficult to detect and quantify (46). All these characteristics limit the use of this method in a large scale study (44).

An indirect method to measure relative telomere length is monochrome multiplex quantitative polymerase chain reaction (qPCR) (52), which is used in the majority of studies on telomere length. This method takes advantage of the different melting temperatures of telomeres and a single-copy gene (albumin) working with a single fluorescent DNA-intercalating dye (SYBR Green). Telomere signals are collected in early cycles and single-copy gene signals are collected in later cycles when the telomere product is fully melted. By comparing the amount of telomere (T) amplification product to that of the single-copy gene (S), a T/S-ratio of standard DNA is formed as an average value of telomere length. The resulting samples are then compared to that ratio to determine if average telomere length is longer (T/S-ratio >1) or shorter (T/S-ratio <1) than the standard (52). Measuring telomere length via qPCR requires only a small amount of DNA and multiple samples can be processed simultaneously, thus saving both time and costs (52). These characteristics have made it the desired method for epidemiological studies (47, 53). A disadvantage of qPCR, however, is that it yields only the average telomere length, and with substantial technical noise (51).

A modification of the monochrome multiplex qPCR method to measure telomere length directly is working with an oligomer standard (53). The synthesized oligomer standard is 84 base pairs long and only consists of TTAGGG repeats. A standard curve is formed by a dilution series of known quantities of the oligomer standard, used to calculate absolute telomere length, and a single-copy gene serves as an amplification control (53). Since all techniques except TRF involve relative measurements, methods based on the oligomer standard allow for a direct comparison of results. This method makes it possible to compare the results directly both between experiments and laboratories (53).

Alternative methods to measure telomere length include variations of fluorescence in situ hybridization (FISH) (54, 55) and single telomere length analysis (STELA) (56), both of which yield indirect estimates of telomere length (47, 51).

The main challenges associated with measuring telomere length can roughly be divided into three categories: (1) variability across results from different methods; (2) a lack of standardization; and (3) the effects of preanalytical conditions, including how DNA has been extracted.

Methods employed to measure telomere length yield substantially distinct results, particularly the two most common used methods: TRF yields the distribution of absolute telomere length, whereas qPCR provides the average relative telomere length. It is therefore difficult to compare results across different studies (51, 57).

Additionally, standardization still remains an issue. For example, in qPCR, which is by far the most commonly used method, choices that all potentially impact on the outcome of an assay include those of: telomere primer sequences, single-copy genes and their concentration, homemade or commercial master mixes, qPCR program conditions, qPCR instruments, data analytics and quality control criteria (47, 58). The lack of standardization therefore results in considerable intra- and inter-lab variation and impacts on reproducibility and comparability of results (51).

Moreover, preanalytical conditions and DNA extraction methods have a substantial influence on the resulting measurements. DNA extracted from the same sample with different methods can lead to different results and can consequently show diverse findings in TLM. Depending on the extraction method applied, relative TLM revealed extensive differences and can even influence epidemiologic studies (59-64).

In combination, these facts make it problematic to design a large-scale epidemiological study investigating telomere length associated with various diseases (51).

1.4 Methods for DNA extraction

Several methods to extract DNA from leukocytes exist. Few are self-prepared, and a variety of commercial kits are available. These methods, however, are not specifically developed to extract DNA for TLM. Many include centrifugations, intense shaking and washing steps, which are not gentle approaches to extract DNA, and could lead to possible shearing of the DNA (60). To achieve the best results in TLMs, DNA should be of high quality and not fragmented (59). Rough treatment of DNA can lead to fragmentation and, consequently, to shorter telomere length (57), since it is generally assumed that telomeric DNA is sensitive to fragmentation (65, 66).

In most studies investigating telomere length to date, genomic DNA has been extracted using the following three methods (57, 60): a column method (51, 59, 60, 63, 67, 68), salting-out of DNA (51, 59-61, 63, 69) and organic extraction with Phenol-Chloroform (60, 61, 67).

All three methods use a proteinase K digest, but the Phenol-Chloroform and the salting-out technique are both liquid-to-liquid-phase methods (60). As for the Phenol-Chloroform method, proteins dissolve in the phenol phase, while DNA remains in the aqueous phase (70). The salting-out technique dehydrates and precipitates proteins, leaving the DNA in the supernatant, and DNA is then precipitated using ethanol (69). The column method takes a different approach: deproteinization occurs, due to the fact that DNA selectively binds to the silica-based column, while other components such as proteins pass through, and DNA is finally washed off the column (71).

For these experiments, five different DNA extraction methods were applied and compared (see Figure 1), namely a commercial kit containing the column method, three methods using the salting-out technique (two commercial kits and one self-prepared method), as well as Phenol-Chloroform extraction.

2. Methods

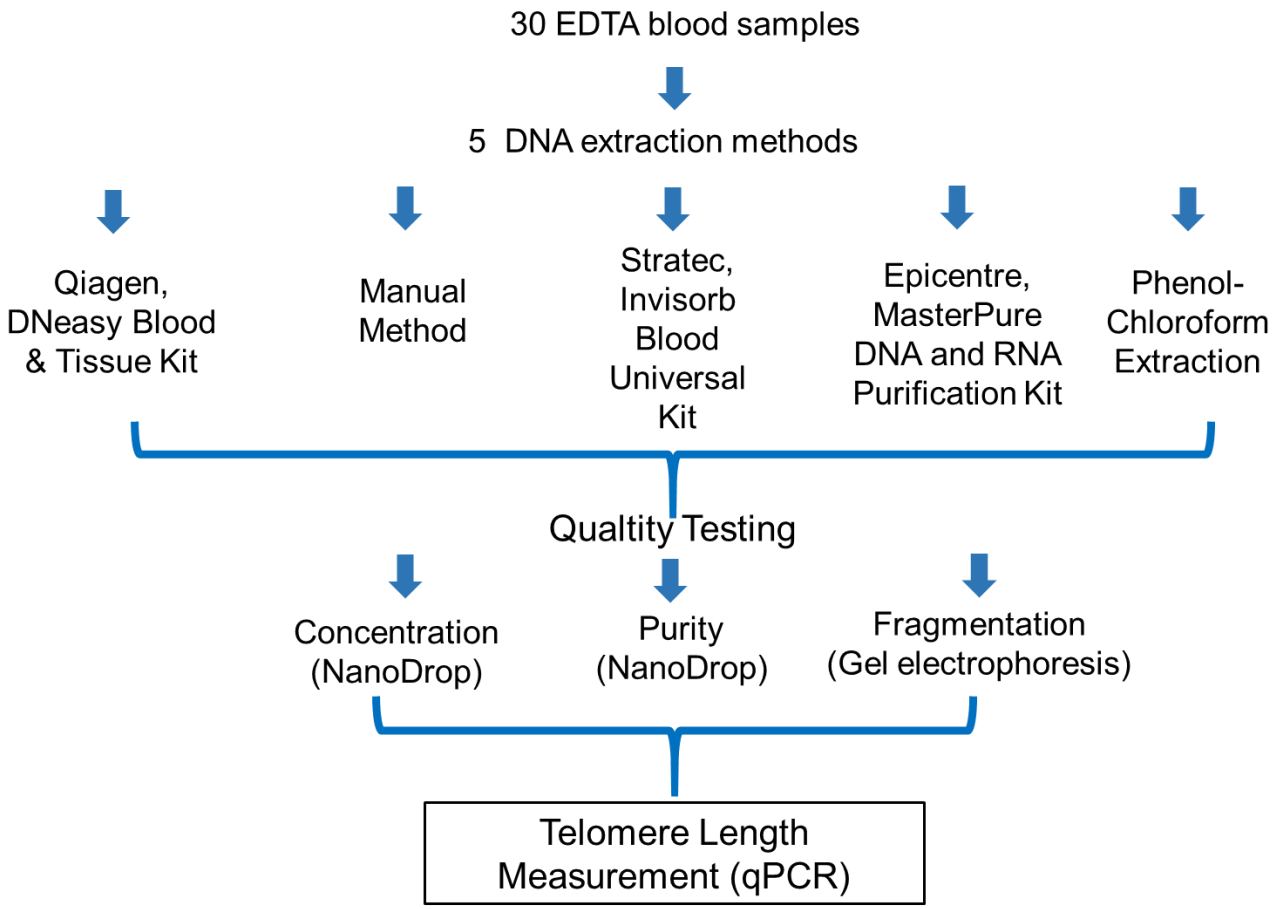


Figure 1. Workflow of experiments. After collection and storage of blood samples, each sample was processed according to the DNA extraction protocols, hence each sample was extracted with five different extraction methods. Quality checks were carried out before performing telomere length measurements by qPCR.

2.1 Blood samples

Blood samples were collected from fifteen volunteers (n=15). All participants gave written informed consent, and this study was approved by the Ethics Committee of Charité Universitätsmedizin Berlin (EA4/093/18). Three age groups were formed to investigate age-dependent telomere length: Group A (young, average age 18 years), Group B (middle-aged, average age 44 years) and Group C (elder, average age 74 years). Each age group consisted of five individuals (see Figure 2).

Ethylenediaminetetraacetic acid (EDTA) blood tubes were obtained from five individuals in each age group via venal puncture on the same day. Blood samples were processed as follows: each sample was handled individually and in addition as a pool of its age group. To form the pool sample, an equal amount of blood from each individual of an age group was combined. After careful mixing of the blood, the pools were subsequently divided up into five pool samples per age group. As a result, each age group contained five individual samples and five pool samples (n=10/age group).

Samples were anonymized and given a sample name corresponding to their age group. For example, age group A (“young”) contained samples A1, A2, A3, A4 and A5 (individual samples), as well as pool samples Pool (A1-A5)-1, Pool (A1-A5)-2, Pool (A1-A5)-3, Pool (A1-A5)-4, and Pool (A1-A5)-5 (see Figure 2). Aliquots were measured to the appropriate amounts for each of the intended five DNA extraction methods, with a small quantity calculated for pipetting loss, and with some left spare for possible repetition of DNA extraction. All samples were labeled and frozen to -80°C.

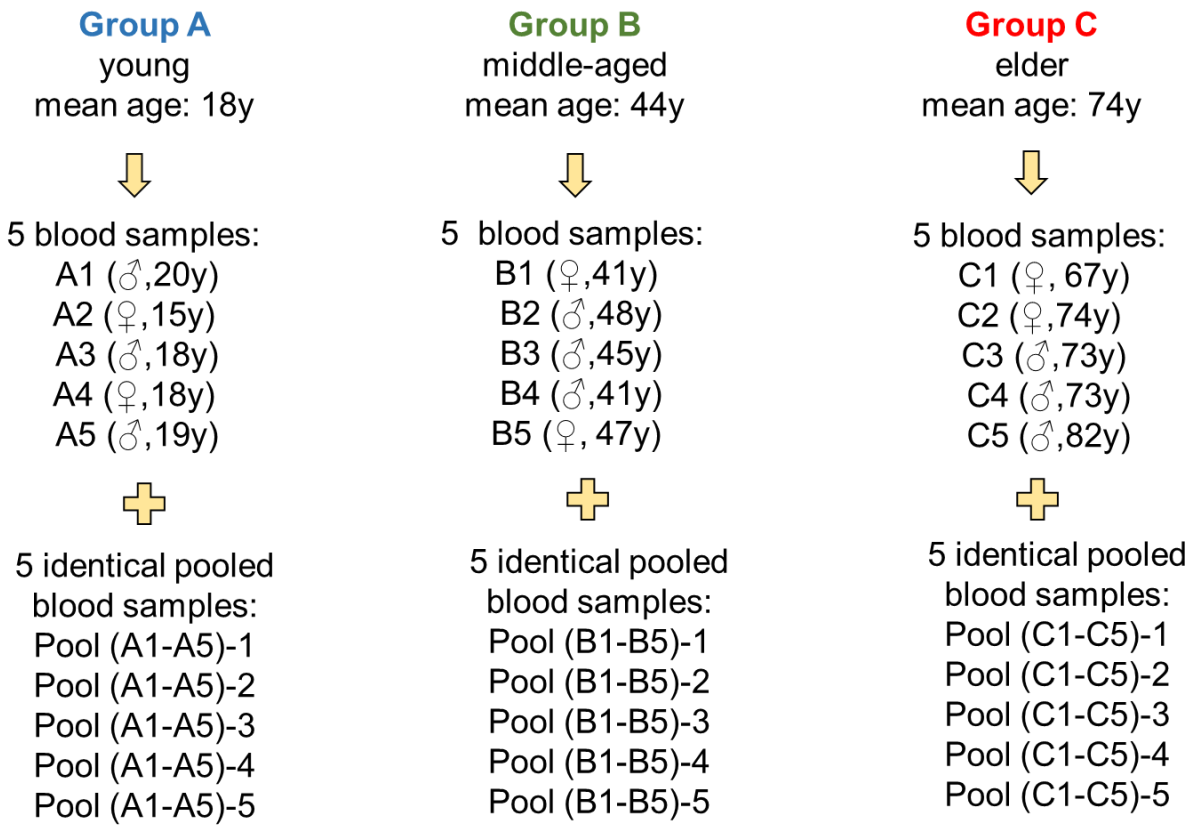


Figure 2. Individual and pool samples stratified by age. Blood samples were assembled depending on the age of individuals. Three age groups were formed: young (age group A), middle-aged (age group B), and elder (age group C). After collection, each sample was processed individually and in addition as a pool of its age group. Five individual blood samples in each age group were combined and subsequently divided into five identical pool samples. Each of the age groups therefore contained five individual samples and five pool samples (n=10).

2.2 DNA extraction

DNA was collected from 30 EDTA blood samples using five DNA extraction methods within a duration of two weeks, giving a total of 150 DNA samples (see Figure 1). Directly before DNA extraction, blood samples were removed from the freezer and underwent a thawing process on ice to avoid DNA degeneration. DNA was frozen to -20°C until telomere length measurement was performed. The following five different DNA extraction protocols were applied:

The first method was a commercial kit: the DNeasy Blood & Tissue Kit from Qiagen, which contained all required reagents and spin columns. The DNA collection was performed according to the manufacturer's protocol (72). The required starting quantity for EDTA blood was 200 µl. In a reaction tube, blood cells were lysed and applied onto a spin column specific to this kit. During centrifugation, DNA selectively bound to the silica-based membrane in the spin column and all other contaminants passed through (71). Through two additional washing steps, all remaining contaminants were removed from the spin column. The DNA was then dissolved from the membrane with 50 µl of elution buffer into a new tube.

The second method was a self-prepared Manual Method, which was modified from the method of Joseph Sambrock and David W. Russel from the Cold spring harbor protocols (73). Reagents were prepared and a starting amount of 600 µl EDTA blood was required. In a reaction tube, erythrocyte lysis was achieved with trihydrochloride, while leucocytes were lysed with ultrasonic waves in a water bath. In the next steps, a proteinase K- and an RNase-digest was performed, and DNA was precipitated with isopropanol and washed with 70% ethanol. After centrifugation, the supernatant was removed, and the DNA was air dried. The DNA pellet was dissolved with 50 µl of nuclease-free water.

The third method was the ready-to-use MasterPure Complete DNA and RNA purification kit from Epicentre. In the kit, the starting quantity of EDTA blood is specified as 200 µl. Extractions were executed following the manufacturer's manual (74). The first step in the protocol is the lysis of erythrocytes, followed by the lysis of leucocytes. Proteins and RNA are removed by protein precipitation reagent and RNase A. DNA is precipitated with isopropanol and separated from the remaining contaminants via centrifugation, leaving the contaminants in the supernatant. The DNA pellet is washed twice with 70% ethanol, air dried, and suspended with 35 µl of an elution buffer.

The fourth method was a Phenol-Chloroform extraction. In a reaction tube 200 µl of phenol-chloroform-isoamyl-alcohol is added to an equal amount of blood sample and mixed. The DNA is dissolved in the aqueous layer and proteins are dissolved in the non-aqueous layer (70). Separation of phases is achieved through centrifugation. The aqueous layer is then removed and combined with chloroform-isoamyl-alcohol solution, centrifuged again, and the aqueous phase on top is once again removed and placed into a new tube. DNA is then precipitated and washed with ethanol, air dried, and resuspended in 50 µl elution buffer (70). This technique is not typically applied in epidemiological studies anymore, as both phenol and chloroform are toxic.

The Invisorb Blood Universal Kit from Stratec, another commercial kit, was the last extraction method tested. Methodologically, this kit is comparable to the MasterPure Complete DNA and RNA purification Kit, and the main difference between these two kits is the quantity of EDTA blood. Extractions were performed following the manufacturer's manual (72), wherein a fixed starting amount of EDTA blood is not specified, and thus it is possible to use a minimum of 1 ml and up to 10 ml as maximum. The chosen starting amount for this test series was 1 ml as it was the most comparable starting amount to the other methods. The first step in the protocol was the lysis of erythrocytes, followed by the lysis of leucocytes and protein removal at 60°C. DNA was precipitated with isopropanol and separated from the remaining contaminants via centrifugation, leaving the contaminants in the supernatant. The DNA pellet was washed with 70% ethanol, air dried, and suspended with 1 ml of an elution buffer.

2.3 DNA quality and quantity testing

Each extracted DNA sample was tested for concentration, purity, and fragmentation (see Figure 1).

In terms of concentration and purity, each sample was measured in triplicate on a NanoDrop spectrophotometer using 1.5 µl of sample for each measurement, and an average concentration out of the three measurements was formed. In preparation for telomere length measurement, an average concentration of around 100 ng/µl was favorable.

Nucleic acid has its highest absorbance at 260 nm, whereas other substance classes have their strongest absorbance at different wavelengths - for example, possible contaminants such as proteins have their strongest absorbance at 280 nm, while other organic contaminants like phenol have their strongest absorbance at 230 nm (75, 76). For purity measurements, two ratios were formed. The first was the 260/280 ratio, where a value of approximately 1.8 is accepted as pure for DNA, while a lower value is an indirect marker for protein contamination. The 260/230 ratio is a secondary measure of DNA purity where a value of 2.0-2.2 is recognized as pure DNA, while a lower value can indicate organic contaminants in the sample (75-77).

To check for possible DNA fragmentation, each sample was loaded onto a 1% agarose gel and separated through electrophoresis (78). For the gel, 100 ml of 1x TRIS-Acetate-EDTA (TAE) Buffer were combined with 1 g of pulverized agarose in a flask, and the mixture was heated in the microwave until the agarose powder was completely dissolved. After cooling the gel to room temperature, 10 µl of SYBR Green gel stain were added and distributed in the entire mixture by swinging the flask multiple times. The gel was then poured into a gel chamber and left to cool down.

In preparation for electrophoresis, 10 µl of each extracted DNA sample were combined with 2 µl of 6x loading dye, and for each gel 15 µl of DNA ladder (Lambda DNA/Hind III Marker) was used (5 µl per pocket). Prior to use, the DNA ladder was heated to 65°C for five minutes and cooled on ice for three minutes. The gel was loaded with the DNA ladder and DNA samples, and the electrophoresis was run for 2.5 hours at 100 volts. Lastly gel pictures were taken with the VersaDoc Imaging System from BioRad and the gel pictures were assessed visually for possible DNA fragmentation. A smear would indicate differently sized DNA pieces, and therefore fragmented DNA, while a single streak would indicate DNA that was not fragmented (79).

2.4 Telomere length measurement

TLM was performed via monochrome multiplex qPCR (52) on three consecutive days on a Bio-Rad CFX384 real-time C1000 thermal cycler. In preparation for TLM, all 150 samples were diluted with nuclease-free water to a concentration of 20 ng/µl each. After dilution, all samples were measured again in triplicate on the NanoDrop spectrophotometer and adjusted to 20 ng/µl if necessary. The positive control (human leukemia cell line 1301) was also adjusted to 20 ng/µl. The positive control and non-template control (water) were prepared in duplicate and were run on every plate.

A standard for each extraction method was formed by combining all samples within one extraction method, hence 30 samples formed the starting mix for each extraction method, resulting in five standard dilution series in total. After combining all samples in each extraction method, the concentration was determined on the NanoDrop spectrophotometer to calculate the exact starting concentration for the standard dilution series. The standard solution was then processed in a 1:3 dilution, as follows: from a starting quantity of 45 µl, 15 µl were transferred to the next tube adding 30 µl of Milli-Q water, mixed well and repeated until 7 standard dilutions were reached. Each dilution was measured in triplicate in each qPCR run.

On each day, the following master mix was prepared: firstly, 1 µl Titanium Buffer, 0.20 µl Titanium Polymerase, 0.20 µl dNTP-mix (10 mM each), 0.075 µl SYBR Green (100x) and 0.525 µl Milli-Q water were combined to a premix of 2 µl per sample. This premix was then combined with 0.1 µl DTT, 2.0 µl Betaine, 0.7 µl telomere primer, 0.7 µl albumin primer and 2.5 µl Milli-Q water. In total, the master mix measured 8 µl per sample. The quantity of master mix was adjusted to the number of samples and in addition 10% of that amount was calculated for pipetting loss.

In preparation for qPCR, a 384-well plate was placed on ice, and 8 µl of master mix was pipetted in each designated sample well. The plate was then centrifuged to spin down the solution to the bottom of the well. Adding to the master mix, 2 µl of each standard dilution was pipetted into the wells, in three wells for each standard dilution. Also, 2 µl of each sample, and positive and non-template controls was pipetted. The plate was then sealed with an adhesive foil and directly before the run, the plate was centrifuged again to ensure that the master mix and samples were well combined on the bottom of the well without any air bubbles. The plate was then put into the qPCR machine.

The following qPCR program was performed: Stage 1: 15 min at 95°C; Stage 2: 2 cycles of 15 s at 94°C, 15 s at 49°C; Stage 3: 32 cycles of 15 s at 94°C, 10 s at 60°C, 15 s at 72°C with T-signal acquisition, 10 s at 85°C, 15 s at 89°C with S-signal acquisition. In the last step, a melt curve is acquired with an increment of 0.5°C for 5 s from 60°C to 95°C with signal acquisition.

To calculate relative telomere length, raw data files were exported from the qPCR machine separately for the telomer primer (T) and albumin primer (S), giving two results for the amplification product for each sample. The T/S-ratio was then formed for each of the standard dilutions, based on the measured starting concentration acting as an average value of telomere length. The T/S-ratio for each sample was then compared to the standard in order to evaluate relative telomer length, which is either longer or shorter than the standard. Since every sample was measured in triplicate, an average T/S-ratio was formed, and a coefficient of variation could be calculated for technical repeats. Telomere length was compared across extraction methods and inter-assay variation was calculated. For pool samples an intra-assay variation could also be formed. Lastly, samples were ranked according to telomere length.

2.5 Materials

For blood sampling the following materials were used: sterile vacutainer blood tubes with EDTA (BD, 367525), adapter (BD, 364815), butterfly needle (BD, 367282), tourniquet, disinfection spray (Softasept, 3887138), cotton swabs and plasters, pipettes and tips, 1.5 ml Eppendorf tubes (Sarstedt, 72.706), gloves.

DNA extractions: pipettes and tips (10, 20, 100, 200 and 1000 µl), Milli-Q water (Adelab Scientific, Milli-Q water purification system), 100% ethanol (Roth, 928.4), isopropanol (Merck, 1.09634.1011), vortex mixer (MSI Minishaker), laboratory centrifuge (Eppendorf, Centrifuge 5415 R), benchtop micro centrifuge (Roth), water bath (GFL, 15318), heat block (Eppendorf, ThermoMixer C, Thermomixer 5436), 50 ml Falcon tubes (Greiner Bio-One, 227261), 15 ml Falcon tubes (Corning, 352096), 1.5 ml Eppendorf tubes (Sarstedt, 72.706), 2 ml Eppendorf tubes (Sarstedt, 72.691), 1.5 ml LoBind Tubes (Eppendorf, 022431021), gloves.

Commercial Kits: Qiagen DNeasy Blood & Tissue Kit (Qiagen, 69504), Invisorb Blood Universal Kit (Stratec Molelcular, 1031150200), Epicentre MasterPure Complete DNA and RNA Purification Kit (Lucigen, MB711400).

Manual Method: EDTA (Merck, 1.08421.100), sodium dodecyl sulfate (Ultra Pure, 811030), Tris 10 mM (Roth, AE15.2), ethanol (Roth, 928.4), isopropanol (Merck, 1.09634.1011), potassium acetate (Roth, T 874.2), acetic acid (Merck, 1.00066.0250), DNase free RNAse (Thermo Fisher, EN0531), proteinase K (Macherey Nagel, 740506), ultrasonic bath (Faust, Transonic 700).

Phenol-Chloroform extraction: phenol-chloroform-isoamyl alcohol (Sigma-Aldrich, 77617), chloroform-isoamyl-alcohol (Sigma-Aldrich, 25666), elution buffer (10mM Tris-HCl, pH:8.5), NH4OAc (Sigma-Aldrich, 09691), Glycogen (Roche, 901 393), ethanol (Roth, 928.4).

Quality Testing: Pipettes and tips, NanoDrop spectrophotometer (Thermo Fisher Scientific, NanoDrop ND 1000), agarose powder (Invitrogen, 1551027), 1x TAE Buffer (50x TAE, Carl Roth, CL86.1), SYBR Green gel stain (Sigma-Aldrich, S9430), Lambda DNA/Hind III Marker (Thermo Fisher Scientific, SM0103), 6x Loading Dye (Thermo Fisher Scientific, SM0103), electrophoresis chamber, power supply (Bio Rad, Power Pac 300) and imaging system (Bio Rad, VersaDoc).

TLM reagents: TE (1mM EDTA, 10mM Tris HCL pH 8.0), Titanium Taq PCR buffer 10 (Clontech, 639141), Titanium Taq Polymerase (Clontech, 639209), dNTPs, set of dATP, dCTP, dGTP, dTTP 10 µmol each, 100 mM (Promega, U1330), Dithiothreitol (DTT) 1M (Sigma, 43816), Betaine (Sigma, 2629), Sybr Green 10000X (Sigma, S9430), Clean (RNAse free) H2O (Fresenius, B230531), gloves.

TLM primer sets:

Telomere primer Set (Telg and Telc) (Biolegio, PAGE purified):

Telg: 5'-ACA CTA AGG TTT GGG TTT GGG TTT GGG TTT GGG TTA GTG T-3'

Telc: 5'-TGT TAG GTA TCC CTA TCC CTA TCC CTA TCC CTA TCC CTA ACA-3'

Albumine primer Set (Albugc and Albdcg) (Biolegio, PAGE purified):

AlbU: 5'-CGG CGG CGG GCG GCG CGG GCT GGG CGG AAA TGC TGC ACA GAA TCC TTG-3'

AlbD: 5'-GCC CGG CCC GCC GCG CCC GTC CCG CCG GAA AAG CAT GGT CGC CTG TT-3'

TLM equipment: Bio-Rad T1000 (Thermal cycler with CFX 384 real-time system), NanoDrop spectrophotometer (Thermo Fisher Scientific, NanoDrop ND 1000), hard-shell 384-well PCR plates (Bio-Rad, HSP3801), PCR plate seal, Micro seal B film (Bio-Rad, MSB1001), 1.5 ml Eppendorf tubes (Sarstedt, 72.706), pipettes and tips.

3. Results

This section presents the results of DNA extraction in terms of quality of extracts, followed by a comparison of TLMs resulting from the different extraction methods.

3.1 Comparison of DNA extraction results

The Qiagen DNeasy Blood & Tissue Kit extracted DNA samples with an average concentration of 74 ng/µl, with the highest concentration at 123 ng/µl in sample Pool (C1-5)-3 and the lowest concentration at 44 ng/µl in sample C5 (see Table 2). The 260/280 ratio showed an average value of 1.79 and the 260/230 ratio an average value of 1.36, indicating a sign of organic contamination (see Table 1). On the agarose gel, all the samples showed a smear (see Figures 3, 4 and 5) as a sign of low quality and possible fragmentation of the DNA.

The Manual Method extracted DNA that exceeded the aimed average concentration of 100 ng/µl by over threefold, with an average concentration of 346 ng/µl. The highest quantity was 573 ng/µl in sample B5 and the lowest 122 ng/µl in sample Pool (B1-5)-1 (see Table 3). The purity measurements showed a 260/280 ratio with an average value of 1.82 and a 260/230 ratio with an average value of 2.10 (see Table 1), both in the acceptable range for pure DNA. However, this method showed long smears on the gel indicating substantial fragmentation (see Figures 3, 4 and 5).

The MasterPure Complete DNA and RNA Purification Kit from Epicentre yielded DNA with an average concentration of 59 ng/µl, with the highest concentration at 96 ng/µl in sample Pool (C1-5)-2 and the lowest concentration at 23 ng/µl in sample Pool (C1-5)-1 (see Table 4). The 260/280 ratio had an average value of 1.76 and the 260/230 ratio an average value of 1.45 (see Table 1), both indicating possible protein and organic contamination. The gel electrophoresis image showed no smears (see Figures 3, 4 and 5), so it is therefore unlikely that major DNA fragmentation had occurred.

With the Phenol-Chloroform extraction method, the average DNA concentration was 53 ng/µl, with the highest concentration at 160 ng/µl in sample A2 and the lowest concentration at 20 ng/µl in sample B2 (see Table 5). The 260/280 ratio had an average value of 0.92 and the 260/230 ratio an average value of 0.18 (see Table 1), indicating severe contamination. The gel electrophoresis image showed no smears (see Figures 3, 4 and 5), but as the concentration was so low, it was hard to assess possible fragmentation.

With the Stratec Invisorb Blood Universal Kit the average DNA concentration was 202 ng/µl, with the highest concentration at 358 ng/µl in sample C4 and the lowest concentration at 117 ng/µl in sample C5 (see Table 6). The 260/280 ratio had an average value of 1.89 and the 260/230 ratio an average value of 2.37 (see Table 1). Gel electrophoresis images showed no smears (see Figures 3, 4 and 5).

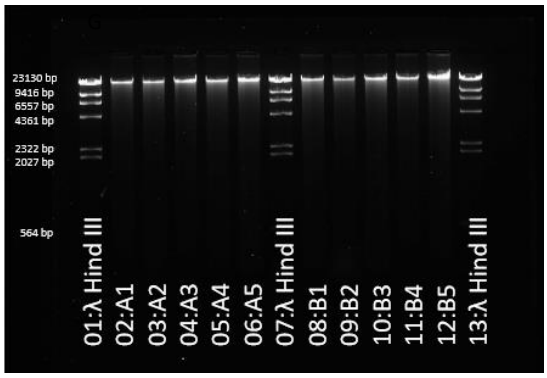
Table 1. DNA quality and quantity results (concentration, purity ratios, fragmentation). Values shown are averages for each extraction method.

Extraction Method	**Average C (ng/µl)**	**260/280 (Δ Optimum) ***	**260/230 (Δ Optimum) ***	**Fragmentation**
Qiagen DNeasy	74.17	1.79 (0.01)	1.36 (0.74)	+
Manual Method	346.00	1.82 (0.02)	2.10 (0.00)	++
Epicentre MasterPure	58.59	1.76 (0.04)	1.45 (0.65)	(+)
Phenol-Chloroform	53.44	0.92 (0.88)	0.18 (1.92)	-
Stratec Invisorb	202.28	1.89 (0.09)	2.37 (0.27)	(+)

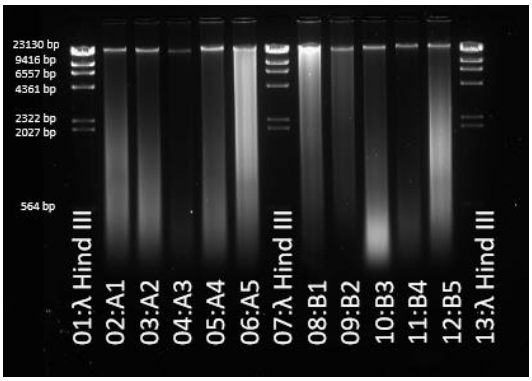
*Difference from optimum value: 260/280=1.8; 260/230=2.1

Desired DNA quality criteria for TLM

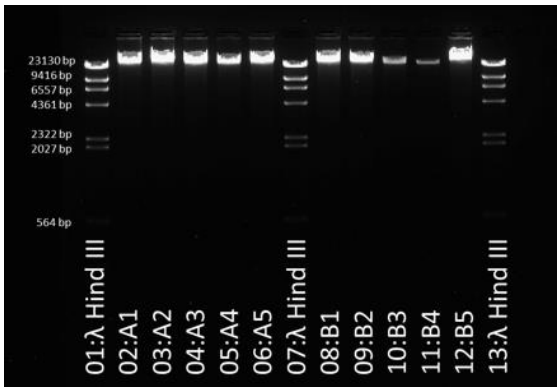
Overall, the Stratec Invisorb Blood Universal Kit exceeded all desired quantity and quality measurements: concentration was within the desired range, purity measurements were acceptable, and there was no sign of severe degradation in the electrophoresis. The Manual Method yielded suitable quality values in all measurements except for possible fragmentation, for which it yielded the overall poorest results on the agarose gel out of the five methods. The Qiagen DNeasy Blood & Tissue Kit extracted DNA with a lower concentration, a poor secondary measure for purity and possibly degraded DNA. The Epicentre MasterPure Complete DNA and RNA purification kit yielded DNA that was low in concentration and the secondary purity measure indicated organic contamination; however, it showed no signs of severe fragmentation. Overall, the Phenol-Chloroform extraction method produced DNA with the poorest results: DNA concentration appeared to be not only very low but also highly contaminated. Furthermore, it was not possible to assess fragmentation on the gel, as the concentration was too low for most of the samples.



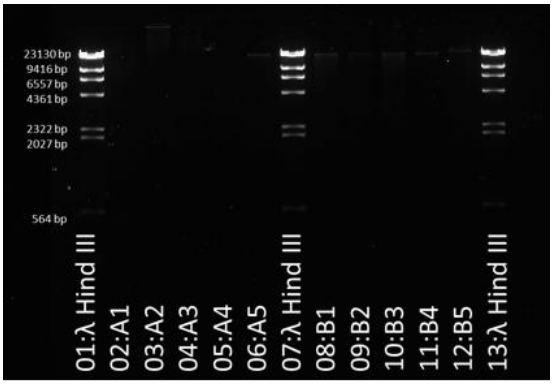
Qiagen DNeasy Blood & Tissue



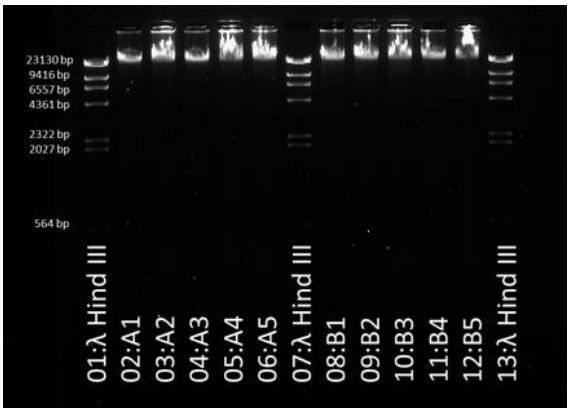
Manual Method



Epicentre MasterPure Complete DNA and RNA purification kit

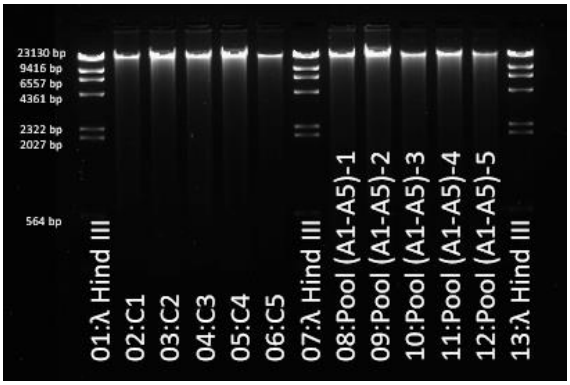


Phenol-Chloroform extraction

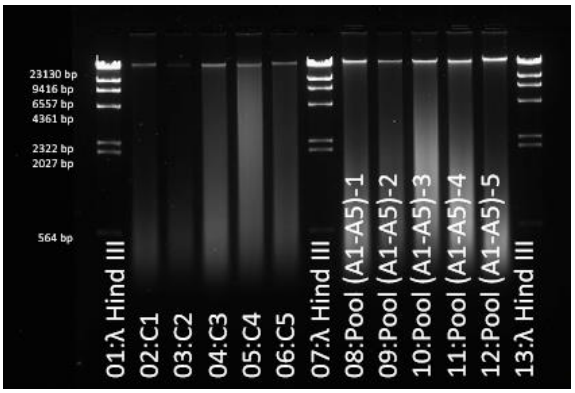


Stratec Invisorb Blood Universal kit

Figure 3. Gel electrophoresis images of ten identical samples extracted with five different DNA extraction methods (individual samples of age groups A and B). Wells 01, 07, 13: Lambda DNA/Hind III Marker, fragments: 23130 bp, 9416 bp, 6557 bp, 4361 bp, 2322 bp, 2027 bp and 564 bp. Wells 02-06: DNA Samples A1, A2, A3, A4, A5; wells 08-12: DNA Samples B1, B2, B3, B4, B5. Each electrophoresis was performed with 1% Agarose Gels at 100 volts for 2.5 hours.



Qiagen DNeasy Blood & Tissue Kit



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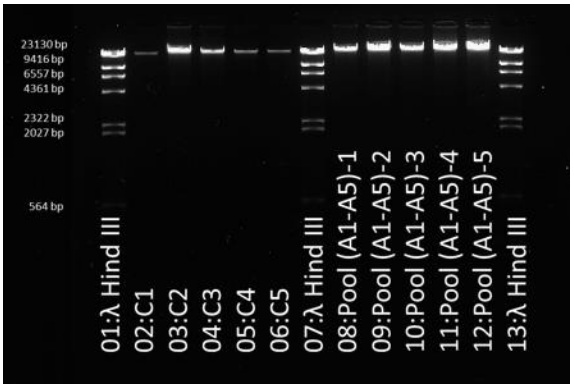
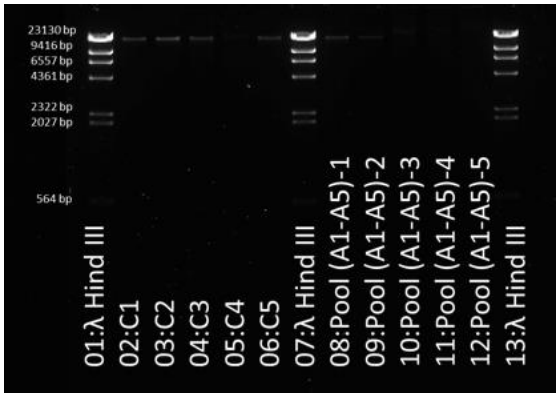
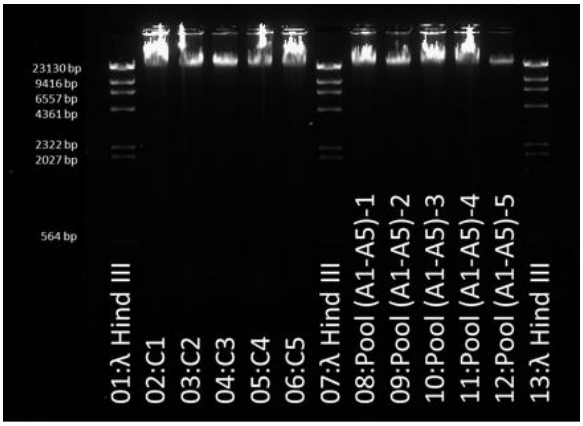


Epicentre Master Pure Complete DNA and RNA purification kit

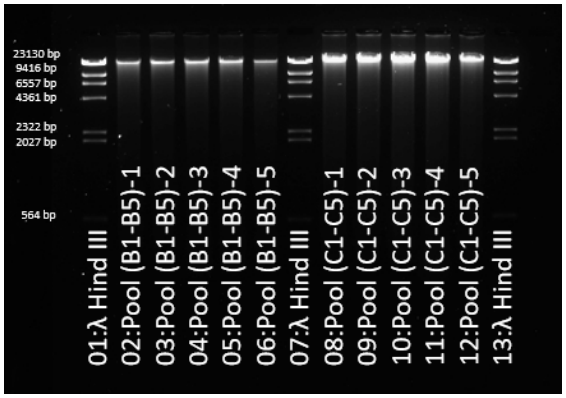


Phenol-Chloroform extraction

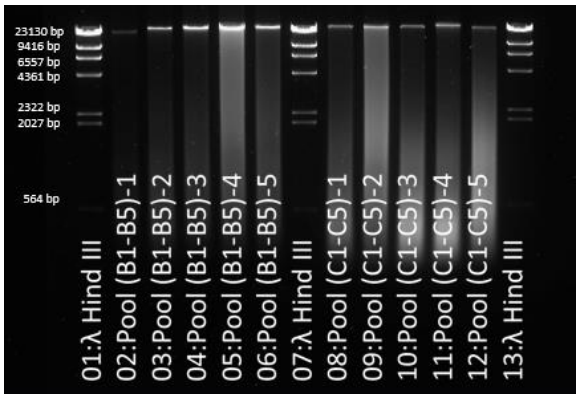


Stratec Invisorb Blood Universal kit

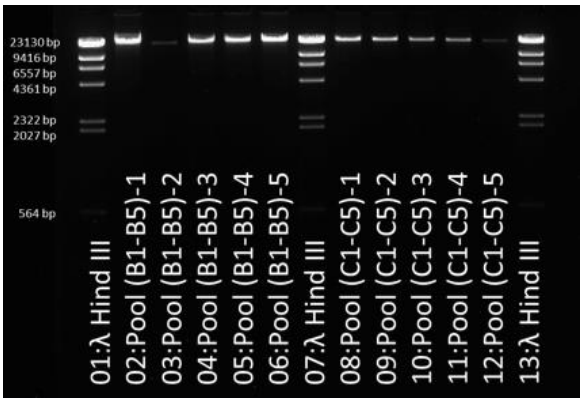
Figure 4. Gel electrophoresis images of ten identical samples extracted with five different DNA extraction methods (individual samples of age group C and pool samples of age group A). Wells 01, 07, 13: Lambda DNA/Hind III Marker, fragments: 23130 bp, 9416 bp, 6557 bp, 4361 bp, 2322 bp, 2027 bp and 564 bp. Wells 02-06: DNA Samples C1, C2, C3, C4, C5; wells 08-12: DNA Samples Pool (A1-5)-1, Pool (A1-5)-2, Pool (A1-5)-3, Pool (A1-5)-4, Pool (A1-5)-5. Each electrophoresis was performed with 1% Agarose Gels at 100 volts for 2.5 hours.



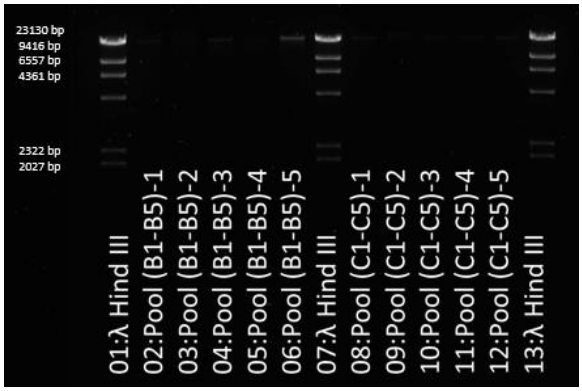
Qiagen DNeasy Blood and Tissue Kit



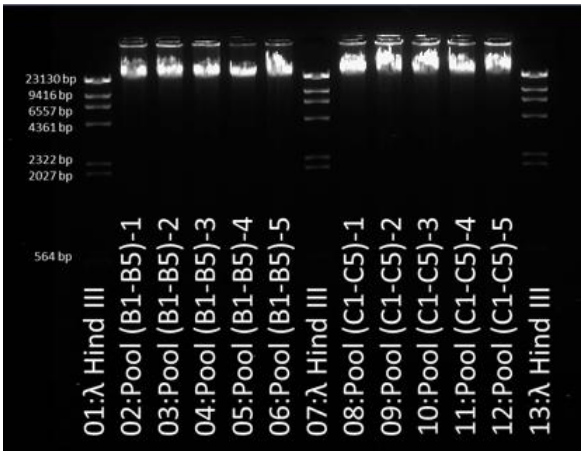
Manual Method



Epicentre MasterPure Complete DNA and RNA purification kit



Phenol-Chloroform extraction



Stratec Invisorb Blood Universal kit

Figure 5. Gel electrophoresis images of ten identical samples extracted with five different DNA extraction methods (pool samples of age groups B and C). Wells 01, 07, 13: Lambda DNA/Hind III Marker, fragments: 23130 bp, 9416 bp, 6557 bp, 4361 bp, 2322 bp, 2027 bp and 564 bp. Wells 02-06: DNA Samples Pool (B1-B5)-1, Pool (B1-B5)-2, Pool (B1-B5)-3, Pool (B1-B5)-4, Pool (B1-B5)-5; wells 08-12: DNA Samples Pool (C1-C5)-1, Pool (C1-C5)-2, Pool (C1-C5)-3, Pool (C1-C5)-4, Pool (C1-C5)-5. Each electrophoresis was performed with 1% Agarose Gels at 100 volts for 2.5 hours.

Table 2. Concentration and purity measurements of 30 samples extracted with the Qiagen DNeasy Blood and Tissue Kit. *

Sample ID	ng/ul	260/280	260/230
A1	49	1.71	1.57
A2	57	1.80	1.55
A3	67	1.70	1.03
A4	61	1.76	1.32
A5	83	1.82	1.62
B1	65	1.77	1.17
B2	59	1.78	1.02
B3	77	1.76	1.01
B4	60	1.86	1.47
B5	97	1.84	1.56
C1	51	1.84	1.64
C2	70	1.77	1.45
C3	74	1.82	1.72
C4	82	1.80	1.36
C5	44	1.71	0.97
Pool (A1-A5)-1	71	1.81	1.45
Pool (A1-A5)-2	97	1.82	1.62
Pool (A1-A5)-3	59	1.73	1.12
Pool (A1-A5)-4	67	1.73	1.11
Pool (A1-A5)-5	51	1.75	1.07
Pool (B1-B5)-1	57	1.82	1.37
Pool (B1-B5)-2	68	1.80	1.17
Pool (B1-B5)-3	69	1.73	0.97
Pool (B1-B5)-4	71	1.80	1.56
Pool (B1-B5)-5	61	1.79	1.31
Pool (C1-C5)-1	107	1.85	1.64
Pool (C1-C5)-2	113	1.81	1.38
Pool (C1-C5)-3	123	1.84	1.77
Pool (C1-C5)-4	122	1.79	1.29
Pool (C1-C5)-5	93	1.81	1.49
Average	**74**	**1.79**	**1.36**

*Each sample was measured in triplicate and average values were determined.

Table 3. Concentration and purity measurements of 30 samples extracted with the Manual Method. *

Sample ID	ng/ul	260/280	260/230
A1	364	1.87	2.28
A2	556	1.88	2.30
A3	160	1.80	2.10
A4	354	1.86	2.14
A5	495	1.86	1.85
B1	353	1.82	2.02
B2	369	1.76	2.17
B3	543	1.86	2.05
B4	249	1.90	2.07
B5	573	1.85	1.68
C1	191	1.73	1.91
C2	140	1.67	2.34
C3	290	1.87	1.99
C4	335	1.86	2.18
C5	238	1.84	1.78
Pool (A1-A5)-1	380	1.74	2.47
Pool (A1-A5)-2	385	1.78	2.41
Pool (A1-A5)-3	344	1.84	2.35
Pool (A1-A5)-4	348	1.88	2.10
Pool (A1-A5)-5	325	1.86	2.19
Pool (B1-B5)-1	122	1.69	2.21
Pool (B1-B5)-2	307	1.78	2.29
Pool (B1-B5)-3	323	1.76	2.21
Pool (B1-B5)-4	318	1.84	2.03
Pool (B1-B5)-5	342	1.83	1.94
Pool (C1-C5)-1	429	1.81	2.11
Pool (C1-C5)-2	382	1.79	1.94
Pool (C1-C5)-3	411	1.81	2.04
Pool (C1-C5)-4	358	1.76	1.93
Pool (C1-C5)-5	394	1.85	1.95
Average	**346**	**1.82**	**2.10**

*Each sample was measured in triplicate and average values were determined.

Table 4. Concentration and purity measurements of 30 samples extracted with the Epicentre MasterPure Complete DNA and RNA purification kit. *

Sample ID	ng/µl	260/280	260/230
A1	89	1.78	1.53
A2	83	1.76	1.37
A3	95	1.81	1.50
A4	70	1.75	1.39
A5	75	1.79	1.55
B1	86	1.79	1.56
B2	66	1.79	1.65
B3	47	1.75	1.12
B4	45	1.76	0.97
B5	70	1.70	1.62
C1	58	1.83	1.11
C2	44	1.70	1.32
C3	30	1.69	2.79
C4	73	1.80	1.13
C5	37	1.85	0.83
Pool (A1-A5)-1	35	1.80	1.59
Pool (A1-A5)-2	58	1.78	1.69
Pool (A1-A5)-3	44	1.73	1.74
Pool (A1-A5)-4	60	1.81	1.58
Pool (A1-A5)-5	58	1.78	1.62
Pool (B1-B5)-1	41	1.68	1.26
Pool (B1-B5)-2	59	1.79	1.05
Pool (B1-B5)-3	33	1.76	1.62
Pool (B1-B5)-4	31	1.75	1.66
Pool (B1-B5)-5	39	1.64	1.56
Pool (C1-C5)-1	23	1.63	1.48
Pool (C1-C5)-2	96	1.84	1.39
Pool (C1-C5)-3	64	1.83	1.27
Pool (C1-C5)-4	72	1.82	1.30
Pool (C1-C5)-5	75	1.77	1.17
Average	**59**	**1.76**	**1.45**

*Each sample was measured in triplicate and average values were determined.

Table 5. Concentration and purity measurements of 30 samples extracted with Phenol-Chloroform extraction. *

Sample ID	ng/µl	260/280	260/230
A1	65	0.68	0.12
A2	160	0.77	0.31
A3	69	0.74	0.13
A4	50	0.69	0.11
A5	127	0.95	0.19
B1	88	0.98	0.19
B2	20	0.70	0.12
B3	21	0.81	0.12
B4	26	1.11	0.19
B5	44	0.66	0.11
C1	92	1.08	0.22
C2	101	1.37	0.32
C3	91	1.10	0.22
C4	67	0.89	0.16
C5	52	1.03	0.18
Pool (A1-A5)-1	37	1.20	0.19
Pool (A1-A5)-2	75	0.92	0.15
Pool (A1-A5)-3	28	0.65	0.10
Pool (A1-A5)-4	32	0.66	0.10
Pool (A1-A5)-5	32	0.68	0.10
Pool (B1-B5)-1	59	0.94	0.16
Pool (B1-B5)-2	30	0.72	0.11
Pool (B1-B5)-3	42	1.08	0.18
Pool (B1-B5)-4	34	0.90	0.15
Pool (B1-B5)-5	26	0.99	0.25
Pool (C1-C5)-1	21	1.26	0.26
Pool (C1-C5)-2	38	0.80	0.12
Pool (C1-C5)-3	31	0.85	0.14
Pool (C1-C5)-4	23	1.20	0.28
Pool (C1-C5)-5	25	1.11	0.29
Average	**53**	**0.92**	**0.18**

*Each sample was measured in triplicate and average values were determined.

Table 6. Concentration and purity measurements of 30 samples extracted with the Stratec Invisorb Blood Universal Kit. *

Sample ID	ng/µl	260/280	260/230
A1	228	1.88	2.37
A2	282	1.89	2.34
A3	162	1.87	2.34
A4	222	1.89	2.39
A5	232	1.87	2.28
B1	158	1.90	2.37
B2	120	1.90	2.43
B3	262	1.89	2.36
B4	154	1.90	2.42
B5	315	1.89	2.35
C1	133	1.90	2.39
C2	244	1.89	2.36
C3	189	1.89	2.35
C4	358	1.89	2.34
C5	117	1.88	2.44
Pool (A1-A5)-1	204	1.88	2.35
Pool (A1-A5)-2	214	1.89	2.37
Pool (A1-A5)-3	173	1.89	2.42
Pool (A1-A5)-4	190	1.89	2.37
Pool (A1-A5)-5	190	1.89	2.36
Pool (B1-B5)-1	163	1.90	2.38
Pool (B1-B5)-2	164	1.89	2.38
Pool (B1-B5)-3	172	1.89	2.41
Pool (B1-B5)-4	151	1.88	2.39
Pool (B1-B5)-5	135	1.90	2.36
Pool (C1-C5)-1	207	1.89	2.36
Pool (C1-C5)-2	230	1.90	2.36
Pool (C1-C5)-3	242	1.89	2.33
Pool (C1-C5)-4	200	1.90	2.38
Pool (C1-C5)-5	257	1.89	2.33
Average	**202**	**1.89**	**2.37**

*Each sample was measured in triplicate and average values were determined.

3.2 Comparison of telomere length measurements

After analyzing the T/S-ratio for all measured samples, three separate approaches were taken: First, it was evaluated how the samples performed in terms of variation over three technical repeats. A variation of less than 10% was considered acceptable. Second, it was analyzed how comparable the telomere length results were between extraction methods for each sample with respect to inter-assay variation. Intra-assay variation was also evaluated for pooled samples. Third, samples were ranked according to telomere length. Results were determined separately for individual samples and identical pool samples.

a) Individual samples

Samples extracted with the Qiagen DNeasy Blood & Tissue Kit showed an average coefficient of variation of 3.91% for technical repeats, whereas samples extracted with the Manual Method showed an average coefficient of variation of 3.24%. Epicentre MasterPure Complete DNA and RNA Purification Kit produced samples with an average coefficient of variation of 4.68% and Stratec Invisorb Blood Universal Kit an average coefficient of variation of 4.61%. All four extraction methods therefore yielded the desired variation (< 10%) across technical repeats (see Table 7). These extraction methods thus performed well in terms of accuracy and reproducibility measurements. Phenol-Chloroform extraction, however, yielded a coefficient of variation across technical repeats of over 70% in age group A, with an average coefficient of variation of 47.83%, and thus far exceeding the desired variation of < 10%. Due to this variation and previous poor results in DNA quality assessment, Phenol-Chloroform-extracted samples lacked comparability with other methods and were henceforth excluded from subsequent comparison (see Table 7).

Regarding T/S-ratios, Qiagen DNeasy Blood & Tissue kit yielded the longest telomeres per age group compared to the remaining three methods, which are all salting-out DNA extraction techniques. Epicentre MasterPure Complete DNA and RNA Purification Kit produced DNA with the shortest average telomere length per age group (see Table 7).

Table 7. Average T/S-ratios and average coefficients of variation for technical repeats per age group and extraction method for individual samples. *

Method	Age group	Average T/S	Average CV %	Average CV %
Qiagen DNeasy	Samples A	1.33	3.59	3.91
	Samples B	1.16	3.50	
	Samples C	1.03	4.63	
Manual Method	Samples A	1.12	3.05	3.24
	Samples B	1.07	3.49	
	Samples C	0.93	3.17	
Epicentre MasterPure	Samples A	1.04	5.79	4.68
	Samples B	0.92	5.00	
	Samples C	1.01	3.24	
Phenol-Chloroform	Samples A	5.55	70.20	47.83
	Samples B	0.50	42.44	
	Samples C	1.64	30.84	
Stratec Invisorb	Samples A	1.16	5.16	4.61
	Samples B	1.13	4.15	
	Samples C	0.99	4.52	

***Each sample was measured for its telomere length on three consecutive days (three technical repeats). Out of these three measurements, an average T/S-ratio was formed for each individual sample, resulting in an average T/S-ratio per age group. In addition, coefficients of variation were calculated and averaged for each age group as well as for the method in general. All results are listed within an extraction method.

Desired coefficient of variation of < 10%.

Telomere length values for individual samples varied depending on the extraction method employed. Samples in age group A showed an average inter-assay variation of 12.91% (see Figure 6). Sample A1 displayed the lowest inter-assay variation of 11%, and samples A2 and A3 had the highest inter-assay variation of 14%. Samples in age group B yielded an average inter-assay variation of 13.75% (see Figure 7), with sample B2 producing the highest inter-assay variation with 19%. Samples B4 and B5 showed the lowest inter-assay variation with 10% each. In age group C, an average inter-assay variation of 10.88% was observed (see Figure 8). Sample C5 displayed an average inter-assay variation of only 6%, but sample C3 had the highest variation of 19%. All individual samples, except sample C4 and C5, had an inter-assay variation in telomere length measurements of higher than 10%. Overall, the average coefficient of variation was 12.51% across 15 individual samples.

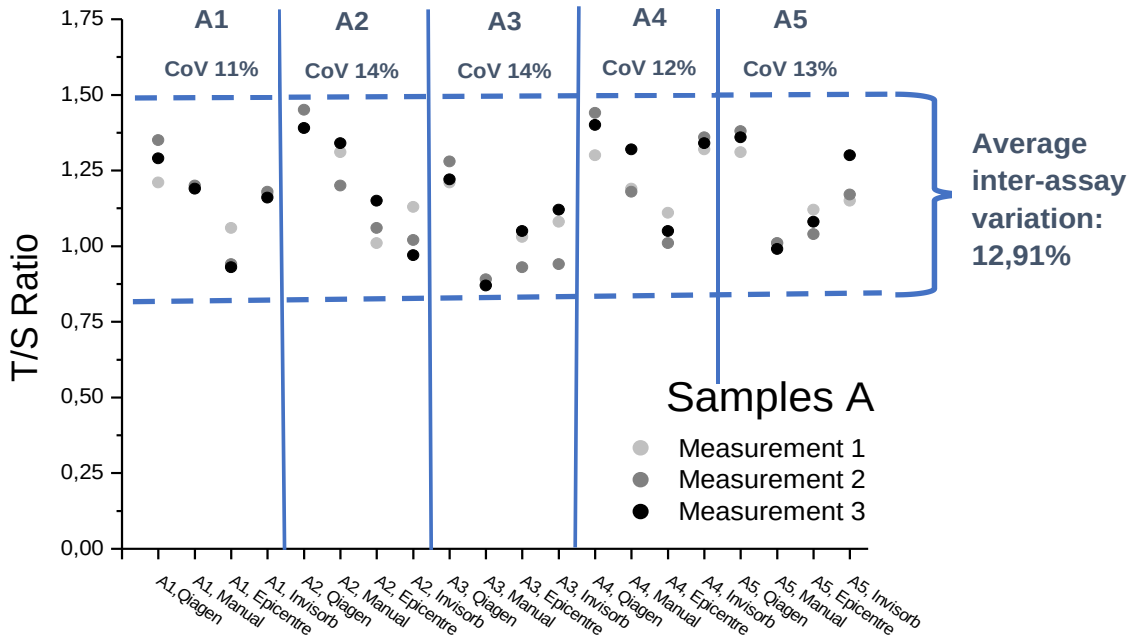


Figure 6. T/S-ratios (y-axis) for individual samples of age group A ("young"): A1, A2, A3, A4, A5, extracted with four DNA extraction methods (x-axis): Qiagen, Manual, Epicentre, Invisorb. Each sample was measured on three consecutive days (Measurements 1, 2, 3). Inter-assay variation was determined for each individual sample across extraction methods, and additionally the average inter-assay variation was calculated for the age group.

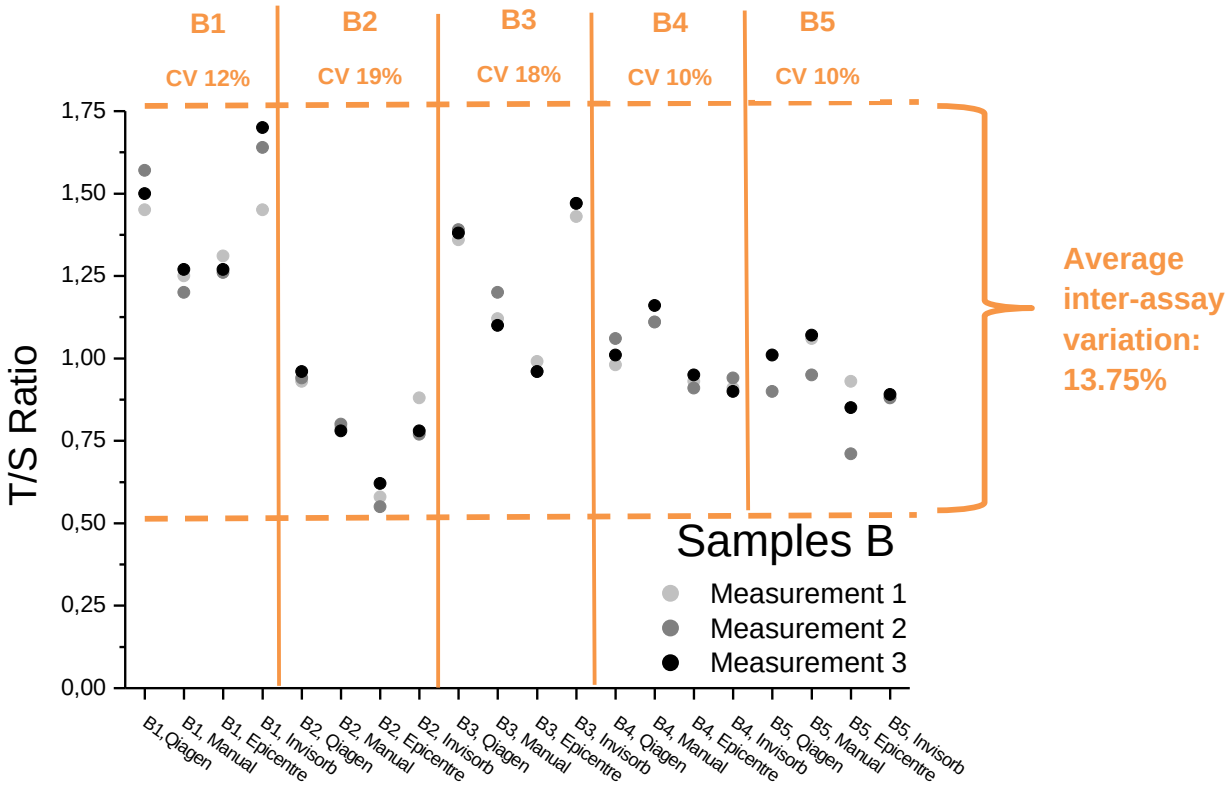


Figure 7. T/S-ratios (y-axis) for individual samples of age group B ("middle-aged"): B1, B2, B3, B4, B5, extracted with four DNA extraction methods (x-axis): Qiagen, Manual, Epicentre, Invisorb. Each sample was measured on three consecutive days (Measurements 1, 2, 3). Inter-assay variation was determined for each individual sample across extraction methods, and additionally the average inter-assay variation was calculated for the age group.

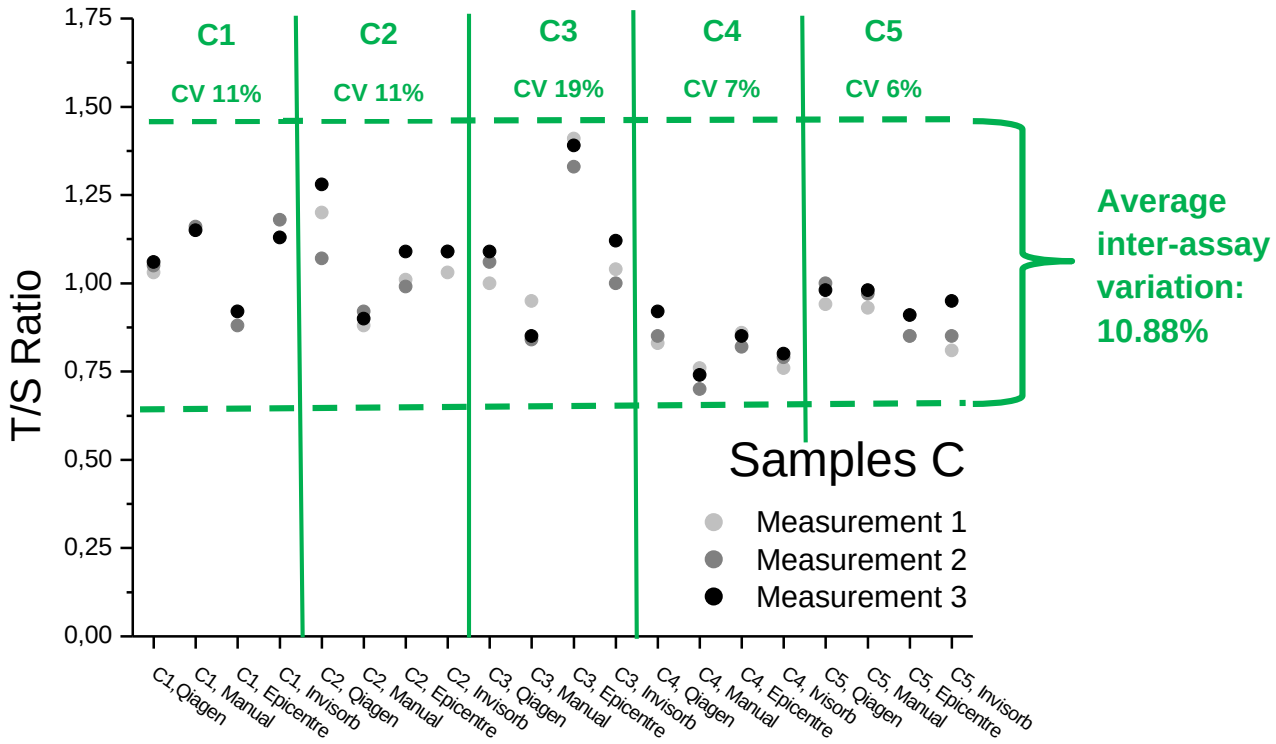


Figure 8. T/S-ratios (y-axis) for individual samples of age group C ("elder"): C1, C2, C3, C4, C5, extracted with four DNA extraction methods (x-axis): Qiagen, Manual, Epicentre, Invisorb. Each sample was measured on three consecutive days (Measurements 1, 2, 3). Inter-assay variation was formed for each individual sample across extraction methods, and additionally an average inter-assay variation was calculated for the age group.

All individual samples were ranked according to their telomere length (see Table 8). Importantly, no consistent ranking existed across the different extraction methods: samples B2, C4 and C5 appeared to have the shortest telomeres, and sample B1 the longest. For telomere lengths that fall between the longest and shortest telomere lengths, the ranking for each extraction method varied greatly. For example, sample B3 had the second longest telomere length when extracted with the Stratec Invisorb Blood Universal Kit, but only ranked 9th when extracted with the Epicentre MasterPure Complete DNA and RNA Purification Kit (see Table 8).

Table 8. Ranking of telomere length of all individual samples according to method (Qiagen, Manual, Epicentre, Invisorb) and average ranking of all methods. *

Individual Sample	**A1**	**A2**	**A3**	**A4**	**A5**	**B1**	**B2**	**B3**	**B4**	**B5**	**C1**	**C2**	**C3**	**C4**	**C5**
Qiagen DNeasy	6	2	7	3	5	1	14	4	11	13	10	8	9	15	12
Manual Method	4	1	13	3	9	2	14	6	7	8	5	11	12	15	10
Epicentre MasterPure	8	4	7	5	3	2	15	9	10	14	11	6	1	13	12
Stratec Invisorb	5	10	9	3	4	1	14	2	11	12	6	7	8	15	13
Average of all Methods	**6**	**4**	**10**	**2**	**5**	**1**	**15**	**3**	**11**	**12**	**8**	**9**	**7**	**14**	**13**

*Order of rank: 15 individual DNA samples were ranked according to their telomere length (rank 1 representing the longest telomere length, rank 15 the shortest telomere length).

a) Pool Samples

For technical repeats, pool samples extracted with the Qiagen DNeasy Blood & Tissue kit showed an average coefficient of variation of 3.04%, comparable to the variation yielded with the Manual Method, which had an average coefficient of variation of 3%. Epicentre MasterPure Complete DNA and RNA Purification Kit yielded samples with an average coefficient of variation of 4.07%, and Stratec Invisorb Blood Universal Kit had an average coefficient of variation of 3% (see Table 9). These variations were within the desired variation of < 10%. Therefore, these extraction methods performed well in accuracy and reproducibility measurements. Overall, the variation for technical repeats was lower than for individual samples.

As previously seen in individual samples, pool samples of the Phenol-Chloroform extraction yielded a coefficient of variation higher than 10%, with an average coefficient of variation of 31.78% across technical repeats. Due to this variation, along with the poor results in DNA quality measurements, Phenol-Chloroform-extracted pool samples were also excluded from further comparison due to the lack of accuracy of this method.

Qiagen DNeasy Blood & Tissue Kit produced the longest average relative telomere lengths for age groups A and B, whereas for age group C, the Manual Method yielded the longest average telomere length. Epicentre MasterPure Complete DNA and RNA Purification Kit extracted DNA with the shortest average telomere length in each age group (see Table 9).

Table 9. Average T/S-ratios and average coefficients of variation for technical repeats per age group and extraction method for pool samples. *

Method	Age group	Average T/S	Average CV %	Average CV %
Qiagen DNeasy	Pool (A1-A5)	1.35	1.16	3.04
	Pool (B1-B5)	1.14	3.24	
	Pool (C1-C5)	1.00	4.70	
Manual Method	Pool (A1-A5)	1.24	2.26	3.00
	Pool (B1-B5)	1.09	3.30	
	Pool (C1-C5)	1.06	3.45	
Epicentre MasterPure	Pool (A1-A5)	1.20	4.91	4.07
	Pool (B1-B5)	1.04	4.09	
	Pool (C1-C5)	0.93	3.21	
Phenol Chloroform	Pool (A1-A5)	1.38	27.80	31.78
	Pool (B1-B5)	0.56	34.04	
	Pool (C1-C5)	0.71	33.51	
Stratec Invisorb	Pool (A1-A5)	1.31	1.68	3.00
	Pool (B1-B5)	1.10	2.65	
	Pool (C1-C5)	1.04	4.67	

***** Each sample was measured for its telomere length on three consecutive days (three technical repeats). Out of these three measurements, an average T/S-ratio was formed for each pool sample, resulting in an average T/S-ratio per age group. In addition, coefficients of variation were calculated and averaged for each age group as well as for the method in general. All results are listed within an extraction method.

Desired coefficient of variation of < 10%.

Pool samples in each age group include 20 DNA samples that were derived from the identical blood pool. After performing telomere length measurement, pool samples in age group A showed an average inter-assay variation of 8.58% across four extraction methods. Within each extraction method, intra-assay variation was also calculated: Qiagen DNeasy Blood & Tissue Kit yielded an intra-assay variation of 9%, Invisorb Blood Universal Kit from Stratec and the Manual Method had the same intra-assay variation of 8%, whereas the Epicentre MasterPure Complete DNA and RNA Purification Kit yielded the lowest intra assay variation of 5% (see Figure 9).

Pool samples in age group B showed an average inter-assay variation of 6.09% across four extraction methods. Considering each extraction method individually, the Manual Method presented an intra-assay variation of 8% and Qiagen DNeasy Blood & Tissue Kit showed an intra-assay variation of 6%. The Epicentre MasterPure Complete DNA and RNA Purification Kit yielded an intra-assay variation of 4% and Stratec Invisorb Blood Universal Kit had the lowest intra-assay variation of 3% (see Figure 10).

Pool samples in age group C showed an average inter-assay variation of 10.88%. The highest intra-assay variation of 15% was seen in samples extracted with the Epicentre MasterPure Complete DNA and RNA Purification Kit, while there was an intra-assay variation of 10% in samples extracted with the Manual Method. With the Stratec Invisorb Blood Universal Kit, an intra-assay variation of 5% could be observed. Qiagen DNeasy Blood & Tissue Kit showed the lowest intra-assay variation of 4% (see Figure 11).

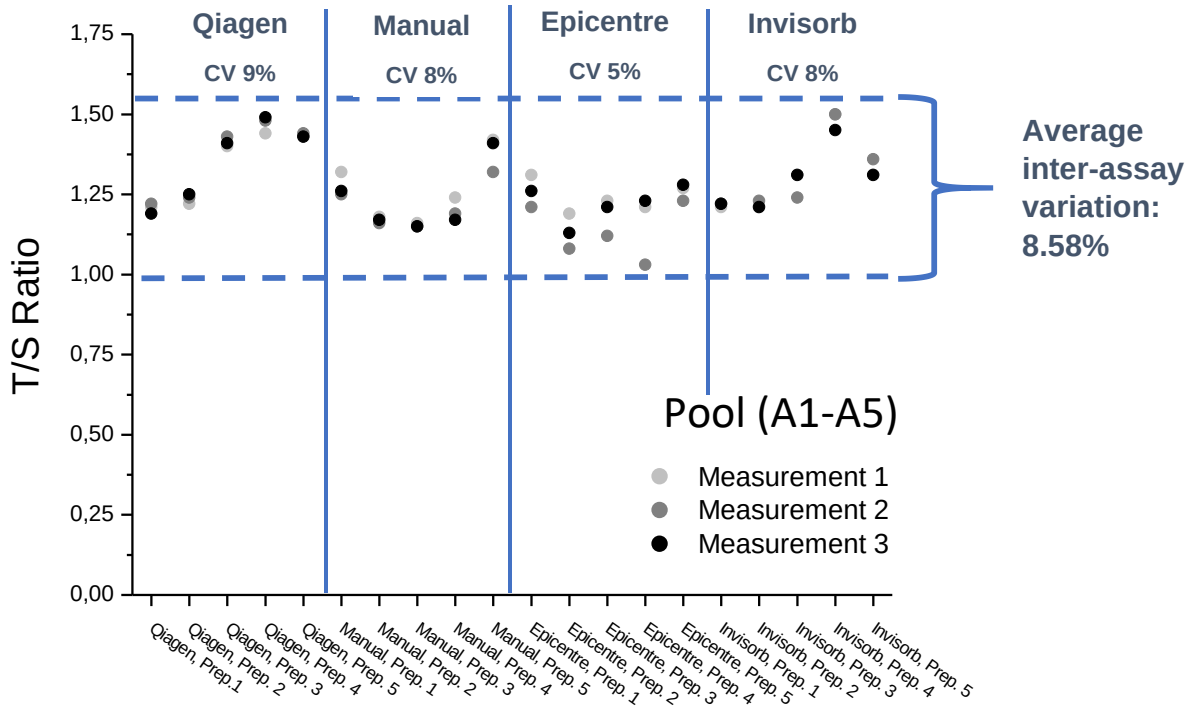


Figure 9. T/S-ratios (y-axis) for identical pool samples of age group A ("young"): Pool (A1-A5)-1, Pool (A1-A5)-2, Pool (A1-A5)-3, Pool (A1-A5)-4, Pool (A1-A5)-5, extracted with four DNA extraction methods (x-axis): Qiagen, Manual, Epicentre, Invisorb. Each sample was measured on three consecutive days (Measurements 1, 2, 3), a coefficient of variation was formed for each extraction method (intra-assay variation), and additionally an inter-assay variation was calculated for the entire age group.

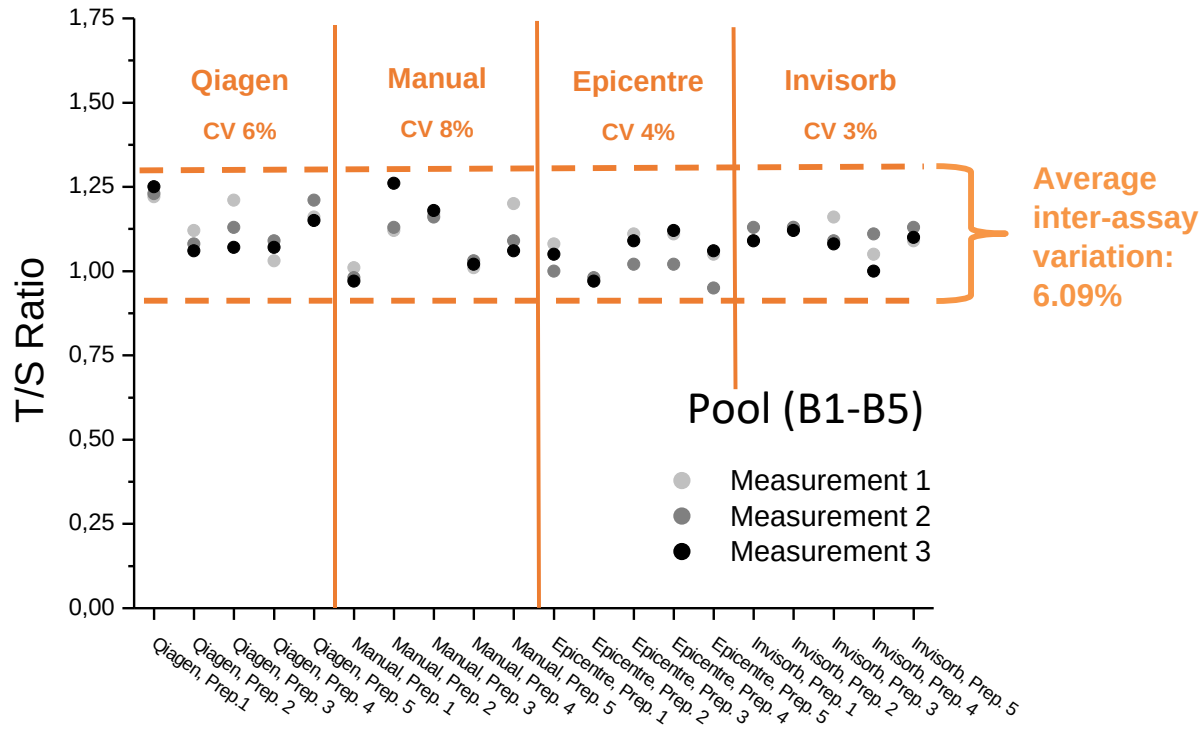


Figure 10. T/S-ratios (y-axis) for identical pool samples of age group B ("middle-aged"): Pool (B1-B5)-1, Pool (B1-B5)-2, Pool (B1-B5)-3, Pool (B1-B5)-4, Pool (B1-5)-5, extracted with four DNA extraction methods (x-axis): Qiagen, Manual, Epicentre, Invisorb. Each sample was measured on three consecutive days (Measurements 1, 2, 3), a coefficient of variation was formed for each extraction method (intra-assay variation), and additionally an inter-assay variation was calculated for the entire age group.

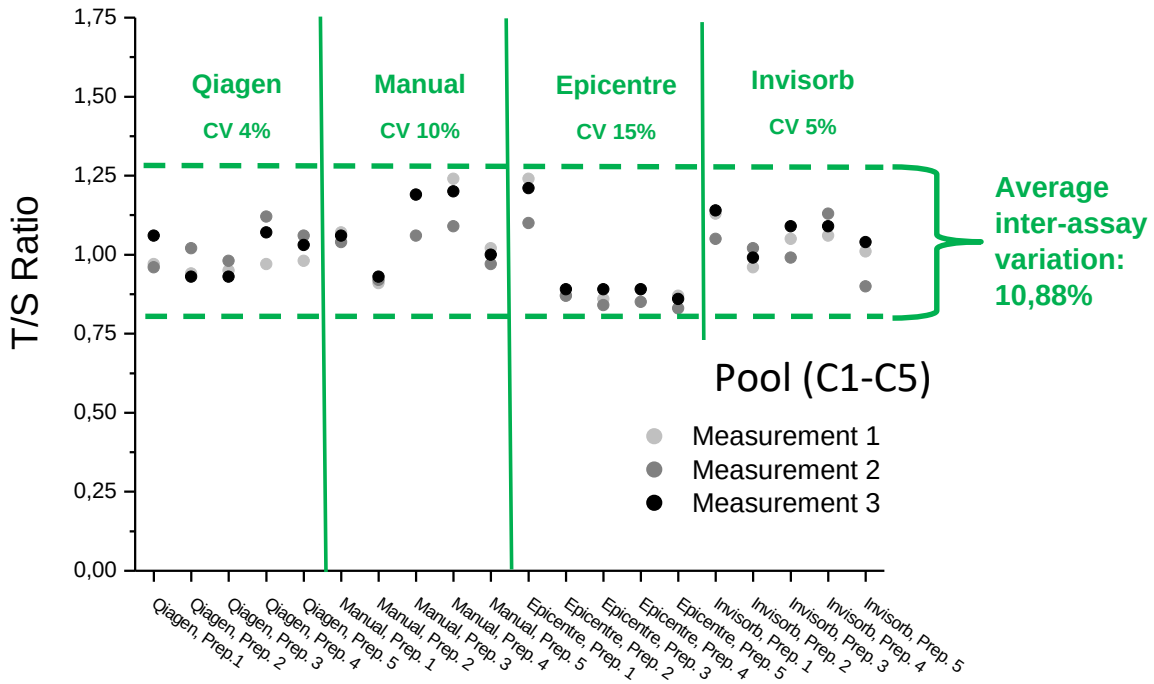


Figure 11. T/S-ratios (y-axis) for identical pool samples of age group C ("elder"): Pool (C1-C5)-1, Pool (C1-C5)-2, Pool (C1-C5)-3, Pool (C1-C5)-4, Pool (C1-C5)-5, extracted with four DNA extraction methods (x-axis): Qiagen, Manual, Epicentre, Invisorb. Each sample was measured on three consecutive days (Measurements 1, 2, 3), a coefficient of variation was formed for each extraction method (intra-assay variation), and additionally an inter-assay variation was calculated for the entire age group.

Similarly to the ranking of individual samples, ordering of pool samples with respect to telomere length produced no consistent ranking across different DNA extraction methods. It could be observed, however, that on average, individuals of age group A have the longest telomeres (ranks 1-5), individuals in age group C have the shortest telomers (ranks 10, 12, 13, 14, 15), and age group B telomeres fall in between the shortest and the longest telomeres (ranks 6, 7, 8, 9, 11) (see Table 10).

Table 10. Ranking of telomere length of all pool samples according to method (Qiagen, Manual, Epicentre, Invisorb) and average ranking of all methods. *

Pool Sample	**A-1**	**A-2**	**A-3**	**A-4**	**A-5**	**B-1**	**B-2**	**B-3**	**B-4**	**B-5**	**C-1**	**C-2**	**C-3**	**C-4**	**C-5**
Qiagen DNeasy	6	4	3	1	2	5	9	8	10	7	13	14	15	11	12
Manual Method	2	5	8	3	1	14	6	7	12	10	11	15	9	4	13
Epicentre MasterPure	2	6	3	5	1	9	11	8	7	10	4	13	14	12	15
Stratec Invisorb	4	5	3	1	2	9	6	7	12	10	8	14	13	11	15
Average of all methods	**4**	**5**	**3**	**2**	**1**	**8**	**9**	**6**	**11**	**7**	**10**	**15**	**13**	**12**	**14**

* Order of rank: 15 pool DNA samples were ranked according to their telomere length (rank 1 representing the longest telomere length, rank 15 the shortest telomere length).

Overall, the average inter-assay variation for telomere length was lower for pool samples (8.25%) than for individual samples (12.51%).

4. Discussion

4.1 Implications of inter-assay variation

Interest in telomere length as a biomarker for aging and age-related diseases is on the rise, and with it the need for reproducible and reliable results. An assumption that DNA extraction may impact TLM existed beforehand, and could be expected due to previous results in literature (57, 59-61, 63). To investigate this matter further, this study systematically evaluated five different DNA extraction methods and their effect on telomere length. It demonstrated that, indeed, depending on the method used to isolate the DNA, telomere length as quantified by qPCR varies substantially to the point that a consistent ranking of relative telomere length of fifteen individuals could not be established.

The experiments revealed that DNA extracted with five different methods exhibited large variations regarding quantity, purity, and fragmentation. After measuring telomere length with qPCR, a high inter-assay variation was observed among the various extraction methods, ranging from 7-19% for individual samples and from 6-11% for pool samples. In pool samples, furthermore, an intra-assay variation of 4-15% was observed. Although telomere length variation was to some extent expected, it is generally assumed that the ranking of telomere length within one cohort is comparable at least, regardless of the applied extraction method. The data described here, however, demonstrate that this is not the case. Rankings obtained with one method were stable to some extent. However, rankings obtained via different methods displayed considerable inconsistencies, so that a person may in fact be classified to have among the longest telomeres in a cohort when extracted with one method but be among the shortest telomeres when extracted with another method.

Preanalytical conditions of DNA, such as low purity measurements, were also found to directly lead to high variability in telomere length measurements. This was particularly apparent for Phenol-Chloroform extraction, which led to variations of at least 28% and up to 70% among technical repeats on three consecutive days. It is debatable whether Phenol-Chloroform extraction is nowadays obsolete, since a variety of commercial kits exist which do not contain toxic components and are not prone to high contamination.

Although the average telomere length per age group varied highly, a decline in telomere length with increasing age was detected with all methods, consistent with earlier results (13).

This may explain why most studies, employing a broad variety of methods, observe an age-dependent decline in telomere length. By contrast, telomere length variation can easily be overlooked in the comparison of samples measured with slightly different procedures. This work provides further evidence of the impact of DNA extraction on measurements of relative telomere length (59-61, 63), therefore extending existing methodological knowledge and contributing to the research of telomere length and their association with age-related diseases. Even with the moderate number of 15 samples, the impact of the DNA extraction method in terms of intra- and inter-assay variation was demonstrated.

One other study investigated different DNA isolation methods along with preanalytical conditions and their effect on TLM (59). In this study, six DNA extraction methods were tested and a variation of preanalytical difficulties was analyzed: the influence of degradation and freezing of the samples. Telomere length was assessed by qPCR and it was shown that not only did the method of DNA isolation significantly affect telomere length but also sample degradation reduced telomere length, with an average decrease of 22%, whereas freezing only had a minor impact (<5 %). Therefore, preanalytical conditions were adjusted and standardized for these experiments: blood drawings as well as aliquoting and freezing the samples were completed on the same day. To prevent DNA degradation, blood samples were thawed on ice and were processed for DNA extraction as soon as the blood was completely melted. In addition, the extracted DNA was tested for degradation with gel electrophoresis before TLMs.

Another study compared DNA isolation methods to measure telomere length on colorectal cancer patients and non-cancer controls (60). DNA preparation was executed using three techniques: a column method, a salting-out of DNA and extraction with Phenol-Chloroform. These experiments revealed longer telomere lengths of DNA samples extracted with the salting-out technique or Phenol-Chloroform than for silica-based column isolation. It was suggested that this might be due to the extraction technique itself, where mixing and vortexing could have possibly sheared DNA. To further investigate these results, and to rule out other factors that might influence TLM by qPCR, the authors performed TLM by Southern blot and confirmed their findings. An association was detected between colorectal cancer risk and relative telomere length, which was more pronounced in the samples extracted with Phenol-Chloroform and the salting-out technique, which led to the conclusion that those two methods provided more accurate results for telomere length than the column-based method, the latter of which could lead to falsely negative associations with colorectal cancer.

The influence of DNA extraction methods on telomere length and its impact on epidemiological studies was also demonstrated in another work (61), where four different experiments were carried out, including the comparison of different DNA isolation methods on different cohorts and a following association analysis for cardiovascular disease. It was revealed that two widely applied DNA extraction methods (Qiagen DNA Blood 200 µl Kit EZ1 and Stratec Invisorb Blood Univsersal Kit) yielded results with major differences in relative telomere length - relative telomere length was roughly 40% lower when measured with EZ1 compared to Invisorb-extracted DNA. The Qiagen EZ1 kit uses magnetic particles which bind DNA, while Invisorb Blood Universal Kit is a salting-out method. Again, it was demonstrated that these differences in relative telomere length could indeed produce false associations of relative telomere length to disease, in this case cardiovascular disease, in that the method which produced shorter relative telomere length (EZ 1Kit) yielded no association with cardiovascular diseases.

The discovery that column-based extracted DNA results in shorter telomere length compared to a salting-out technique are inconsistent with the results of this work. Here, the silica-column based extraction produced rather slightly longer average telomere lengths (T/S=1.17) than the three salting-out techniques that were applied, with a difference of 4-15% depending on salting-out technique variation. This difference was more pronounced for individual samples than for pooled samples, which might be explained by the overall higher variation across individual samples. This was also true for experiments carried out by Denham et al. (63), where a column-based extraction method yielded longer telomere length compared to a non-commercial salting-out technique.

4.2 Telomere length as a biomarker of age and age-related diseases

White blood cells are the sample of choice for the majority of telomere length research and were also used here, as blood is obtained easily by venal puncture and is also suitable for repeated sample collection over time (25). Blood leucocytes present a highly heterogenous cell population including different cell subpopulations such as lymphocytes, granulocytes, and monocytes (80). Even in healthy individuals, the composition of peripheral blood leucocytes may differ greatly depending on stress exposures (81), and it is receptive to other transient changes in the immune system such as inflammation, which induces proliferation in leucocytes (80). Newly released leucocytes have telomere length similar to hematopoietic stem cell progenitors, while mature leucocytes exhibit much shorter telomeres (82). Notably, the timing of blood collection should be chosen carefully, since telomere length may vary

after acute infections, injuries, surgeries or immunosuppressive medication (58). It remains uncertain if telomere length in leucocytes is representative of telomere lengths of other tissues and whether it can provide information about pathophysiological changes in different organs, so that more studies are needed to establish this correlation (80).

The qPCR protocol used in this study provides information about the average telomere length, but not the distribution of shorter and longer telomeres (46, 83). While average telomere length provides valuable information and a general idea of telomere attrition, it has been documented that the shortest telomere in individual cells - rather than average telomere length - regulates telomere function and therefore chromosome stability (14, 84). In addition, it has also been shown that qPCR results are less reliable for the shortest and longest telomeres, which have been the target of many studies (85). Moreover, average telomere length is limited to providing associations with certain diseases but cannot prove cause-and-effect relations (46, 86). Other methods like quantitative FISH and STELA can offer further information about the length of telomeres in individual chromosomes (46, 47). Combining several techniques to comprehensively assess telomere length appears to be a logical consequence, as each technique can provide different aspects of telomere length and structure.

Due to intra- and inter-laboratory technical variations, however, comparability and reproducibility of telomere length determined with different methods is very poor. As a solution, a set of telomere length standards would improve not only telomere length quantification between methods but also inter-laboratory comparability, particularly when multiple research laboratories are involved in studies (51). To date, most research laboratories use their own protocols based on varying levels of reagents, controls and equipment (25, 51, 58), and also measure samples in duplicates, triplicates or quadruplicates (61). Standardized protocols would thus greatly facilitate comparison and reproducibility of results.

QPCR is the most widely employed method in telomere length research these days, which is why it was chosen in this study. Numerous factors can, however, critically influence TLM by qPCR (25, 58). As shown here, the choice of DNA extraction method is certainly among those factors, but also sample source, sample storage, DNA storage, qPCR assay conditions, reference standards and data analysis methods can play a significant role, as shown elsewhere (58). QPCR is susceptible to the influence of residual solvents or salts, which are included in most extraction methods (60). It is therefore crucial to implement strict purity thresholds, to establish exact standard operation procedures and to exclude samples that do not meet the purity thresholds and possibly other criteria (61). To distinguish which DNA extraction method produces the most accurate results for telomere length measurement, it is desirable to compare telomere length results among different extraction techniques by executing them simultaneously, as similarly proposed in the literature (58). It is then vital to maintain the same extraction method throughout studies, as also proposed in the literature (61). Additionally, preanalytical aspects need to be taken into careful consideration, including sample collection, sample storage and DNA storage until telomere length assessment (59). As part of the future standardization of TLM techniques, research laboratories may, moreover, regularly participate in ring trials to ensure the same experimental conditions and eliminate inter-laboratory variation, with controls for short, intermediate, and long telomeres as well as set limitations for coefficient of variation.

The focus of this work was to assess the impact of different DNA extraction methods on telomere length quantification. It revealed substantial intra- and inter-assay variation among DNA extraction methods and that consistent ranking of telomere length cannot be established as a consequence. In addition to the methodological differences of DNA extraction methods, inter-assay variation may further originate from various steps during qPCR, resulting in a combined number of factors affecting study results.

4.3 Future outlook

Age-related studies produce an extensive amount of data on telomere length and their association with age-related diseases. It is therefore critical not to lose sight of the actual implications of telomere length. As humans grow older and age-related diseases become more prevalent, telomere length may become a routine research area or even a clinical marker in the future to be tested regularly, providing the examiner with valuable insights into the dynamics of biological age and its likely association with certain diseases. The question of whether information on personal telomere length will affect patients has to be shown in future studies. For these studies and for more fundamental research investigation, it is therefore of critical importance to improve technical and methodological assessment of TLM while its clinical significance is still being established, so as to achieve accurate, reproducible, and inter-laboratory comparable results.

In conclusion, various experimental factors need to be taken into consideration and optimized before conducting large epidemiological studies on telomere length. In addition to standardizing TLM itself, standardization of collection and storage of samples, as well as careful choice of DNA extraction methods will be crucial. With all this in mind, telomere length has the potential to yield deep insights into aging and the complex molecular processes underpinning it.

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„Ich, Katharina Sonja Kim, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: Comparison of Different DNA Extraction Methods and their Impact on Telomere Length Measurement/ Vergleich verschiedener DNA-Extraktionsmethoden und ihr Einfluss auf Telomerlängenmessung selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.
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Ich versichere ferner, dass ich die in Zusammenarbeit mit anderen Personen generierten Daten, Datenauswertungen und Schlussfolgerungen korrekt gekennzeichnet und meinen eigenen Beitrag sowie die Beiträge anderer Personen korrekt kenntlich gemacht habe (siehe Anteilserklärung). Texte oder Textteile, die gemeinsam mit anderen erstellt oder verwendet wurden, habe ich korrekt kenntlich gemacht.

Meine Anteile an etwaigen Publikationen zu dieser Dissertation entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit dem/der Erstbetreuer/in, angegeben sind. Für sämtliche im Rahmen der Dissertation entstandenen Publikationen wurden die Richtlinien des ICMJE (International Committee of Medical Journal Editors; www.icmje.og) zur Autorenschaft eingehalten. Ich erkläre ferner, dass ich mich zur Einhaltung der Satzung der Charité – Universitätsmedizin Berlin zur Sicherung Guter Wissenschaftlicher Praxis verpflichte.

Weiterhin versichere ich, dass ich diese Dissertation weder in gleicher noch in ähnlicher Form bereits an einer anderen Fakultät eingereicht habe.

Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§§156, 161 des Strafgesetzbuches) sind mir bekannt und bewusst.“

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Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

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